Technische Universität München
TUM School of Life Sciences

Investigations into metabolic properties of non-*Saccharomyces* yeasts for the production of beers with novel flavor profiles

Yvonne Methner

Vollständiger Abdruck der von der TUM School of Life Sciences der Technischen Universität München zur Erlangung einer

Doktorin der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

Vorsitz: Prof. Dr. Corinna Dawid

Prüfer*innen der Dissertation:

1. Hon.-Prof. Dr.-Ing. Fritz Jacob
2. Prof. Dr. Wolfgang Liebl

Die Dissertation wurde am 10.05.2023 bei der Technischen Universität München eingereicht und durch die TUM School of Life Sciences am 29.08.2023 angenommen.

“In den kleinsten Dingen zeigt die Natur die allergrößten Wunder.“

- Carl von Linné -

Acknowledgements

First and foremost, I would like to thank my doctoral advisor and supervisor, Prof. Dr. Fritz Jacob, who made this work and the outstanding topic possible for me in the first place and provided me with his full support and advice. I thank you for encouraging me in my ideas, giving me freedom in researching and placing your trust in me.

Moreover, I would like to thank Prof. Dr. Wolfgang Liebl who volunteered to review this dissertation and Prof. Dr. Corinna Dawid for acting as chief examiner. Furthermore, I would like to thank Dr. Martina Gastl, who took over the position as director from Prof. Dr. Fritz Jacob as he went into his well-deserved retirement. Thank you for allowing me to continue pursuing my thesis as an external doctoral student and for your full support.

My special thanks go to Dr. Mathias Hutzler and Dr. Martin Zarnkow, who always had an open door as well as an open ear for me. The in-depth professional discussions with you and your critical feedback have significantly advanced this work. I would like to thank you for allowing me to pester you with all my questions at any time and for taking your time. Furthermore, I thank you for your humanity and that you never lost your confidence that I will successfully complete this work. This trust helped me not to give up on my goal and it was a great pleasure to work with you. In addition, I would like to thank you, Mathias, for passing on your passion for yeast research to me and for opening the ways for me to continue pursuing this passion in my current profession.

I would also like to thank Dr. Hubertus Schneiderbanger and Dario Cotterchio. Thank you for supporting me at all times with questions and for the pleasant cooperation with you.

While writing this thesis, many analyses were necessary, for which I needed support, which I received from my colleagues at the Weihenstephan Research Center for Brewing and Food Quality. In the first place, I would like to thank the entire teams of Mathias and Martin. Many thanks especially to Margit Grammer, Monika Mayerhofer and Franziska Elisath for your active support in microbiology. You were always there for my small as well as bigger questions and concerns and helped me whenever possible. Furthermore, I could learn a lot from you and I am very grateful for the good cooperation and your inexhaustible helpfulness. I would also like to thank my colleagues at the Yeast Center, especially Dominique Stretz, Sebastian Hans and Sophia Rott for their support in my work. In this context, I would also like to thank Margit Bauer, who gave me a lot of time through her work in the scullery. From Martin's team, I would like to express my special thanks to Friedrich Ampenberger, who supported me in the pilot brewery with advice and action and from whom I was able to learn a lot. My special thanks goes to my doctoral student colleagues Luis, Oli, Andi and Philipp, who have since become friends. I thank you for the many valuable professional discussions, for the smooth collaboration on different projects and furthermore for the great time we had together outside of work. Without you, it would have been only half as nice and I do not want to miss the time with you.

A special thanks goes to Hubert Walter and his entire CTA team for their active support during the analyses. Thank you Hubert, for answering all my technical questions and for going above and beyond when my IT did not want to work the way I would have liked. I would like to emphasize the good cooperation with Florian Mallok when it came to my numerous tastings, with Sabine Margeth, who never lost track of my countless samples, and with Markus Schmidt, without whose support I would probably still be analyzing my samples today. The same is true for Susan Illing from instrumental analysis, to whom I would like to express my special thanks. Thank you Susan for always thinking along

with me, for sparing no extra effort and work to ensure flawless results, and for also mentally being there. A big thank you to the entire instrumental analysis team for the good cooperation and support and, on behalf of the entire team, especially to Prof. Dr. Mehmet Coelhan and Karl-Heinz Bromig.

I also thank a number of students who supported my doctoral thesis as part of their master's and diploma theses. I would like to emphasize Konstantin Lettner, Daniel Hofmeister, Sebastian Hans and Paul Fallscheer.

Furthermore, thank you to the secretariat, who supported me with organizational and administrative work. I would especially like to thank Georgine Widmann, Sylvia Kugler, Jutta Vilser, Natalie Böck and Hülya Dilek for their great cooperation.

In this context, I would also like to express my gratitude to the AiF and in particular to Dr. Erika Hinzmann, who financially supported this work. I would also like to thank our partner research institution, the Leibniz Institute of Food Systems Biology at the Technical University of Munich, for the collegial and successful collaboration. My personal thanks here goes to Dr. Stephanie Frank, who coordinated the research project together with me and always took time for fruitful discussions.

Additionally, I would like to express my gratitude to my collaborative partners in the various research projects conducted as part of this thesis. Many thanks especially to Prof. Dr. Frank Endres, Dr. Alexandra Prowald, Dr. Frederico Magalhães, Prof. Dr. Diego Libkind, Mailen Latorre and Nadine Weber for the successful and pleasant collaboration.

Another thanks goes to Sarah Silva, who improved the linguistic quality of this work in English and who always took her time even at short notice.

Last but not least, I would like to thank my family and friends who accompanied me on the way of the dissertation. First and foremost, I would like to thank my husband and also best friend Kai from the bottom of my heart. Thank you for carrying me through this time, for supporting me, for being my rock, for always believing in me, for being there for me with tireless patience, and for the fact that my sometimes higher emotional amplitudes did not even stop you from proposing to me and marrying me during this time. I would also like to address a special thank you to my parents Rika and Frank. Thank you for awakening my interest in this field at an early age, for always supporting me and being there for me. Moreover, I thank my mother-in-law Jutta for always being there for me during this time.

List of publications

Publications in international peer-reviewed journals related to this thesis

Publication 1

Methner, Y.; Hutzler, M.; Matoulková, D.; Jacob, F.; Michel, M. (2019). **Screening for the Brewing Ability of Different Non-*Saccharomyces* Yeasts.** Fermentation 5 (4): 101. DOI: 10.3390/fermentation5040101.

Publication 2

Methner, Y.; Hutzler, M.; Zarnkow, M.; Prowald, A.; Endres, F.; Jacob, F. (2022). **Investigation of Non-*Saccharomyces* Yeast Strains for Their Suitability for the Production of Non-Alcoholic Beers with Novel Flavor Profiles.** Journal of the American Society of Brewing Chemists 80 (4): 341-355. DOI: 10.1080/03610470.2021.2012747.

Publication 3

Methner, Y.; Magalhães, F.; Raihofer, L.; Zarnkow, M.; Jacob, F.; Hutzler, M. (2022). **Beer fermentation performance and sugar uptake of *Saccharomycopsis fibuligera*–A novel option for low-alcohol beer.** Frontiers in Microbiology 13: 1011155. DOI: 10.3389/fmicb.2022.1011155.

Publication 4

Methner, Y.; Dancker, P.; Maier, R.; Latorre, M.; Hutzler, M.; Zarnkow, M.; Steinhaus, M.; Libkind, D.; Frank, S.; Jacob, F. (2022). **Influence of Varying Fermentation Parameters of the Yeast Strain *Cyberlindnera saturnus* on the Concentrations of Selected Flavor Components in Non-Alcoholic Beer Focusing on (*E*)-*β*-Damascenone.** Foods 11 (7): 1038. DOI: 10.3390/foods11071038.

Publication 5

Methner, Y.; Weber, N.; Kunz, O.; Zarnkow, M.; Rychlik, M.; Hutzler, M.; Jacob, F. (2022). **Investigations into metabolic properties and selected nutritional metabolic byproducts of different non-*Saccharomyces* yeast strains when producing nonalcoholic beer.** FEMS Yeast Research: foac042. DOI: 10.1093/femsyr/foac042.

Further publications in peer-reviewed journals

Endres, F.; Prowald, A.; Fittschen, U. E. A.; Hampel, S.; Oppermann, S.; Jacob, F.; Hutzler, M.; Laus, A.; Methner, Y.; Zarnkow, M. (2022). **Constant temperature mashing at 72 °C for the production of beers with a reduced alcohol content in micro brewing systems.** European Food Research and Technology. DOI: 10.1007/s00217-022-03968-2

Michel, M.; Cocuzza, S.; Biendl, M.; Peifer, F.; Hans, S.; Methner, Y.; Pehl, F.; Back, W.; Jacob, F.; Hutzler, M. (2020). **The impact of different hop compounds on the growth of selected beer spoilage bacteria in beer.** Journal of the Institute of Brewing: 4: 354-361. DOI: 10.1002/jib.624.

Publications in non-peer reviewed journals

Hutzler, M.; Methner, Y.; Jacob F. (2022). **Special yeasts for nonalcoholic and low-alcohol beers**. Brauwelt international. 23-26: 21-24.

Raihofer, L.; Methner, Y.; Hutzler, M.; Zarnkow, M.; Jacob, F. (2021). **Raman-Spektroskopie in der Lebensmittel- und Getränkeanalytik.** Brauwelt. 45-46: 1166-1169.

Hutzler, M.; Methner, Y.; Jacob, F. (2021). **Spezialhefen für alkoholfreie und alkoholarme Biere.** Brauwelt. 25-26: 633-636.

Endres, F.; Prowald, A.; Fittschen, U. E. A.; Hampel, S.; Oppermann, S.; Jacob, F.; Zarnkow, M.; Hutzler, M.; Methner, Y.; Laus, A. (2021). **Vergleichendes isothermes Maischen bei 72 °C.** Brauwelt. 9-10: 223-227.

Hutzler, M.; Zarnkow, M.; Hans, S.; Stretz, D.; Meier-Dörnberg, T.; Methner, Y.; Schneiderbanger, H.; Jacob, F. (2020). **New Yeasts – New Beers.** Brewing and Beverage Industry International: 16-23.

Hutzler, M.; Zarnkow, M.; Hans, S.; Stretz, D.; Meier-Dörnberg, T.; Methner, Y.; Schneiderbanger, H.; Jacob, F. (2020). **Neue Hefen – neue Biere.** Brauindustrie: 8-13.

Methner, Y.; Ampenberger, F.; Zarnkow, M.; Michel, M.; Jacob, F.; Hutzler, M. (2020). **Wenig Alkohol mit Einfachbier.** Brauwelt. 10:265-270.

Hutzler, M.; Narziß, L.; Methner, Y.; Stretz, D.; Jacob, F.; Michel, M. (2019). **Auferstehung der Lagerbier-Hefe *S. pastorianus* Franconia – TUM 35.** Brauwelt. 40-41:1161-1165.

Conference contributions

Methner, Y.; Laus, A.; Hutzler, M.; Zarnkow, M.; Endres, F.; Jacob, F. **Isotherme Maische plus Spezialhefen – alternativer Weg zu alkoholfreiem Bier.** 9. Seminar Hefe und Mikrobiologie, Online-Seminar, 2022.

Methner, Y.; Hutzler, M.; Zarnkow, M.; Jacob, F. **Aromahefen – Maltose-negative Hefen zur Herstellung alkoholfreier Biere.** 15. Weihenstephaner Praxisseminar, Online-Seminar, 2021.

Methner, Y.; Hutzler, M.; Zarnkow, M.; Jacob, F. **Investigation of Non-*Saccharomyces* Yeast Strains for Their Suitability for the Production of Non-Alcoholic Beers with Novel Flavor Profiles.** American Society of Brewing Chemists (ASBC), Virtual Meeting, 2021.

Methner, Y.; Hutzler, M.; Zarnkow, M.; Jacob, F. **Neuartige Aromahefen.** 8. Seminar Hefe und Mikrobiologie, Online-Seminar, 2021.

Hutzler, M.; Methner, Y. **Spezialhefen für alkoholfreie und -arme Biere. Special yeasts for non- and low-alcoholic beers.** Virtual BrauBeviale@stage, 2020.

Contents

List of Tables

List of Figures

Abbreviations

4-VG	4-vinyl guaiacol
4-VP	4-vinyl phenol
AATase	alcohol acetyl transferase
Abbr.	abbreviation
Acetyl CoA	acetyl-coenzyme A
Adh	alcohol dehydrogenase
ADP	adenosine diphosphate
ATF	alcohol acetyl transferase
ATP	adenosine triphosphate
BIOHAZ	Biological hazards
CO_2	carbon dioxide
DMS	dimethyl sulfide
DMSO	dimethyl sulfoxide
EFSA	European Food Safety Authority
EHT	ethanol hexanoyl transferase
EU	European Union
FAN	free amino nitrogen
Fructose-1,6-bisP	fructose-1,6-bisphosphate
FDC	ferulic acid decarboxylase
Glucose-6-P	glucose-6-phosphate
GRAS	Generally Recognized As Safe
H_2S	hydrogen sulfide
IPA	India Pale Ale
LAB(s)	low-alcohol beer(s)
M	Molar
Mal	maltose
Malt	maltotriose
MALDI-TOF	matrix-assisted laser desorption ionization-time of flight
MCFA	medium-chain fatty acid

NAB(s)	non-alcoholic beer(s)
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
OAV(s)	odor activity value(s)
OD	optical density
PAD	phenylacrylic acid decarboxylase
PCR	polymerase chain reaction
P_i	inorganic phosphate
POF	phenolic off-flavor
QPS	qualified presumption of safety
rep-PCR	repetitive element palindromic PCR
RSM	Response Surface Methodology
SO_2	sulfur dioxide
sp.	species
spp.	species pluralis
TCA	tricarboxylic acid cycle
TRBA	Technical Rules for Biological Agents
TUM	Technical University of Munich
US	United States
USA	United States of America
var.	variety
v/*v*	volume per volume
YM	yeast and mold
YP	yeast peptone
YPD	yeast extract-peptone-dextrose

Summary

In recent years, increased attention has been paid to the fermentation of beers using non-*Saccharomyces* yeasts. In compliance with the German Purity Law, the potential modification options initially seem limited with regards to diversifying beer flavor. Although numerous special malts and aroma hops have already been investigated in order to develop a greater variety of products, yeasts have a decisive influence on the beer flavor. The scientific breakthrough by Emil Christian Hansen, who succeeded in isolating a single cell of the brewing yeast *Saccharomyces pastorianus* in 1883, laid the foundation for the domestication of *Saccharomyces* yeasts for beer brewing, yet the aroma diversity of beers has progressively decreased as co-culture fermentations were no longer carried out. However, non-*Saccharomyces* yeasts do have the potential for producing sensorially appealing flavors during the fermentation of brewer's wort, which is demonstrated by beers traditionally produced with non-*Saccharomyces* yeasts, such as Berliner Weisse or Lambic beers.

A wide variety of yeasts is used in numerous food and beverage fermentations, often making a decisive contribution to the overall flavor. Some of these yeasts could also be suitable for the fermentation of brewer's wort to generate outstanding aromas. As research in this field is currently still in its infancy and offers far-reaching potential, 20 different yeast species known from versatile food and beverage fermentations were investigated for their suitability for beer brewing as part of this work. The focus was on diversifying the beer flavor in order to generate novel and at the same time appealing flavor profiles. This applied to regular beers containing alcohol as well as non-alcoholic and low-alcohol beers. In contrast to regular beers containing alcohol, non-alcoholic beers have experienced steady market growth in recent years. In addition, there is a greater need for flavor diversification in this sector due to the continuing challenge of eliminating wort aromas from non-alcoholic beers for flavor optimization. Therefore, this thesis focuses on non-*Saccharomyces* yeasts suitable for the production of non-alcoholic beers, while non-*Saccharomyces* yeasts for the production of regular beers are only discussed in the first publication.

A yeast needs to possess certain metabolic properties to be suitable for beer brewing. This includes the ability to metabolize the carbohydrates present in the brewer's wort and a sufficient tolerance to hops and, in the case of the production of a regular beer with alcohol, sufficient tolerance to ethanol. The kind of wort sugars the respective yeast strain can utilize for its cell metabolism determines whether a regular beer or a non-alcoholic or low-alcohol beer can be produced.

In this thesis, a characterization method on a microtiter plate scale was first developed due to the limited data available on the use of non-*Saccharomyces* yeasts for beer production. By applying this method, extensive screening could be conducted within a relatively short period of time. A total of 110 different yeast strains of 18 yeast species were investigated in selective media to find out which wort sugars could be metabolized and whether there was sufficient hop and ethanol tolerance. With regard to flavor formation, the yeasts were inoculated into wort for an initial odor test and subjected to a test to detect phenolic off-flavors. Provided that there was sufficient hop and ethanol tolerance, no phenolic off-flavors were detected and the odor test revealed a positive initial impression, the yeasts were used for subsequent fermentation trials on a small 2-liter scale. Five promising yeast strains of three different species were tested, which were able to metabolize maltose in the microtiter plate test and thus showed potential for the production of regular beers containing alcohol. Three beers fermented with *Saccharomycopsis fibuligera* yeast strains stood out positively due to their fruity flavors, which were reminiscent of plum and berries.

Since an outstanding number of yeast strains, which were maltose- and maltotriose-negative, had developed pleasant fruity flavors in the odor test, 15 of these yeast strains were selected in the course of the thesis for further fermentation trials on a small 2-liter scale that showed potential for the production of non-alcoholic beers. Three beers fermented with *Cyberlindnera saturnus* yeast strains stood out with particularly positive flavor characteristics reminiscent of pear, cool mint sweets, and banana. One of the three yeast strains, C. sat 247 released a pleasant berry flavor as well as a subtle apple flavor in addition to the aromas mentioned. The non-alcoholic beer of another *Saccharomycopsis fibuligera* yeast strain was also convincing with berry and plum-like flavors. Although the *S. fibuligera* yeast strain S. fib Lu27 was able to metabolize maltose in a selective medium within a very short time, the strain proved to be weak in fermentation in brewer's wort, as the beer remained below 0.5% (*v*/*v*) ethanol at an original gravity of 7 °P and a fermentation temperature of 20 °C.

Based on the anomalies regarding sugar utilization of the *S. fibuligera* yeast S. fib Lu27, this strain as well as the *S. fibuligera* strain S. fib SF4 were investigated for their sugar metabolism. Existing literature indicates that this yeast species possesses extracellular glucosidases that can hydrolyze both maltose and maltotriose before diffusing into the cell. During this research, it was found that both yeast strains can also actively take up maltose into the cell, whereas no specific maltotriose permease was found. Furthermore, due to the filamentous growth of *S. fibuligera*, single cells of the strain S. fib SF4 were isolated and re-cultured, which indeed resulted in a significant reduction of mycelial growth in direct comparison to the mother culture and could represent a technological advantage for industrial application due to the improved flocculation behavior. At the same time, no genetic differences were detected in a $(GTG)_5$ rep-PCR fingerprint and the flavor profile of the beer remained unchanged.

The flavor profile of the beer fermented with *C. saturnus* C. sat 247 was also investigated further on the basis of its strikingly positive sensory properties, since the mentioned berry- and apple-like flavors could not be attributed to any specific metabolites. By applying instrumental analytics, (*E*)-*β*-damascenone was detected, which correlated with the flavors determined from sensory analysis in a multivariate data analysis. Additionally, the influence of varying fermentation temperatures, yeast pitching rates and original gravities on the flavor properties of beers fermented with the yeast strain C. sat 247 was tested on a small 2-L scale. Optimization of the beer to achieve maximum fruitiness while minimizing undesirable aroma components and ethanol content was reached at a fermentation temperature of 16.1 °C, original gravity of 10.5 °P and a pitching rate of 5×10^6 cells/mL.

The increasing scientific and industrial interest in the use of non-*Saccharomyces* yeasts for beer brewing requires a safe and reliable application of the yeasts. For this reason, in the final part of this thesis, 16 yeast strains that showed suitability for the production of non-alcoholic beers were tested for nine beer-relevant biogenic amines and for their tolerance in gastric juice at 37 °C in vitro. While the selected biogenic amines remained below the limit of quantification of 5.0 mg/L without exception, six yeast species revealed viabilities above 90% in the tolerance test. This underscores the need for comprehensive safety testing before yeasts are applied in brewing beer. However, non-alcoholic beers can also have positive health aspects. Based on this, four selected yeast strains of the species *C. saturnus*, *Kluyveromyces marxianus* and *S. ludwigii* and their respective non-alcoholic beers were tested for B vitamins. Although these were found in nutritionally relevant amounts to supplement a balanced diet, they most likely originated from the raw materials and were not synthesized by the yeasts themselves.

Zusammenfassung

In den letzten Jahren wurde verstärkt Augenmerk auf die Fermentation von Bieren mittels Nicht-*Saccharomyces* Hefen gelegt. Vor dem Hintergrund des Deutschen Reinheitsgebotes scheinen die Optionen an potentiellen Modifikationen zunächst begrenzt, wenn es um die Diversifizierung des Bieraromas geht. Zwar wurden bereits zahlreiche Spezialmalze und Aromahopfen untersucht, um eine größere Produktvielfalt zu entwickeln, allerdings nehmen die Hefen einen entscheidenden Einfluss auf das Bieraroma. Nachdem mit dem wissenschaftlichen Durchbruch durch Emil Christian Hansen, dem es im Jahr 1883 gelang, eine Einzelzelle der Brauhefe *Saccharomyces pastorianus* zu isolieren, der Grundstein für die Domestizierung von *Saccharomyces* Hefen für das Bierbrauen gelegt war, nahm die Aromavielfalt der Biere fortschreitend ab. Dass jedoch Nicht-*Saccharomyces* Hefen durchaus Potential für die Herstellung sensorisch ansprechender Aromen während der Fermentation von Bierwürze haben, beweisen traditionell mit Nicht-*Saccharomyces* Hefen hergestellte Biere wie beispielweise Berliner Weiße oder Lambic Biere.

In zahlreichen Lebensmittel- und Getränkefermentationen kommen unterschiedlichste Hefen zum Einsatz, die oftmals einen entscheidenden Eintrag zu der Aromagebung leisten. Einige dieser Hefen könnten auch für die Fermentation von Bierwürze geeignet sein, um herausragende Aromen zu generieren. Da die Forschung in diesem Bereich zurzeit noch in den Kinderschuhen steckt und ein weitreichendes Potential bietet, wurden im Rahmen dieser Arbeit 20 verschiedene Hefespezies, die aus unterschiedlichsten Lebensmittel- und Getränkefermentation bekannt sind, auf ihre Tauglichkeit zum Bierbrauen untersucht. Der Fokus lag hierbei auf der Diversifizierung des Bieraromas, um neuartige und gleichzeitig ansprechende Aromaprofile zu generieren. Dies betrifft gleichermaßen alkoholhaltige Vollbiere sowie alkoholfreie bzw. alkoholarme Biere. Im Gegensatz zu den Vollbieren verzeichnen alkoholfreie Biere allerdings in den letzten Jahren ein stetiges Marktwachstum. Zudem besteht in dem Bereich ein größerer Bedarf an einer Aromadiversifizierung, die nicht zuletzt eine Aromaoptimierung beinhaltet aufgrund der nach wie vor existierenden Herausforderung, Würze-Aromen aus alkoholfreien Bieren zu eliminieren. Aus diesem Grund wird in dieser Arbeit ein Fokus auf Nicht-*Saccharomyces* Hefen gelegt, die zur Herstellung alkoholfreier Biere geeignet sind, während Nicht-*Saccharomyces* Hefen zur Produktion von Vollbieren nur eingangs thematisiert werden.

Damit die Anwendbarkeit einer Hefereinzucht für die Bierherstellung gegeben ist, muss diese bestimmte Stoffwechseleigenschaften aufweisen. Dies schließt ein, dass sie die in der Bierwürze vorhandenen Kohlenhydrate verstoffwechseln kann und eine ausreichende Toleranz gegenüber Hopfen und im Falle der Herstellung eines alkoholhaltigen Bieres ebenso eine ausreichende Toleranz gegenüber Ethanol aufweisen muss. Abhängig davon, welche Würzezucker der jeweilige Hefestamm für seinen Zellstoffwechsel nutzen kann, wird gesteuert, ob ein Vollbier oder ein alkoholfreies bzw. alkoholarmes Bier hergestellt werden soll.

Basierend auf der eingeschränkten Datenlage zum Einsatz von Nicht-*Saccharomyces* Hefen für die Bierherstellung wurde im Rahmen dieser Arbeit zunächst eine Charakterisierungsmethode im Mikrotiterplatten-Maßstab entwickelt, mit deren Hilfe ein umfangreiches Screening in verhältnismäßig kurzer Zeit durchgeführt werden konnte. Insgesamt wurden 110 verschiedene Hefestämme aus 18 Hefespezies in Selektivmedien getestet, um herauszufinden, welche Würzezucker metabolisiert werden können und ob eine ausreichende Hopfen- und Ethanoltoleranz besteht. Hinsichtlich der Aromabildung wurden die Hefen für einen ersten Geruchstest sowohl in Würze inokuliert, als auch einem Test unter-

zogen, um phenolische Fehlaromen nachzuweisen. Sofern eine ausreichende Hopfen- und Ethanoltoleranz gegeben war, keine phenolischen Fehlaromen detektiert wurden und der Geruchstest einen ersten positiven Eindruck vermittelte, wurden die Hefen für anschließende Gärversuche im 2-Liter Kleinmaßstab herangezogen. Zunächst wurden fünf vielversprechende Hefestämme aus drei verschiedenen Spezies getestet, die im Mikrotiterplatten-Test Maltose verstoffwechseln konnten und somit für die Herstellung alkoholhaltiger Biere Potential aufwiesen. Aufgrund ihres fruchtigen, an Pflaume und Beeren erinnernden Aromas, stachen dabei drei Biere positiv hervor, die mit *Saccharomycopsis fibuligera* Hefestämmen fermentiert wurden.

Da im Geruchstest auffallend viele Hefestämme angenehm fruchtige Aromen entwickelt hatten, die Maltose- und Maltotriose-negativ waren, wurden im Verlauf der Arbeit 15 Hefestämme für weitere Gärversuche im 2-Liter Kleinmaßstab ausgewählt, die Potential für die Herstellung alkoholfreier Biere zeigten. Mit besonders positiven Aromaeigenschaften, die an Birne, Eisbonbon und Banane erinnerten, stachen drei Biere hervor, die mit *Cyberlindnera saturnus* Hefestämmen fermentiert wurden. Einer der drei Hefestämme (C. sat 247) erzeugte neben den genannten Aromen zusätzlich ein angenehmes Beeren- sowie ein leichtes Apfelaroma. Auch das alkoholfreie Bier eines weiteren *Saccharomycopsis fibuligera* Hefestammes überzeugte mit beeren- und pflaumenartigen Aromen. Obwohl der *S. fibuligera* Hefestamm S. fib Lu27 Maltose als Selektivmedium innerhalb kürzester Zeit verstoffwechseln konnte, erwies sich der Stamm in Bierwürze als gärschwach, denn das Bier blieb unter 0,5 Vol.% bei einer Stammwürze von 7 °P und einer Fermentationstemperatur von 20 °C.

Basierend auf den Auffälligkeiten hinsichtlich der Zuckerverwertung der *S. fibuligera* Hefe S. fib Lu27 wurden dieser Stamm sowie der *S. fibuligera* Stamm S. fib SF4 auf ihren Zuckerstoffwechsel hin untersucht. Aus bestehender Literatur geht hervor, dass diese Hefespezies extrazelluläre Glucosidasen besitzt, die sowohl Maltose als auch Maltotriose hydrolisieren können, bevor sie in das Zellinnere diffundieren. Im Rahmen der Forschungsarbeiten stellte sich heraus, dass beide Hefestämme zudem Maltose aktiv ins Zellinnere aufnehmen können, wohingegen keine spezifische Maltotriose-Permease gefunden wurde. Des Weiteren wurden aufgrund des filamentösen Wachstums der *S. fibuligera* Einzelzellen des Stamms S. fib SF4 isoliert und erneut kultiviert, was im direkten Vergleich zur Mutterkultur tatsächlich zu einer deutlichen Reduktion des Mycelwachstums führte und einen technologischen Vorteil für die industrielle Anwendung aufgrund einer verbesserten Flokkulation bedeuten könnte. Gleichzeitig wurden im Rahmen eines $(GTG)_5$ rep-PCR Fingerprints keine genetischen Unterschiede festgestellt und das Aromaprofil blieb erhalten.

Auch das Aromaprofil des Biers, fermentiert mit *C. saturnus* C. sat 247, wurde aufgrund seiner auffallend positiven sensorischen Eigenschaften weitergehend untersucht, denn das erwähnte beeren- und apfelartige Aroma konnte auf keine spezifischen Metabolite zurückgeführt werden. Mithilfe von instrumenteller Analytik wurde (*E*)-*β*-Damascenon detektiert, welches im Rahmen einer multivariaten Datenanalyse mit den aus der Sensorik ermittelten Aromen korrelierte. Zudem wurde im 2-L-Kleinmaßstab der Einfluss variierender Gärtemperaturen, Hefeanstellzahlen und Stammwürzen auf die Aromaeigenschaften von Bieren getestet, die mit dem Hefestamm C. sat 247 fermentiert wurden. Eine Optimierung des Bieres mit maximaler Fruchtigkeit bei gleichzeitig minimalen unerwünschten Aromakomponenten und minimalem Ethanolgehalt wurde bei 16,1 °C Gärtemperatur, 10,5 °P Stammwürze und einer Hefeanstellzahl von 5×10^6 Zellen/mL erreicht.

Das zunehmende wissenschaftliche, aber auch industrielle Interesse zur Anwendung von Nicht-*Saccharomyces* Hefen zum Bierbrauen setzt einen unbedenklichen und sicheren Einsatz der Hefen voraus.

Aus diesem Grund wurden im abschließenden Teil dieser Arbeit 16 Hefestämme, die zur Herstellung alkoholfreier Biere Taugklichkeit zeigten, auf neun bierrelevante biogene Amine und in vitro auf ihre Toleranz in Magensaft bei 37 °C untersucht. Während die ausgewählten biogenen Amine ohne Ausnahme unterhalb der Bestimmungsgrenze von 5,0 mg/L blieben, wiesen sechs Hefespezies im Toleranztest eine Viabilität von über 90% auf. Dies unterstreicht die Notwendigkeit einer umfassenden Sicherheitsprüfung, bevor die Hefen zum Bierbrauen eingesetzt werden. Doch auch positive gesundheitliche Aspekte können von alkoholfreien Bieren ausgehen. Darauf basierend wurden vier ausgewählte Hefestämme der Spezies *C. saturnus*, *Kluyveromyces marxianus* und *S. ludwigii* und die damit fermentierten alkoholfreien Biere auf B-Vitamine untersucht. Diese wurden zwar in ernährungsphysiologisch relevanten Mengen gefunden, um eine ausgewogene Ernährung zu ergänzen, allerdings stammten sie höchstwahrscheinlich aus den Rohstoffen und wurden nicht von den Hefen selbst synthetisiert.

1 Introduction

Beer flavor is defined by the raw materials malt, hops, yeast, and water. The influence of flavor-active substances from yeast fermentation is greatest compared to the flavor input from malt and hops [145, 260, 262]. Although some studies have been conducted in recent years on the use of non-*Saccharomyces* yeasts in brewer's wort and their impact on beer flavor, there are still significant knowledge gaps due to the great biodiversity of yeast species that might be used to produce beer. In contrast, the potential of different malt and hop varieties to diversify beer flavor has been widely studied until now. Today, beers are produced almost exclusively by fermentation with the yeast species *Saccharomyces cerevisiae* and *Saccharomyces pastorianus*, which are referred to as domesticated culture yeasts. Since it is assumed that with just about 1500 ascomycete yeasts (*Saccharomycotina*) only a fraction of the existing yeasts have been characterized so far [37], there is still huge potential in this field of research. Additionally, each yeast species comprises numerous strains that can differ qualitatively and quantitatively in their flavor synthesis [91, 113, 138]. Thus, it is not surprising that the flavors formed by microorganisms during the fermentation of foods and beverages can be both pleasant and desirable, but also cause unpleasant off-flavors. In brewing, flavors play a major role for producers as well as consumers in terms of flavor expression and flavor quality as the brewer’s goal is to offer a flawless beer from a sensory point of view. For this reason, microorganisms are applied specifically for flavor formation, whether in the context of co-culture fermentations for specialty beers such as Belgian gueuze [336] or specific yeast species in mono-cultivation such as *S. cerevisiae* for top-fermented beers [333]. The domesticated brewer's yeasts *S. cerevisiae* and *S. pastorianus* have been extensively studied with respect to flavor formation in beers [204, 228], different technologies have been used and even hybrids of *Saccharomyces* have been produced to expand the flavor diversity of lager beers [59, 171, 214]. Nevertheless, investigations into the suitability and flavor formation of non-*Saccharomyces* yeasts for beer brewing are still in their infancy and offer great potential [148].

The growing craft beer scene, which has revolutionized the global beer market in the last two decades and is experiencing steady growth, shows that there is a clear need for product diversification of beers [15, 115]. For example, the number of craft beer breweries in the United States (US) has steadily increased from about 1,500 to more than 9,100 between 2007 and 2021 [45]. Specialty malts and different hop varieties play a role in this diversification, but the use of non-*Saccharomyces* yeasts is becoming increasingly popular to influence flavor, acidity, mouthfeel and even the color of the beers [51]. Although regular beer containing alcohol and low-alcohol beer (LAB) as well as non-alcoholic beer (NAB) are discussed in this work, the focus is on LAB and NAB, as their production in Germany showed an increasing trend between 2005 and 2021, more than doubling from just under two million hectoliters to over four million hectoliters [313]. Globally, the NAB and LAB market is also growing and experienced a 20% growth from 2011 to 2016 [27].

Based on the aforementioned gaps in knowledge and the growing market of craft beer, LAB and NAB, this thesis deals with investigations of non-*Saccharomyces* yeasts for their suitability for brewing beer with emphasis on LAB and NAB. A prerequisite of this research work was that all beers should be produced in consideration of the German Purity Law of 1516. Since bottom-fermented beers may only be produced by using *Saccharomyces pastorianus*, non-*Saccharomyces* yeasts belong to the top-fermented yeasts. The top-fermented beer segment also traditionally includes mixed fermentations such as Berliner Weisse or Belgian beer styles. These are fermented with non-*Saccharomyces* yeasts of the *Brettanomyces*/*Dekkera* genus, among others [144]. According to current interpretations, the German

Purity Law does not conflict with the use of non-*Saccharomyces* yeasts, as they are generally allowed to be classified as top-fermenting yeasts during fermentation regardless of their flocculation behavior. Accordingly, in this thesis the production of regular beers containing alcohol, as well as LAB and NAB by using non-*Saccharomyces* yeasts during fermentation are taken into account. Consequently, the carbohydrate metabolism of different yeasts is investigated. Besides the primary metabolites, studies are focused on the secondary metabolites, which are responsible for the diversification of the beer flavor. The safe use of the yeasts is also discussed in the context of this research work.

The publications of this thesis are divided into five parts:

1. In the first part of this thesis, a screening of a wide range of 110 different non-*Saccharomyces* yeasts was carried out to investigate their suitability for beer brewing. A screening method was developed to test a large number of yeasts for their suitability for beer brewing in a relatively short period of time. The most promising yeast strains were tested in small-scale fermentations with regard to fermentation performance and flavor formation. The focus was set on the production of beers containing alcohol.
2. Numerous promising yeast strains emerged from the first part of this thesis, which were potentially suitable for the production of NABs. Hence, in the second part, a selection of 15 promising yeast strains were investigated in small-scale fermentation trials for their fermentation performance and flavor formation in brewer's wort using the maltose-negative yeast *Saccharomycodes ludwigii* as the reference strain.
3. Following on from parts 1 and 2, two *Saccharomycopsis fibuligera* yeast strains were found to produce exceptionally positive flavors during wort fermentation. However, the carbohydrate metabolism of this yeast species stood out as both NAB and alcoholic beer could be produced. Accordingly, the carbohydrate metabolism was to be elucidated in the third part of this thesis. In addition, the yeast species *S. fibuligera* has the phenotypic characteristic of filamentous growth, which could lead to challenges in clarification on an industrial scale. Therefore, by performing a single cell isolation, a reduction in filamentous growth was achieved without the positive flavor properties of the beer being negatively affected by this procedure.
4. Following on from part 2 of this thesis, the flavor of one beer fermented with the yeast strain *Cyberlindnera saturnus* stood out as particularly positive by exhibiting berry and apple notes. Since these flavor impressions could not be clearly assigned to designated secondary metabolites, this was to be clarified in the fourth part of the thesis.
5. Both food safety as well as health benefits play a significant role when using microogranisms in food fermentations. For the majority of non-*Saccharomyces* yeasts investigated in this thesis, the beers have not yet been studied with regards to this topic. Consequently, this was the research subject of the fifth part of this thesis.

In the following general introduction, which provides the corresponding basic knowledge for this thesis, a brief look is taken at the history of yeasts for beer brewing. Subsequently, the 20 different yeast species are specified, which were investigated for their suitability for brewing beer as part of this thesis. An overview is given of whether studies already exist for the individual yeast species on the subject of beer brewing or whether they have been investigated for cider or wine fermentation. In addition, it is shown in what kind of further beverage or food fermentations the yeasts occur taking their traditional use into account. This overview is supplemented by a list of other non-*Saccharomyces* yeasts that have already been investigated for their suitability for beer brewing. Since NABs are the focus of

this thesis, an overview of the production of NABs is given, before the prerequisites that non-*Saccharomyces* yeasts must fulfill in order to be suitable for the fermentation of brewer's wort are addressed. The hop and ethanol tolerance of the yeasts, their carbohydrate metabolism, flavor formation and their safe use, as well as their potential health benefits, are discussed.

1.1 How yeasts evolved in beer brewing before the domestication of *Saccharomyces* yeasts

Historically, fermentation in beer brewing occurred spontaneously by microbes from the environment and without any control over the microbial fermentation [314]. This can be attributed to the fact that the origin of beer brewing is dated about 10,000 years ago [10, 191]. The environment represented a broad spectrum, as microbes could be introduced from surrounding air, raw materials, brewing equipment or via insects [16]. In addition, microorganisms could also be introduced by the brewers themselves and influenced the overall flavor of the beer [82]. Due to the variable environmental conditions, the processes of beer brewing were correspondingly inconsistent, which was not only inefficient but also often resulted in beers with unpleasant off-flavors [314]. Nevertheless, *Saccharomyces* yeasts often dominated spontaneous fermentations, especially in the production of beer and bread, while non-*Saccharomyces* yeasts only showed rapid proliferation during the first days of fermentation in brewer's wort, resulting in a high flavor production at the beginning of the fermentation process [60, 80]. The ability of *Saccharomyces* yeasts to dominate during fermentation was an important basis for the establishment of *Saccharomyces* yeasts in beer brewing.

What enables *Saccharomyces* yeasts to assert themselves in brewer's wort? There are numerous arguments in favor of this, such as rapid growth, efficient glucose suppression, the ability to produce and consume ethanol, and stress tolerance to different environmental factors such as high ethanol and low oxygen concentrations. Other yeasts also possess some of these abilities, however, these abilities are uniquely combined and regulated in *Saccharomyces* yeast [261]. The ability of *Saccharomyces* yeasts to produce high concentrations of ethanol helps prevent microbial spoilage. In addition, the yeast works preferentially in fermentation metabolism and is Crabtree-positive. As a result, the presence of glucose suppresses cellular respiration and carbohydrates, such as glucose, can be degraded to ethanol without fully oxidizing them to carbon dioxide even in the presence of oxygen [262]. Although oxygen is often still present at the beginning of fermentation, yeast cells prefer fermentation to cellular respiration and consequently exhibit high fermentative activity [314].

In general, yeasts can use respiration and fermentation for cellular energy metabolism to form adenosine triphosphate (ATP). As long as oxygen is available, Crabtree-negative yeasts stay within the respiration pathway whereas Crabtree-positive yeasts can also activate their fermentation metabolism in the presence of oxygen due to genetic events which consequently leads to ethanol formation [257]. Figure 1 gives an overview under aerobic conditions of the metabolic pathways of a Crabtree-positive *S. cerevisiae* (red lines) in direct comparison to a Crabtree-negative *Kluyveromyces lactis* (green lines). The uptake of glucose and the glycolysis with its phosphorylation to glucose-6-phosphate (Glucose-6-P) and conversion to fructose-1,6-bisphosphate (Fructose-1,6-bisP) proceeds identically in the Crabtree-positive and -negative yeasts. *K. lactis* as a Crabtree-negative yeast subsequently degrades fructose-1,6-bisphosphate directly to pyruvate and further to Acetyl-CoA in the mitochondria before it enters the tricarboxylic acid cycle (TCA cycle) and the respiratory chain. This pathway requires oxygen and pyruvate is fully degraded to carbon dioxide (CO_2) while energy is built in the form of ATP. Ethanol, in turn, can be converted to acetaldehyde by alcohol dehydrogenase (Adh) in the presence of oxygen. The Crabtree-positive *S. cerevisiae* can thereby enter alcoholic fermentation even in the presence of

oxygen using fermentation and respiration pathway simultaneously. Ethanol accumulates and is recycled for ATP production [257, 262].

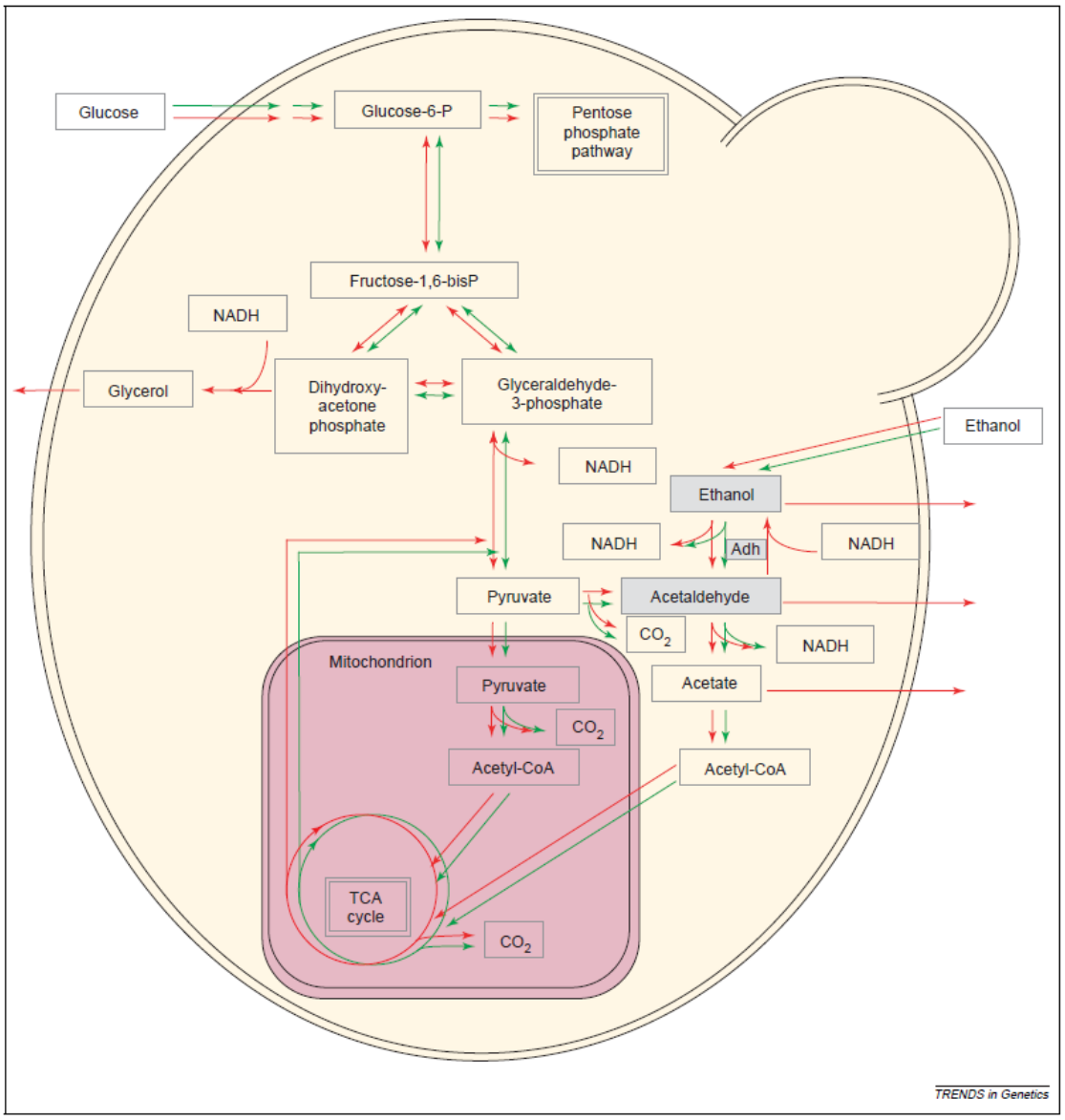


Figure 1. Different metabolism pathways under aerobic conditions of *Saccharomyces cerevisiae* (red) as Crabtree-positive yeast and *Kluyveromyces lactis* (green) as Crabtree-negative yeast according to Piškur et al. [262]. *S. cerevisiae* is able to accumulate ethanol whereas *K. lactis* degrades hexoses in a straight line to adenosine triphosphate (ATP) and carbon dioxide (CO_2).

Emil Christian Hansen, an employee of the Carlsberg Laboratory in Copenhagen, set a decisive milestone for the brewing industry by isolating a single cell of *S. pastorianus* (formerly *Saccharomyces carlsbergensis*) for the first time in 1883. This yeast strain was then used for the production of lager beer [22]. In fact, today, lager beers fermented with *S. pastorianus* yeast strains account for 90% of beers produced worldwide [118], and *Saccharomyces* yeasts are applied in general as starter cultures in about 99% of the commercially available beers all over the world [17]. Therefore, in the context of beer brewing, *Saccharomyces* yeasts play the dominant role. The two main species mentioned are traditionally designated, clustered by their flocculation behavior or flotation (yeast containing foam) and their applied fermentation temperatures [57, 144, 280]. *S. cerevisiae* is mainly used for the production of top-fermented ale and weiss beers while *S. pastorianus* is applied for the fermentation of

bottom-fermented lager beers [118, 154, 191]. At the same time, flocculation is another reason for the domestication of *Saccharomyces* yeasts, as it enables the cells to adhere to each other and form aggregates that sediment quickly. This is both a technologically simple and economically cost-effective way to separate cells from beer in the context of industrial beer production. The flocculation behavior depends on numerous external factors such as nutrient conditions, dissolved oxygen, pH, fermentation temperature and storage conditions of the yeast [340]. Moreover, the flocculation genes of the individual yeast strains play a crucial role, so the flocculation varies strongly among different *S. cerevisiae* strains [65, 342]. In addition to flocculation behavior, *Saccharomyces* yeasts can also be subdivided according to growth and fermentation temperature. Optimal growth and fermentation temperatures for *Saccharomyces* yeasts are generally between 25 and 30 °C. Nevertheless, the bottom-fermenting yeast *S. pastorianus* is traditionally applied at 4–15 °C due to its cryotolerance [120], while the temperature for the top-fermenting yeast *S. cerevisiae* is adjusted to 14–25 °C [57].

As the starter cultures in industrial processes are therefore very well defined in today's industrial brewing processes, the variety is limited [314]. Although the use of defined starter cultures and thus the limited variety offers the advantage of consistent, standardized processes with maximum efficiency on an industrial scale, a reduced variety of flavors is a negative consequence [118, 191, 235, 262, 314]. To overcome this disadvantage, the microbial diversity from traditional fermentations with a wide spectrum of flavors can serve as a model and can be combined with the high technological knowledge and standardized processes from modern brewing.

1.2 The return of non-*Saccharomyces* yeasts in beer brewing

Yeasts can produce flavors directly, but they can also produce flavor-active substances from odorless precursors in the medium during fermentation [314]. To increase flavor diversity during the fermentation process, investigations have been carried out in recent years using a variety of non-*Saccharomyces* yeasts to produce regular beers containing alcohol, LAB and NAB. This often involved the use of non-*Saccharomyces* yeast species already known from traditional spontaneous food and beverage fermentations. Cocoa, for example, is still spontaneously fermented today, and there are also some wines being produced by spontaneous fermentation [314]. Although the commercialized beer market operates with a limited selection of domesticated *Saccharomyces* yeasts, as described in section 1.1, beers are still being produced by so-called spontaneous fermentation today. In Africa, for example, many traditional beers, mainly based on sorghum, are spontaneously fermented with a variety of different yeasts and bacteria [17, 133]. *S. cerevisiae* often dominates, while lactic acid bacteria represent the second most prevalent category [17, 39]. Nevertheless, non-*Saccharomyces* yeasts play a crucial role in the flavor of the beers. Some yeast species identified from traditional African beers are characterized in more detail in the following section 1.2.1. These species are given different names depending on their region of origin in Africa. Even in Europe it is possible to find beers that are traditionally produced by spontaneous fermentation. Belgium lambic and geuze beers, for example, are traditionally produced by means of spontaneous fermentation, and so-called "coolship ales" from the USA are also subject to spontaneous fermentation. The term "lambic" stands for a spontaneous fermentation process by microbes (yeasts and bacteria) present in the brewery. Both *Saccharomyces* and non-*Saccharomyces* yeasts play a crucial role during the spontaneous fermentation of lambic beers. At the beginning of fermentation there is great microbial diversity of bacteria and yeasts. During the main fermentation, *Saccharomyces* spp. are active before *Brettanomyces* spp. gain predominance during maturation [39, 40, 46, 57]. *Brettanomyces* spp. are commonly found in spontaneous beer fermentations to produce Belgian lambic or Berliner Weisse [215]. The yeast species is responsible for the formation

and release of specific volatile flavor compounds such as ethyl esters, fatty acids and phenolic substances that give the beers their characteristic flavor [186]. *Brettanomyces* yeasts possess the ability to degrade dextrin via the enzyme *α*-glucosidase to produce highly attenuated beverages [314]. Additionally, *Brettanomyces* possesses glycoside hydrolase activities and is therefore capable of releasing glycosidically bound flavor precursors from hops, which are subsequently converted into a flavor-active compound [76].

In recent years, traditionally produced, spontaneously fermented beers have once again become the model for creating novel, more complex, and improved flavor profiles in brewing using non-*Saccharomyces* yeasts and thereby generating innovative products and new beer styles [23, 55, 57, 314]. Due to the often low fermentation yields when using non-*Saccharomyces* yeasts for brewer's wort fermentation, the yeasts are ideally suited to producing LAB and NAB [57]. Beyond the more complex flavor and ethanol reduction in beer, non-*Saccharomyces* yeasts have potential for the production of low-calorie beers, functional beers, and bioflavoring approaches due to their various different enzymatic activities although this is still an under-explored field in brewing practice. In contrast, the use of non-*Saccharomyces* yeasts for the production of sour beers, such as American coolship ales, is already widespread. *Dekkera*/*Brettanomyces* are used for these purposes as they are known from Belgian lambics and are perfectly suitable for co-fermentations with other microbes, such as acetic acid or lactic acid bacteria [23, 40, 74].

1.2.1 Overview of non-*Saccharomyces* yeasts investigated in this thesis

In this thesis, 20 different non-*Saccharomyces* yeast species were investigated for their suitability for beer brewing with emphasis on their flavor formation. The literature sources depicted in Table 1 initially served as a knowledge basis for the selection of the yeast species and were further completed in the course of this work. When selecting the yeast species, it was decisive that they were already known from other existing food or beverage fermentations for reasons of safe use. Most yeast species have already been tested for beer brewing, so this was the focus of the literature research. Often, the appointed yeasts species have already been applied in several food or beverage fermentations. However, existing literature references are only mentioned beyond brewing in Table 1 if multiple applications were covered in one study or if there were no studies on brewing applications. If no studies on brewing applications could be found, studies into wine/rice wine and cider were considered. Table 1 thus provides a literature overview of the yeast species investigated in this dissertation and states whether they have already been studied in beer, wine/rice wine and/or cider.

Table 1. Investigated yeast species of this thesis with a focus on brewing application and related literature references.

Yeast species	Application	Literature
Cyberlindnera fabianii	Beer, rice wine	[30, 156, 187, 330, 331]
Cyberlindnera misumaiensis	Beer, cider	[30, 71, 173]
Cyberlindnera saturnus	Beer, wine	[30, 94, 182, 189, 242]
Debaryomyces hansenii	Beer, wine	[44, 119, 242, 284, 308, 323]
Hanseniaspora uvarum	Beer, wine, cider	[5, 9, 119, 139, 156, 270]
Kazachstania servazzii	Beer	[119, 156]
Kluyveromyces lactis	Beer, wine, cider	[119, 127, 163, 242, 329]
Kluyveromyces marxianus	Beer	[119, 156, 198, 225, 242]
Lachancea kluyveri	Wine	[157]
Metschnikowia sp.	Beer, wine, cider	[21, 93, 147, 242, 269, 270, 273]

Nakazawaea holstii	Wine	[83]
Pichia kluyveri	Beer, wine, cider	[6, 127, 139, 157, 236, 274]
Saccharomycodes ludwigii	Beer, wine, cider	[31, 43, 50, 87, 95, 103, 118, 127, 156, 207, 242, 256, 270, 314, 335]
Saccharomycopsis fibuligera	Rice wine, liquor	[3, 183, 187, 350, 352]
Schizosaccharomyces pombe	Beer, malt extract, wine	[32, 50, 188, 295, 335]
Torulaspora delbrueckii	Beer, malt extract, wine	[11, 50, 53, 54, 56, 66, 77, 93, 119, 139, 147, 156, 163, 219, 295, 316, 317, 321, 322]
Torulaspora microellipsoides	Beer, wine	[112]
Wickerhamomyces anomalus	Beer, wine	[52, 55, 66, 147, 242, 295, 308]
Zygosaccharomyces rouxii	Beer	[77, 103, 112, 304]
Zygotorulaspora florentina	Beer, wine	[55, 87, 139, 244]

Table 1 reveals that there are several studies related to brewing for some yeast species, while other species are only mentioned sporadically in the context of beverage fermentations. In the following, the 20 yeast species are each briefly characterized by providing an overview of the food and beverage fermentations in which they have been found or applied to date. The focus is on beverage fermentations and initial indications of flavor development are given. A more detailed overview of the flavor formation during fermentation of brewer's wort can be found in section 1.4.3. In addition, a brief insight is given into the clinical relevance, since a safe use of yeasts for food fermentation from a health perspective is critical. In section 1.4.4, this topic is addressed in more detail. The synonyms of the following yeast species as well as basic information and clinical importance are based on "The Yeasts Database" [38]. In general, based on the classification of fungi into risk groups in the Technical Rules for Biological Agents (TRBA 460) published by the Federal Institute for Occupational Safety and Health, all yeasts considered below belong to the risk group 1, unless otherwise indicated. A classification in risk group 1 means that the yeast is unlikely to cause disease in humans. For individual yeast species, additional comments from TRBA 460 are given where indicated [48].

Cyberlindnera fabianii

Formerly known as *Pichia fabianii*, *Hansenula fabianii* or *Lindnera fabianii,* the yeast species *Cyberlindnera fabianii* was first isolated in 1942 as a contaminant from industrial food fermentations. In 1948, *C. fabianii* was isolated from a fermentation plant where it was already used for flavor formation by adding esters to alcoholic beverages [347]. Traditionally, *C. fabianii* is used in fruit juice fermentation to produce masau from *Ziziphus mauritiana* (Indian jujube) in Zimbabwe [245]. Furthermore, the yeast species can also be found in traditional loog-pang-lao, which is a starter for the production of rice wine [187]. With regard to rice wine, *C. fabianii* is also known as dried yeast starter [30]. Recently, the yeast species was even isolated from rye malt sourdough [156] and is described as being a typical sourdough resident [344]. Several brewing trials in brewer's wort have been conducted with *C. fabianii* in monocultivation as well as in co-cultivation with *S. cerevisiae* [30, 156, 331]. The co-cultivation with *S. cerevisiae* resulted in a more complex beer flavor as *C. fabianii* produces more esters than *S. cerevisiae* in a direct comparison despite its limited fermentation capacity, resulting in a lower ethanol concentration [330, 331]. *C. fabianii* does not possess the metabolic properties to grow in the absence of oxygen [331]. Although there are several studies into human infections, the clinical importance seems to be low as *C. fabianii* is reported to rarely cause human infections [35, 360, 362]. TRBA 460 also states that *C. fabianii* is detected or suspected as a pathogen in individual cases. However, cases of illness usually

occur only in immunocompromised individuals. However, it is pointed out that identification of the species is often unreliable [48].

Cyberlindnera misumaiensis

Cyberlindnera misumaiensis, also found under its synonyms *Pichia misumaiensis*, *Hansenula misumaiensis* and *Lindnera misumaiensis* was first described in an abstract as *H. misumaiensis* by Sasaki and Yoshida in 1958 although a valid description was never published [174]. To date, studies in the food fermentation field on this yeast species are rare. It is only known that *C. misumaiensis* was isolated from apple must/cider [71, 173] and that first brewing trials were carried out in brewer's wort [30]. Due to the limited data availability, its clinical importance is unknown. *C. misumaiensis* is not listed in the TRBA 460.

Cyberlindnera saturnus

Cyberlindnera saturnus, further called *Saccharomyces saturnus*, *Willia saturnus*, *Hansenula saturnus*, *Williopsis saturnus* or *Lindnera saturnus* has the ability to produce esters, especially isoamyl acetate during fermentation. In 2008, the production of isoamyl acetate was detected during fermentation of sugar beet molasses [354]. Two years later, *C. saturnus* was used for the production of papaya wine. During fermentation, a number of volatile compounds were formed, including fatty acids, alcohols, and esters, with esters being the most dominant [182]. During the same year, grape must for wine production was fermented using *C. saturnus* in co-cultivation with *S. cerevisiae*. The concentrations of acetic acid, ethyl acetate and isoamyl acetate were higher in the co-cultivation experiments compared to the control fermentation, which was carried out with *S. cerevisiae* only. [94]. Brewing trials were also carried out in recent years with *W. saturnus* var. *mrakii* [189], which is now known as *C. mrakii* [30], to investigate the flavor formation during fermentation. The clinical importance for this yeast species is unknown. *C. saturnus* is not listed in the TRBA 460.

Debaryomyces hansenii

To be found among the synonyms *Saccharomyces hansenii, Debaryozyma hansenii*, *Pichia hansenii*, *Torulaspora hansenii*, *Debaryomyces tyrocola* var. *hansenii*, *Debaryomyces hansenii* is one of the most abundant yeast species in dairy products, especially on the surface of cheese [104, 105], in meat products [44, 264] and during the fermentation of soy sauce [306]. In addition, *D. hansenii* has been reported to play a role in the fermentation of sauerkraut [284] as well as fruits, wine, and beer [44]. In beer brewing, *D. hansenii* occurs in traditional spontaneously fermented lambic beers but does not take a predominant role [308].As a starter culture of “Tchoukoutou”, a Beninese African sorghum beer, *D. hansenii* is also present during mixed fermentation along with further microbes although the species could only be detected in low concentrations of less than 5%. In Tanzania, a similar beer is traditionally brewed which is called “pombe” [323]. *D. hansenii* was already investigated in brewer’s wort. However, no significantly high concentrations could be found among the analyzed flavor substances 3-methylbutanol, 2-phenylethanol, 3-methylbutyl acetate, 2-phenylethyl acetate, ethyl caprylate and ethyl decanoate [119]. Nevertheless, a distinctive feature of this yeast species is its osmotolerance and ability to grow in media with up to 4 M (Molar) sodium chloride (NaCl) [44]. The clinical importance of *D. hansenii* is unknown.

Hanseniaspora uvarum

Previously called *Kloeckeraspora uvarum* or *Kloeckera apiculata*, *Hanseniaspora uvarum* appears in quite a broad range of different food fermentations. The yeast species is especially known for its use in winemaking. During the production of wine with further microbes, *H. uvarum* was described as a

major non-*Saccharomyces* yeast during fermentation. The yeast species possesses enzymatic activity, which can be of advantage for wine production due to technological effects. The relevant enzymes are pectinases, proteases and glycosidases, which can be released during fermentation to improve clarification, stabilization and flavor. Hence, the yeast also contributes significantly to the formation of wine flavor. A desirable increase in esters, especially acetate esters in mixed fermentations and an increased production in fusel alcohols and ethyl esters (C6-C10) have been reported [139]. A possible disadvantage is the formation of acetic acid in higher concentrations during fermentation [5, 139]. Still, the formation of acetic acid is most likely to be strain specific as suggested in a study by Moreira et al. [229] it was found that only the concentration of acetate esters increased in co-cultivation with *S. cerevisiae*, while fusel alcohols and ethyl esters decreased [139]. Besides grape must fermentation, *H. uvarum* is moreover involved in the fermentation of apple must to produce cider. Thus, the yeast species has already been isolated from the cider industry and is known as indigenous Asturian cider yeast [270]. During the fermentation of apple must, *H. uvarum* positively contributes to the flavor formation by producing appreciable amounts of esters and higher alcohols [9]. Furthermore, the yeast is known from coffee bean [202] and from sourdough [156, 344] fermentations. Given its versatile fermentation capabilities, it is unsurprising that *H. uvarum* has been tested multiple times in brewer's wort fermentations [119, 156, 270]. It should be emphasized that the yeast species was described as having the ability to decarboxylate ferulic acid to 4-vinyl guaiacol (4-VG) [156]. Regarding the clinical importance, infections were very rarely reported. Garcia-Martos et al. reported an isolation of *H. uvarum* from clinical material (stool and infected nails) in three cases [116]. *H. uvarum* is not listed in the TRBA 460.

Kazachstania servazzii

Only limited information exists in literature about *Kazachstania servazzii* (synonym *Saccharomyces servazzii*) in the context of food and beverage fermentations. It has been described that the yeast species has an important role in creating the flavor of Japanese radish pickles during fermentation [324] and it is typically present in sourdoughs [156, 344]. Meanwhile, *K. servazzii* has been tested for the fermentation of brewer's wort and revealed promising results. During pilot fermentations in co-culture with *S. cerevisiae*, tasters described an enhanced fruity character [119]. The clinical importance of *K. servazzii* is unknown. *K. servazzii* is not listed in the TRBA 460.

Kluyveromyces lactis

Kluyveromyces lactis is known by a variety of synonyms, so they will not be fully enumerated here. Among others, the yeast species can be found under the synonyms *Torulaspora lactis* and *Saccharomyces lactis*. *K. lactis* is regularly present in mold surface-ripened cheese. When grown in a cheese model medium, this species appears to produce fruity notes [329]. By fermenting a culture medium (broth) containing 5% glucose, 0.25% yeast extract and 4000 ppm of vitamins B_1 and H_1, *K. lactis* formed 73 mg/L isoamyl alcohol, 72 mg/L 2-phenylethanol and 22 mg/L acetoin. Moreover, 2-phenylethyl acetate, isobutanol, isobutyric acid and isovaleric acid could be measured in significant amounts [155]. Studies in brewer's wort [119], grape and apple must were also conducted and promising pleasant flavor substances were perceived in beer as well as in wine and cider [127]. It was further reported that *K. lactis* can transform several monoterpene alcohols from beer and wine and synthesizes, for example, citronellol and linalool from geraniol [163]. Its clinical importance is unknown.

Kluyveromyces marxianus

As for *K. lactis*, there are numerous synonyms for *Kluyveromyces marxianus*, which are not listed here. The synonym *Saccharomyces marxianus* is commonly found in older literature references. Basically, *K. marxianus* possesses a high biotechnological potential. The yeast species is described as producing

different enzymes, single-cell protein, flavor compounds, ethanol and can reduce lactose among other compounds. It tolerates a broad substrate spectrum as well as a broad spectrum of temperatures, reveals high growth rates, and is less prone to fermentation when exposed to excess sugar [101]. *K. marxianus* can be found predominantly during the souring phase of the fermentation process of "bili bili", which is a traditional sorghum beer from Chad [198]. Furthermore, the yeast species was isolated from traditional opaque beer beverages brewed in Zimbabwean households [225]. There are also initial studies with *K. marxianus* for the fermentation of brewer's wort [119, 156]. Further studies also link *K. marxianus* to a wide variety of food and beverage fermentations. For instance, the yeast species is described as typically occurring in sourdoughs [344] and it was found in rye and in barley sourdough [156]. Moreover, studies have been conducted on how *K. marxianus* influences cocoa flavor during co-fermentation with other microorganisms [73]. Further flavor studies were conducted in cassava bagasse and in a semi-synthetic medium. During solid state fermentation with cassava bagasse as the growth medium, ethyl acetate and acetaldehyde were the major compounds produced [203] while investigations in semi-synthetic medium led to isoamyl alcohol, 2-phenylethyl alcohol and isobutyric acid formation [97]. Regarding the clinical importance, *K. marxianus* was only infrequently isolated in clinical material leading to vaginitis [117].

Lachancea kluyveri

Formerly known as *Saccharomyces kluyveri* or *Torulaspora kluyveri*, there is hardly any information on *Lachancea kluyveri* regarding food and beverage fermentations. The yeast species was only reported on grapes and in wine fermentations [157] while its clinical importance is unknown.

***Metschnikowia* sp.**

In the context of this thesis, the investigated *Metschnikowia* yeast strains could not be clearly assigned to a species. The reason for this is that *Metschnikowia* sp. cannot be unambiguously assigned to a species using standard genetic methods (ITS and/or 26S sequencing), as there are too few polymorphisms [143]. To find out the exact species, further housekeeping genes would have to be added for sequencing. As in many beverage fermentations, *Metschnikowia pulcherrima* is the main species reported and is described here. It can be found in spontaneous grape must fermentations due to its natural occurrence on grapes and predominates in the first stages of fermentation until an ethanol concentration of 3-4% is reached [269]. The enological potential of *M. pulcherrima* as a starter culture was tested in several studies as mono- and co-cultivations with *S. cerevisiae* and a synergistic effect on the flavor formation was reported when fermenting in co-cultivations [21, 147, 273]. Additionally, *M. pulcherrima* was found as an indigenous yeast in cider and was investigated for its potential for brewing beer. The result was a low fermentation capacity and comparatively low production of volatile compounds (phenols, esters) and acids in comparison to other applied non-*Saccharomyces* yeasts [270]. As with most of the other yeast species discussed in this thesis, the clinical importance for *M. pulcherrima* is unknown.

Nakazawaea holstii

Nakazawaea holstii (synonyms: *Pichia holstii*, *Hansenula holstii*, *Endomycopsis holstii*) could be identified during the fermentation of sauerkraut [284] and in Cornalin musts during spontaneous wine fermentation [83]. Apart from this, data is poor within the context of food and beverage fermentations. The clinical importance of *N. holstii* is also unknown.

Pichia kluyveri

Previously known as *Hansenula kluyveri*, *Pichia kluyver*i takes part in a wide variety of food and beverage fermentations. The yeast species was identified in “tchapalo“, a traditional sorghum beer from Côte d’Ivoire, however, it seems to play a subordinate role as it is not regularly found during this fermentation process [236]. Nevertheless, *P. kluyveri* is highly suited to producing LAB or NAB as it only ferments glucose and fructose while simultaneously releasing high amounts of desirable flavors [274]. Meanwhile, it has been tested in further brewing trials for the production of beer where it significantly enhanced the fruitiness [139]. For the flavor evaluation in fermentations of wine, beer and cider, an olfactory "sniff" test was performed on plates of glucose wort, grape must and apple juice. A strong, pleasant banana flavor was detected, which can be attributed to isoamyl acetate. Furthermore, there was a notable formation of acetate esters, phenyl ethyl acetate (rose, floral), butyl acetate and hexyl acetate [127]. Basically, *P. kluyveri* is known to occur during the fermentation of wine [6, 83, 157]. It is able to produce significant amounts of the volatile thiols 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexan-1-ol acetate (3MHA) in Sauvignon Blanc wine [6]. Moreover, *P. kluyveri* plays a role during cocoa and coffee bean fermentation. Crafack et al. reported that the cocoa flavor was influenced positively to reveal a sweet, fruity cocoa flavor when integrating *P. kluyveri* in the fermentation process [73]. During coffee bean fermentation of Arabica coffee, the yeast species was beneficial due to its pectin-degrading enzymes [202]. The clinical importance of *P. kluyveri* is unknown. *P. kluyveri* is not listed in the TRBA 460.

Saccharomycodes ludwigii

Saccharomycodes ludwigii is already used commercially to produce NAB in the industry [31, 43, 118]. As early as 1933, there was a patent for the production of a beer with low alcohol content by using *S. ludwigii* for fermentation [128]. Kunz and Methner also mentioned *S. ludwigii* in a 2009 patent describing a process for producing a non-alcoholic malt-based beverage, which could also be a NAB [172]. In 1992, Narziss et al. conducted a study comparing a beer produced with limited fermentation against a beer fermented with *S. ludwigii*. The beer produced with *S. ludwigii* revealed comparatively higher concentrations of esters and higher alcohols. With the aid of biological acidification, the pH value was decreased to 4.6, and off-flavors such as diacetyl or wort off-flavors could be slightly masked [241]. In recent years, numerous studies have been performed on the production of NAB by using *S. ludwigii* [50, 103, 156, 207, 256, 314, 335]. The potential of producing beers with desirable flavors was stated [205], however, the sensory profiles can differ based on the specific yeast strain selected [103]. Accordingly, there have been approaches to isolate *S. ludwigii* as an indigenous yeast for the fermentation of cider and this was subsequently applied in beer brewing [270]. Apart from investigating the fermentation performance of *S. ludwigii* in brewing, the yeast species was evaluated for the fermentation of cider and wine as a mono- or co-starter culture [87, 95, 127]. *S. ludwigii* does not appear to have any clinical importance. The growth of this species is weak or absent at 37 °C. Therefore, it is unlikely to cause human infections [41]. *S. ludwigii* is not listed in the TRBA 460.

Saccharomycopsis fibuligera

Endomyces fibuliger, *Pichia fibuliger*, *Endomycopsis fibuliger* and *Saccharomyces fibuliger* are synonyms for *Saccharomycopsis fibuligera*. Traditionally, this yeast species is used for fermented masau (*Ziziphus mauritiana*) fruit juice in Zimbabwe like *C. fabianii* [245]. *S. fibuligera* is a major amylolytic yeast in indigenous food fermentations which are based on starchy substrates like rice or cassava [136]. It was isolated from nuruk, which is a traditional microbial mixed-strain starter that is primarily cultivated on rice, wheat, barley, and rye [158]. As an amylolytic fermentation starter, it is used to ferment

rice for the traditional Indonesian food “ragi” and the traditional Thai “Loog-pang”. Due to its amylolytic properties, *S. fibuligera* is used to produce Indonesian sweet, low-alcoholic snacks called Tapé ketan, Tapé ketella/peujeum that are based on rice and cassava tubers. Moreover, it is involved in the fermentation of rice-based alcoholic beverages: Tapuy from the Philippines, Jnard/Jaanr/Thumba from Nepal/India and Bhutan based on finger millet/rice/maize/wheat. *S. fibuligera* ferments saccharified rice starch to alcohol by releasing amylolytic enzymes [3]. Still, the production of ethanol remains at relatively low levels [187]. However, the yeast is characterized not only by its amylolytic abilities, but also its property of flavor enhancement. A study has been carried out to improve the flavor of sweet rice wine where ethyl butanoate, ethyl hexanoate, phenyl ethyl alcohol and 1-octen-3-one reached high OAVs (odor activity values) and were consequently found responsible for the key flavors [352]. The quality of liquor could also be improved as *S. fibuligera* synthesizes flavors (mainly esters) and alcohols during fermentation [350]. In yeast extract-peptone-dextrose (YPD) cultivation medium containing glucose, 2-phenyl ethanol, 2-phenylethyl acetate, and ethyl phenyl acetate, were mainly detected [183]. Recently, even a potential for coffee bean fermentation was mentioned due to pectinase enzyme production of *S. fibuligera* [130]. Its clinical importance is unknown. *S. fibuligera* is not listed in the TRBA 460.

Schizosaccharomyces pombe

First isolated from an ancient beer called “pombe” by Lindner in 1893 [188], today, *Schizosaccharomyces pombe* is still one of the main flavor-contributing species in many mixed fermentations of traditional African beers. “Pombe” means beer in the Swahili language [50]. The traditional beer “pito” in Ghana is prepared from sorghum grains (*Sorghum bicolor* (L) Moench, *Sorghum vulgare*) [285, 295]. Its traditional history from ancient and African beer brewing makes it a promising candidate for further investigations. Callejo et al. found out that *S. pombe* is able to metabolize malic acid across the maloalcoholic fermentation pathway with ethanol and CO_2 as final products instead of lactic acid, which is the final product in bacterial malolactic fermentation [50]. *S. pombe* can be used to produce beers containing alcohol [50]. However, studies in which *S. pombe* was used for the production of NAB have also been described in literature [335], which indicates a strain-dependent carbohydrate utilization. *S. pombe* has also been investigated for the fermentation of red wine. It produced less urea and more pyruvic acid than *Saccharomyces* sp. while selected strains broke down malic acid completely. Strong acetic acid production is judged as negative in terms of red wine quality. Furthermore, yeast strains that produced relatively more pyruvic acid were associated with a more favorable color of the red wine [32]. Apart from beer and wine, *S. pombe* was reported to be present in Kombucha together with other yeast species [318]. As for most other yeasts investigated in this thesis, the clinical importance for *S. pombe* is unknown.

Torulaspora delbrueckii

Formerly known as *Saccharomyces delbrueckii*, *Torulaspora delbrueckii* was first assumed to be a suitable yeast strain for beer brewing by King and Dickinson in 2000 [163]. A year earlier, *T. delbrueckii* was mentioned in relation to the traditional brewing of pito made from sorghum or millet in Ghana [295]. Thus, the barley-sorghum craft beer production has already been studied [93]. Regarding the research on *T. delbreuckii* in the context of the suitability for beer brewing, a study in the journal *Yeast*, which was conducted by Hutzler's research group, was pioneering [219]. Based on this, numerous further studies have been conducted in the context of beer brewing with mono- and co-culture fermentations with *S. cerevisiae* with reported outcomes that the fermentation performance of *T. delbrueckii* strongly depends on the strain as selected ones are able to metabolize maltose from brewer’s wort

[31, 53, 54, 56, 66, 119, 139, 156, 220, 316, 317, 321, 322]. Co-inoculation with *S. cerevisiae* led to an enhanced flavor profile of the beer [321]. This could be attributed to an increase in aromatic compounds such as ethyl hexanoate, *α*-terpineol and 2-phenylethanol emphasizing fruity and citrus notes with no reported off-flavors [54]. In the same way, *T. delbrueckii* was investigated in winemaking as mono- and co-cultivation with *S. cerevisiae*, forming fresh flower and fruity flavors when applying the mono-culture. A significant production of organic acids and 2-phenyl ethanol could be analyzed while acetic acid and glycerol stayed within acceptable levels. A greater aromatic intensity and complexity could be recognized in co-fermentation than from mono-culture fermentation [11, 147, 317]. In addition, *T. delbrueckii* is typically found in sourdoughs and was isolated from rye and rye malt sourdough [156, 344]. A distinctive feature is that *T. delbrueckii* possesses a high concentration of intracellular trehalose which enhances survival under environmentally stressful conditions [77]. There is a minor clinical importance for this species though it was isolated from a skin lesion [61] and the anamorph *Candida colliculosa* was reported to have caused tricuspid valve endocarditis [162].

Torulaspora microellipsoides

Torulaspora microellipsoides (synonyms: *Saccharomyces microellipsoides*, *Zygosaccharomyces microellipsoides*) was described as intensively producing isobutyl and isoamyl alcohols in a standard growth medium [113]. Nevertheless, it is questionable whether the yeast species is suitable for beverage fermentations. Dashko et al. concluded in their study about winemaking that *T. microellipsoides* showed poor fermentation dynamics due to an inefficient sugar consumption and low ethanol production levels while simultaneously exhibiting major organoleptic defects [78]. However, during microwine production, which was conducted on a microscale in 24-well microplates each containing 5 mL of must, it was possible to detect phenylethyl alcohol levels above the threshold level, which could have a positive flavor impact [112]. In addition, *T. microellipsoides* was examined for the fermentation of cashew apple juice [114]. The clinical importance of this yeast species is unknown. *T. microellipsoides* is not listed in the TRBA 460.

Wickerhamomyces anomalus

Previously named *Saccharomyces anomalus*, *Endomyces anomalus*, *Pichia anomala*, *Willia anomala*, *Hansenula anomala*, *Wickerhamomyces anomalus* takes part in a wide range of food and beverage fermentations. With regards to beer brewing, it was found during the fermentation of traditional pito in Ghana [295]. Moreover, *W. anomalus* plays a role in traditional spontaneously fermented lambic sour beers at a later stage of six months fermentation duration [308] and has already been investigated in the process of beer brewing [55, 66]. *W. anomalus* was further assessed to be promising for winemaking due to its wide range of enzymatic activities, high stress tolerance, killer activity, and utilization of organic acids [147]. During winemaking in sequential inoculation with *S. cerevisiae* fruity flavors could be enhanced resulting from higher levels of acetates and ethyl esters. Higher levels of lineal alcohols caused herbaceous notes whereas lower concentrations of organic acids led to an increase in the aromatic quality compared to mono-culture fermentation with only *S. cerevisiae*. The wines from the mixed fermentation were preferred in the sensory evaluation due to the fruity and floral notes [52]. In addition, *W. anomalus* contributes to cocoa bean fermentation [165] and can be found in sourdoughs [344] while investigations on coffee bean fermentation revealed a production of the enzyme pectinase [130, 131]. Although the importance of *W. anomalus* in the context of food and beverage fermentations is extensive and there is a traditional use, reports from clinical specimens can be commonly found in literature, for example fungal keratitis [160]. TRBA 460 describes a possible allergenic

effect for *W. anomalus*. However, this is not synonymous with a higher sensitizing potential compared to other species [48].

Zygosaccharomyces rouxii

Also known by the designations *Saccharomyces rouxii* and *Torulaspora rouxii*, *Zygosaccharomyces rouxii* was identified as an indigenous yeast from traditional balsamic vinegar [305]. In recent years, the yeast species was investigated in several studies for its potential to ferment brewer's wort mainly in the context of LAB and NAB. However, Francesco et al. report that most of their applied *Z. rouxii* strains produced ethanol concentrations above 1.2% (*v*/*v*) in a wort with 12 kg/hL original gravity, consequently exceeding the ethanol limit for NAB. A reduction in the original gravity would therefore be necessary in order for it to be eligible for the production of NAB. Previous reports from Sohrabvandi et al. demonstrate the successful application of *Z. rouxii* in the production of NAB. They used *S. cerevisiae* and then *Z. rouxii* during the fermentation and even outlined a decrease in off-flavors with *Z. rouxii*. [103, 304]. Aside from brewing, *Z. rouxii* was also investigated in relation to winemaking [112] and it is known from soy sauce production where the yeast species produces flavor-active substances, such as esters and higher alcohols while forming few undesired acids [181]. A further advantage is the high concentration of intracellular trehalose of *Z. rouxii,* which enhances its survival under environmentally stressful conditions [77]. Further physiological aspects can be found in a review by a research group of the Technical University Munich, Germany [220]. The clinical importance of the yeast species is unknown.

Zygotorulaspora florentina

Zygotorulaspora florentina (synonyms: *Zygosaccharomyces florentinus*, *Torulaspora florentina*) is known from the fermentation of grape must where it was described as forming low concentrations of acetaldehyde and ethyl acetate. In contrast, during mixed fermentations with *S. cerevisiae*, 2-phenyl ethanol which causes a desirable floral, rose flavor and phenyl ethyl acetate seemed to strongly relate to *Z. florentina* [87]. Holt et al. studied a *Z. florentina* yeast strain, which was isolated from grape must, for the fermentation of brewer's wort and discovered beers with a phenolic profile [139]. Although Nikulin et al. detected phenolic compounds in beer fermented with *Z. florentina* as well, they could not be perceived during sensory evaluations [244]. Another finding was that the lactic acid concentration of beer fermented with *Z. florentina* was higher compared to the reference *S. cerevisiae* [55]. There is no literature on the clinical importance. *Z. florentina* is not listed in the TRBA 460.

1.2.2 Further non-*Saccharomyces* yeasts investigated for beer brewing

Apart from the 20 investigated yeast species in this thesis, there are a large number of further studies in which numerous other yeast species were examined for their suitability for beer brewing or in a broader beer-related context. Since there is a clear focus on the 20 characterized yeasts, only a brief overview of literature sources is listed, in which other yeast species were discussed against the background of beer brewing:

- *Barnettozyma californica* isolated from fruit [112]
- *Candida ethanolica* [323]
- *Candida glabrata* [225]
- *Candida membranifaciens* isolated from fibre pulp [119]
- *Candida tropicalis* [236, 295]
- *Candida zemplinina* [56]
- *Cyberlindnera jadinii* isolated from fruit of *Solanum quitoense* [30]

- *Cyberlindnera subsufficiens* isolated from coconut and soil [30]
- *Eromothecium coryli* [119]
- *Galactomyces geotrichum* [127] isolated from soil [112]
- *Hanseniaspora guilliermondii* isolated from juice [112]
- *Hanseniaspora osmophila* [270]
- *Hanseniaspora valbyensis* isolated from Kombucha [31]
- *Hanseniaspora vineae* isolated from Kombucha [31], isolated from plant [112]
- *Issatchenkia occidentalis* [225]
- *Kazachstania gamospora* [112]
- *Kazachstania zonata* [127]
- *Kloeckera apiculata* [295]
- *Kluvyeromyces africanus* [295]
- *Kodamaea ohmeri* [236]
- *Lachancea fermentati* [335] isolated from Kombucha [28], isolated from Kvass [119]
- *Lachancea thermotolerans* [50, 55, 86, 322, 361]
- *Lindnera meyerae* [127]
- *Meyerozyma caribbica* [236]
- *Naumovia dairenensis* isolated from salad [119]
- *Pichia angusta* [335]
- *Pichia fermentans* [156]
- *Pichia kudriavzevii* [323]
- *Rhodotorula* [225]
- *Scheffersomyces stipitus* isolated from insect larva [119]
- *Sporobolomyces holsaticus* [225]
- *Starmera caribaea* [127] isolated from plant [112]
- *Wickerhamomyces subpelliculosus* isolated from sugar [112]
- *Yarrowia lipolytica* [127]
- *Zygosaccharomyces bailii* [56] isolated from Kombucha [31]
- *Zygosaccharomyces kombuchaensis* isolated from Kombucha [31], isolated from fungus [112]
- *Zygotorulaspora mrakki* isolated from silage [119]

In addition to the yeast species listed, several reviews have been published which provide summaries of non-*Saccharomyces* yeasts being investigated for the production of beer. Those that deserve a special mention are reviews of Basso et al. [23] which focus on *Dekkera*/*Brettanomyces*, *W. anomalus* and *T. delbrueckii* as well as a further review [220] summarizing characteristics of *Dekkera*/*Brettanomyces* and *T. delbrueckii* and furthermore *Candida shehatae*, *C. tropicalis*, *S. ludwigii*, *Z. rouxii* and *P. kluyveri*. Bellut et al. [27] focused on *S. ludwigii*, *Candida* spp., *Cyberlindnera* spp., *Pichia* spp., *T. delbrueckii* and *Zygosaccharomyces* spp. for the production of LAB and NAB in addition to further mentioned yeast species.

1.3 Non-*Saccharomyces* yeasts for the production of non-alcoholic beers (NABs)

As there was a focus on NAB in this thesis, the definition of NAB needs to be clear. Within the framework of the legal situation and consumer understanding in Germany, there is no primary regulation for the sales designation of "non-alcoholic beer", but the maximum limit of 0.5% (*v*/*v*) is based on the Weinverordnung §47 para. 1 [233, 346]. In the European Union (EU), the definition of NAB varies. While a majority of states tolerate a maximum limit of 0.5% (*v*/*v*), the range in France and Italy extends

to 1.2% (*v*/*v*) [129]. Above 1.2% (*v*/*v*), the amount of ethanol must be indicated in accordance with Regulation (EU) No. 1169/2011. According to Regulation (European Community) No. 1924/2006, claims regarding positive health effects may only be made for ethanol concentrations below 1.2% (*v*/*v*). The *Mitteleuropäische Brautechnische Analysenkommission* (MEBAK®) specifies that a NAB with an ethanol content of < 0.54% (*v*/*v*) may be voluntarily declared with the claim ≤ 0.5% (*v*/*v*). Once the claim < 0.5% (*v*/*v*) is chosen, it must be ensured that the analyzed extreme value of ethanol is < 0.49% (*v*/*v*) [152]. Only beers with an ethanol concentration of less than 0.05% (*v*/*v*) may be claimed to be 0.0% (*v*/*v*) ethanol [233].

What are the reasons for consuming NAB? From a historical perspective, the First and Second World Wars led to a shortage of raw materials. As a result, the original gravity of beers was significantly reduced resulting in low alcohol concentrations in beer. Furthermore, from 1919 to 1933, a ban was imposed in the United States of America (USA) on the production and consumption of alcohol. This in turn led to an increase in the production of LABs [43, 216, 302]. In the late 20th century, many brewers sought to expand their product range to offer alternative products for specific circumstances, such as athletes, car drivers and pregnant women. There was also an interest in conquering markets where alcohol was banned due to religious restrictions. Due to the fact that alcohol consumption poses health risks, the consumption of NABs scores points by not having a negative impact on health and also ensures a lower calorie intake when directly compared with beers containing alcohol [43]. At the beginning of the product launch of NAB and LAB, they were not immediately accepted by consumers. It was only between 2011 and 2016 that the global market grew by 20% [27]. Sales in the NAB segment will be approximately EUR 28.5 billion worldwide in 2022, and the market is forecasted to grow by approximately EUR 40 billion in 2025, which would correspond to annual sales growth of approximately 12% (CAGR 2022-2025) [312].

The processes for the production of NAB can be fundamentally divided into the two categories of biological and physical production processes [43]. These in turn can be further broken down into subcategories. The most common procedures are presented in Figure 2.

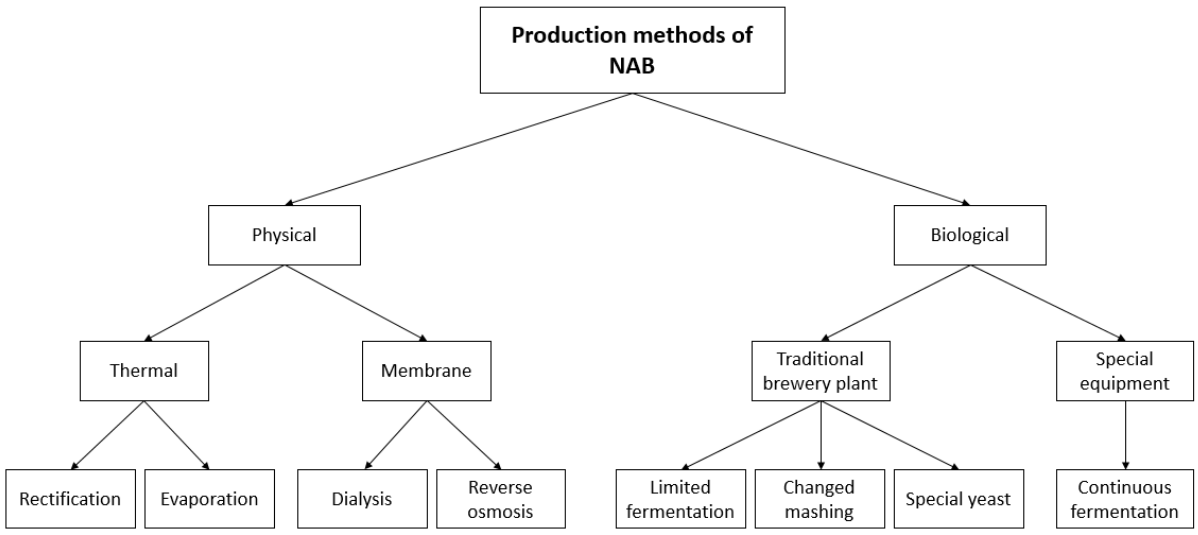


Figure 2. Most common production methods for non-alcoholic beers (NAB). Scheme according to Brányik et al. [43]

In this thesis, only the biological production process was applied. The physical methods will therefore not be discussed further. More detailed information on the physical methods can be found in the following literature sources: [43, 151, 197, 227, 233, 303]. Based on Figure 2, in the biological processes

for the production of NAB, the production of alcohol is limited by working with lower original gravities, which results in fewer fermentable sugars available for the yeasts. Either the mashing process can be modified to reduce the level of fermentable sugars or the fermentation process can be varied. The latter methods can involve: arrested batch fermentation, cold contact method, continuous fermentation (immobilized yeasts) or the use of alternative non-*Saccharomyces* yeast strains [151]. The method of alternative yeast strains is selected in this work.

The technology to produce NAB focusing on the microbiological method has the advantage over the physical methods that no subsequent ethanol reduction is necessary. Hence, it is a simple application for breweries and a cost-effective technology by eliminating the high costs and maintenance required for the equipment of physical production methods [146, 233]. By using selected maltose- and maltotriose-negative non-*Saccharomyces* yeast strains for the production of NAB, their natural fermentation limit in wort can be exploited [31]. Premature removal or inactivation of the yeast, as in the case of arrested batch fermentation, is not necessary. During arrested batch fermentation, ethanol formation is suppressed which leads to undesirable wort flavors caused by remaining wort aldehydes [348]. In contrast, maltose- and maltotriose-negative non-*Saccharomyces* yeasts like *S. ludwigii*, *T.delbrueckii*, *C. fabianii*, *H. uvarum* and *P. fermentans* were reported to degrade wort aldehydes during fermentation albeit strain-specific [156, 169, 243, 314]. Due to the weak fermentation performance, beers with low concentrations of ethanol can thus be produced. However, larger amounts of residual sugars remain than in conventional beers [353]. Besides the advantages mentioned above, the use of non-*Saccharomyces* yeasts for the production of NAB requires meticulously hygienic work, as there is a risk of microbial contamination due to the high levels of fermentable residual sugars [57, 219]. The risk of contamination is increased by the low activity of the yeast, the low ethanol content of the beer and the often-insufficient pH drop during fermentation. Therefore, pasteurisation is absolutely necessary [1, 30, 207, 232]. The often insufficient pH drop requires an additional acidification/pH adjustment of the wort to approximately pH 5.0 [247, 320]. The pH of a pale wort is between 5.0 and 5.7 at an original gravity of 12 °P, while the beer pH of bottom-fermented beers is between 4.35 and 4.60, where a low pH is advantageous for taste and shelf life [240]. Since lower original gravities are set for the production of NAB using non-*Saccharomyces* yeasts, often between 6-9 °P depending on the specific sugar utilization of the yeast strain, the pH of the wort is at a higher reference range [31, 230, 241, 274]. The pH of NAB can also vary depending on the yeast strain. Only a few non-*Saccharomyces* yeast species are able to induce a sufficient pH drop, so the beers often remain above pH 4.5. The use of lactic acid, sour malt or sour wort is therefore recommended to ensure microbiological safety and to exclude spoiling bacteria [13, 31, 343]. Some non-*Saccharomyces* yeasts, such as *L. fermentati*, have the ability to produce larger amounts of lactic acid during fermentation of brewer's wort, resulting in a sufficient pH drop in the NAB [29]. Conversely, it is also essential to exclude any cross-contamination of non-*Saccharomyces* yeasts with *Saccharomyces* yeast cultures for regular beer production in the brewery.

In summary, non-*Saccharomyces* yeasts in particular are ideally suited to the production of NAB, as biological production processes involve little technological effort and time, while offering a great potential for flavor diversity without wort off-flavors. In the past few years there has been increased research in this area, which has demonstrated novel flavor profiles when using non-*Saccharomyces* yeasts. Since flavor diversity using non-*Saccharomyces* yeasts affects both NAB and beers containing alcohol, flavor formation and diversity will be discussed in section 1.4.3. This diversity offers great potential for creating novel, more individual beer styles by using existing brewing methods.

1.4 Prerequisites for the selection of suitable yeast strains for beer brewing

In order to be suitable for beer brewing, yeasts must fulfill a number of requirements, such as sufficient hop and ethanol tolerance and growth at wort pH. For *Saccharomyces* yeasts, it is known that cell growth can occur at a pH of 4.5–6.5. However, this may differ for non-*Saccharomyces* yeasts and therefore needs to be investigated. During fermentation, yeasts acidify their environment through a combination of proton excretion, direct secretion of organic acids, and dissolution of carbon dioxide. In addition to oxygen, selected macronutrients and micronutrients are essential for yeast cell growth. These growth factors are present in the brewer's wort, and the yeasts must be able to metabolize them at least partially [46].

Furthermore, it is crucial for the suitability for beer brewing that the selected yeast strains possess the ability of forming positive flavor compounds during fermentation as fermentation by-products, resulting in a pleasant beer aroma with positive sensory characteristics. Wort flavors, which can usually be attributed to aldehydes, should no longer be present in the beer. Thus, yeasts should be able to eliminate wort aldehydes during the fermentation process. Consequently, the use of non-*Saccharomyces* yeasts offers the potential to generate novel positive flavor characteristics during fermentation, leading to a greater diversity and differentiation of beer flavors.

Once the physiological requirements are met, the yeast strains must also be legally approved for the production of beer. It is essential that the application of the yeast strains is safe. This includes many years of traditional use in food and beverage fermentations and the exclusion of the formation of harmful substances such as biogenic amines during fermentation. It must be further ensured that the yeast strains are non-pathogenic. Provided safe use can be assured, potential positive health aspects may be considered, such as probiotic properties or the synthesis of B vitamins.

In the following sections, the aspects listed will be discussed more in detail.

1.4.1 Inhibition of yeast cell metabolism by hop substances and ethanol

The female cones of the hop plant (*Humulus lupulus* L.) are traditionally utilized as raw material for brewing beer imparting the beer flavor and bitterness [161]. Moreover, hops are used for physicochemical as well as microbial stability and are known to exhibit both antibacterial and antifungal effects [135, 161, 272]. Therefore, a prerequisite for the suitability for beer brewing is that the yeasts must be phenotypically capable of tolerating the hop constituents [23]. The antimicrobial properties of hops can be mainly attributed to the effect of hop *iso*-*α*- and *β*-acids. Hop *iso*-*α*-acids (humulone homologs) are formed during wort boiling by isomerizing *α*-acids to *iso*-*α*-acids and have an essential influence on the bitterness of the beer [20, 89]. They act as proton ionophores that disperse the transmembrane proton gradient, lower the cytoplasmic pH, and suppress the proton motive force leading to an inhibition of certain beer-spoilage bacteria [25, 39]. *β*-acids (lupulone homologs) are reported to have an even stronger antimicrobial effect than *iso*-*α*-acids [135, 309]. Hop *iso*-*α*- and *β*-acids are said to inhibit the growth of gram-positive but not gram-negative bacteria [272, 299]. Schneiderbanger summarized the inhibition mechanisms from various studies [289] and pointed out that the inhibition is caused by oxidative stress of *iso*-*α*-acids in bacterial cells [25]. In addition, the *iso*-*α*-acid acts as a proton ionophore and destructs the transmembrane pH gradient of bacterial cells. Bacterial cell leakage is caused by the hop acids, impeding nutrient uptake and damaging the DNA [289, 343]. *β*-acids display poor solubility in beer as they are not isomerized during wort boiling. Consequently, they are not conveyed into beer and only become relevant in case cold hopping is performed prior to fermentation [279].

Studies on the *Saccharomyces* yeasts *S. cerevisiae* and *S. pastorianus* have shown their resistance to hop *iso*-*α*-acids and state that they are highly tolerant against hop *iso*-*α*-acids, provided that the common concentrations of bitter acids in the beer are not significantly exceeded [135, 287]. Concentrations of *iso*-*α*-acids occurring in beer are summarized in the dissertation by Michel [217], while a detailed overview of the bitter components in beer, their threshold values and typical concentrations can be found in Schönberger and Kostelecky [290]. Furthermore, it was found that the tolerance of eukaryotes against *iso*-*α*- and *β*-acids is significantly higher than that of prokaryotes [135, 218]. Initial studies on the hop tolerance of non-*Saccharomyces* yeasts have been carried out, showing sufficiently high tolerance for different *Hanseniaspora* and *Zygosaccharomyces* genera as well as for the yeast species *L. thermotolerans*, *S. ludwigii* and *T. delbrueckii* to be suitable for the production of beer [31, 86, 219]. Still, there is a need for further research in this field as the influence of hop acids on a large number of non-*Saccharomyces* yeasts, in contrast to *Saccharomyces* species, remains unknown [39, 135, 279]. It should be mentioned that besides hop bitter acids, hop oils and polyphenols also have a relevant antimicrobial influence in beer [161, 351]. However, since no investigations on these were carried out in the context of this thesis, they will not be discussed more in detail.

Apart from sufficient hop tolerance, the ability of yeasts to tolerate ethanol plays a decisive role in the production of alcoholic beers. During fermentation, the increasing ethanol concentration is one of the greatest stress factors of the yeast cells, so there is a need to examine whether the yeast can adapt to the ethanol influence [85]. Ethanol has a toxic effect on yeast physiology. The cell membranes seem to be the main target for ethanol. The sensitivity of yeasts to ethanol and the effects of ethanol vary. Gibson et al. [121] have compiled the possible effects from various sources, which are listed below: Inhibition of cell growth, reduced cell size/viability/respiration/glucose uptake/fermentation, enzyme inactivation, lipid modification, loss of proton motive force across the plasma membrane, increased membrane permeability, lowering of cytoplasmic pH, induction of respiratory-deficient mutants. Furthermore, ethanol exposure has a similar effect as increasing temperatures. Accordingly, these two stress factors have a synergistic effect. Membrane permeability increases in both cases, while proton driving force, intracellular pH and fermentation rates (due to an inhibition of glycolysis) are reduced [259].

1.4.2 Diversity of sugar metabolism pathways in yeasts

A yeast's fermentation performance depends on the uptake of specific carbohydrates to maintain their energy metabolism and form cellular biomass [140, 191]. A pale brewer's wort is composed of 7-9% hexoses, 3% sucrose, 43-47% maltose, 11-13% maltotriose, 6-12% lower dextrins, 19-24% higher dextrins and 3-4% pentosans [240]. For *Saccharomyces* yeasts, the uptake of wort sugars is known to occur through a plasma membrane consisting of mannan, among other compounds, with intervening periplasmic space [36, 193, 199]. The uptake of sugars occurs hierarchically and is controlled by complex regulatory mechanisms. The basic limiting factor for sugar metabolism is the transport of sugars into the yeast cell, and the proteins involved in the transport are encoded by various genes [79, 265].

Glucose, as the preferred carbohydrate, serves as an energy source while also playing the role of a molecular signal, as it is responsible for the induction of gene transcription, which is necessary for efficient glucose metabolism. At the same time, the available glucose suppresses the gene expression of proteins that encode the uptake of other carbohydrate sources [36, 140]. For *S. cerevisiae*, a family of hexose transporters, namely Hxt1p to Hxt17p and Gal2p, is known to interact and regulate sugar uptake. Furthermore, two relevant glucose sensors can be found in interaction with the specified trans-

porters in literature: Snf3p and Rgt2p [140]. During fermentation of brewer's wort, for both *S. cerevisiae* and *S. pastorianus*, glucose and fructose first pass through the cell wall of the yeasts into the cell interior by facilitated diffusion. Two glucose uptake systems exist in the form of low-affinity and high-affinity transporters. The latter is suppressed at high glucose concentrations [191]. The further wort sugars can only be assimilated after the uptake of glucose and fructose, provided the yeast possesses the appropriate transporters and enzyme systems [99, 195, 265]. Verstrepen et al. even describe that glucose is preferred over fructose, since the two monosaccharides are transported into the cell interior via the same carriers and there is a higher affinity for glucose [341]. Sucrose, in turn, is hydrolyzed extracellularly to glucose and fructose by the enzyme invertase released by the yeast cell before it can be metabolized. Consequently, in addition to glucose, sucrose is also consumed early, provided the yeast cell possesses the gene responsible for the formation of an invertase [341]. Considering studies regarding *Saccharomyces* yeasts, only after the available glucose, fructose and sucrose are utilized, maltose and finally maltotriose are assimilated by specific maltose and maltotriose transporters as well as maltase (*α*-glucosidase) as the expression of maltose utilization genes (MAL genes) is suppressed in the presence of glucose [36, 194, 315]. The uptake mechanism for maltose is divided into two systems. With the aid of an energy-dependent maltose permease, energy is released by hydrolyzing ATP to adenosine diphosphate (ADP) and phosphate (P_i). This allows maltose to be actively transported across the cell membrane into the cell interior where it is subsequently hydrolyzed to glucose molecules by a *α*-glucosidase [191, 315]. For maltotriose, a separate energy-dependent transport by a maltotriose-specific permease exists. It is a different permease than the maltose permease. However, both permeases are driven by ATPase-controlled proton gradients [75, 310] and the *α*-glucosidase inside the cell is identical to that for maltose [72, 79, 191]. The glucose molecules are then degraded to pyruvate via glycolysis (Embden-Meyerhof-Parnas pathway), which is similar for all yeasts [240, 268]. Nevertheless, carbohydrate ratios play a significant role during fermentation, as higher viability was demonstrated for brewing yeasts in maltose media compared to glucose media (Stewart 2006).

Figure 3 depicts the transport of the wort sugars into the yeast cell and is modified according to Stewart [315].

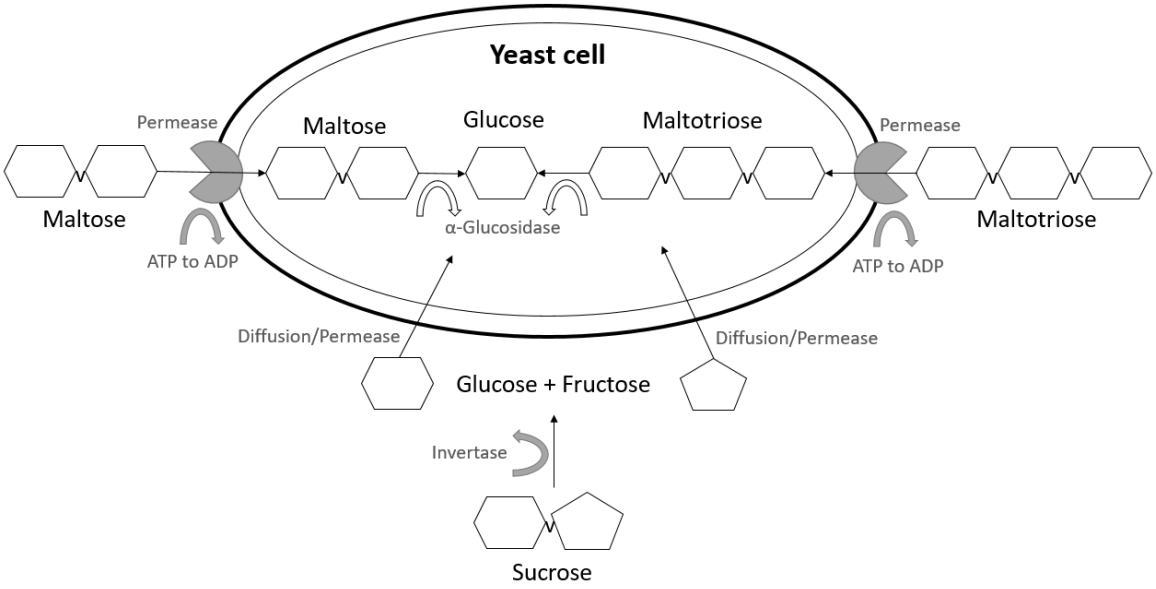


Figure 3. Transport mechanisms of the wort sugars glucose, fructose, sucrose, maltose and maltotriose into the yeast cell and enzymes responsible for the hydrolysis of the di- and trisaccharides to monosaccharides.

Although there are reliable sources on the fermentation ability of glucose, sucrose and maltose by various yeast species published by Kurtzman [175], it often varies within the species as to the kind of carbohydrates they utilize. Consequently, the sugar transport systems and genes encoding for the transporters vary amongst different yeast species and even between different yeast strains. A general overview according to Kurtzman [175] of which wort sugars can be fermented by the yeast species investigated in this thesis can be found in Table 2. Depending on the yeast strain, the assimilation of sugars from fermentation may vary [175]. Since the focus of this thesis was on the fermentation properties of the yeasts, assimilation will not be discussed further.

Table 2. Fermentation performance of wort carbohydrates glucose, sucrose and maltose according to Kurtzman (ed.) [175].

Yeast species	**Glucose**	**Sucrose**	**Maltose**
Cyberlindnera fabianii	+	+	w/s
Cyberlindnera misumaiensis	+	-	-
Cyberlindnera saturnus	+	+	-
Debaryomyces hansenii	w/-	w/-	w/-
Hanseniaspora uvarum	+	-	-
Kazachstania servazzii	+	-	-
Kluyveromyces lactis	+	v	v
Kluyveromyces marxianus	+	+	-
Lachancea kluyveri	+	+	-
Metschnikowia pulcherrima	+	-	-
Nakazawaea holstii	+	-	-
Pichia kluyveri	+	-	-
Saccharomycodes ludwigii	+	+	-
Saccharomycopsis fibuligera	+/w	+/w	+/w
Schizosaccharomyces pombe	+	+	v
Torulaspora delbrueckii	+	v	v
Torulaspora microellipsoides	+	+	-
Wickerhamomyces anomalus	+	+	v
Zygosaccharomyces rouxii	+	v	+/w
Zygotorulaspora florentina	+	+	+

(+) positive, (-) negative, (v) variable, (w/-) weak or negative, (w/s) weak and slow, (+/w) positive or weak

As already mentioned in section 1.3, the production of NAB using non-*Saccharomyces* yeasts makes use of the property of specific yeasts not being able to metabolize, or only to a very limited extent, the sugars maltose and maltotriose, which are proportionally the most abundant in brewer's wort [7, 31, 240]. Due to the lack of the ability to metabolize these sugars, ethanol production is reduced to a minimum [43]. Often, the reason is the absence of the enzyme maltase or the maltose or maltotriose permeases. However, the current state of knowledge in this regard is still limited [79, 220, 227, 265]. Nevertheless, some yeasts can metabolize sugars under aerobic conditions which they cannot utilize under anaerobic conditions. This so-called Kluyver effect is species-specific and affects different sugars. For example, *S. cerevisiae* is Kluyver-effect positive for trehalose, while *K. lactis* is Kluyver-effect positive for maltose utilization [109].

1.4.3 Flavor formation and diversification in beer

While ethanol and carbon dioxide are formed as primary metabolites by a conversion of wort sugars during the fermentation process, intermediates and by-products are produced which can contribute to the beer flavor [57]. Consumers predominantly make their choice based on the flavor properties of the beer [17]. The term “flavor” is composed of the components odor, aroma, taste and mouthfeel [14]. Beers have a complex aroma, which is comprised of a variety of flavor substances. Microorganisms possess the ability to produce a broad range of different secondary metabolites with many of them having an important impact on flavor even though the secondary metabolites are produced at very low concentrations [250, 333]. A multitude of flavor compounds have already been identified in beers, whose formation during fermentation is based on complex biochemical conversion processes in yeast metabolism. The flavor metabolites are unstable over time and therefore alter during storage [250, 337]. The main flavor-active substances produced by yeasts during fermentation can be divided into several groups [17, 57, 220, 250]:

1. Esters
2. Higher/fusel alcohols
3. Carbonyl compounds (aldehydes and ketones/vicinal diketones)
4. Organic and fatty acids
5. Sulfuric compounds
6. Phenols

Figure 4 shows the different metabolic pathways in yeast cells for the formation of flavor-active substances.

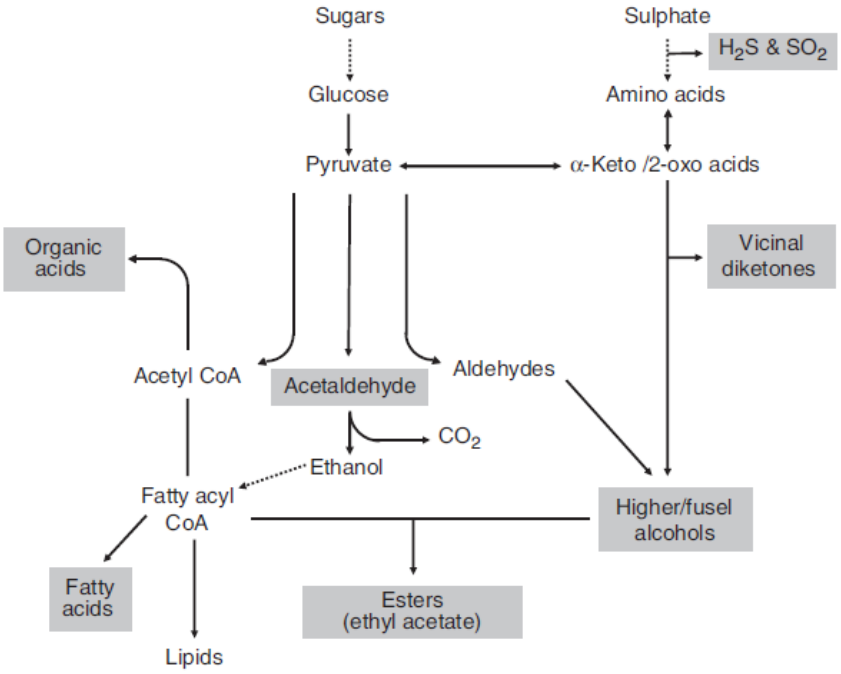


Figure 4. Metabolic pathways of yeasts for the formation of flavor-active substances according to Lodolo et al. and adapted from Hammond [132, 191]; CoA: Coenzyme A; CO_2: carbon dioxide; H_2S: hydrogen sulfide; SO_2: sulfur dioxide.

Figure 4 shows that sugars from brewer‘s wort are mainly hydrolysed to glucose, which is degraded and converted to pyruvate during glycolysis. Pyruvate can be further degraded or converted via different metabolic pathways either to aldehydes, acetaldehyde or to acetyl-CoA. Acetyl-CoA is an important

precursor for the formation of organic and fatty acids as well as esters. In addition to sugar, nitrogenous and sulfur compounds, which are also components of the brewer's wort, are required for yeast growth. Although most of the free amino nitrogen (FAN) present in the wort serves for yeast growth, the FAN significantly affects the higher alcohols, esters, vicinal diketones, and hydrogen sulfide (H_2S) formation [57, 191, 240].

Verstreepen et al., Lodolo et al., Michel et al., and Baiano and Petruzzi among others, have provided reviews of relevant flavor-active secondary metabolites in beer, which are summarized and replenished hereafter [17, 191, 220, 337].

Esters

For ester synthesis by *S. cerevisiae*, alcohols and acids must be available. The alcohols can be present either in the form of ethanol or as more complex fusel alcohols from amino acid metabolism, while the acids are activated fatty acids in the yeasts [49]. Furthermore, the formation of volatile esters is based on enzymatic condensation reactions catalyzed by different groups of ester synthases. In this process, an activated fatty acid, designated as acetyl-coenzyme A (acetyl CoA), reacts with a higher alcohol [357–359]. Accordingly, the concentrations of acetyl CoA and fusel alcohols as well as the total activity of the enzymes responsible for ester catalyzation are decisive for the formation of esters [339]. Genes encoding ester synthesis are ATF1 and ATF2 (encoding alcohol acetyl transferases I and II, also called AATase I and II), Lg-ATF1 (AATase) and EHT1 (ethanol hexanoyl transferase) [88, 106–108, 196, 224, 238, 333, 339, 355, 356]. It is suggested that there may be further genes involved in ester synthesis [201].

Flavor-active esters can be divided into the two groups acetate esters and medium-chain fatty acid (MCFA) or ethyl esters and are formed intracellularly in yeast during fermentation contributing to the fruity and/or floral flavors of beer [17, 338]. According to Verstrepen et al., Meilgaard und Nykänen, the esters that mainly convey a fruity sensory impression are ethyl acetate, isoamyl acetate, phenylethyl acetate, which belong to the group of acetate esters, and ethyl caproate, ethyl caprylate and ethyl decanoate, which belong to the group of ethyl esters [208, 246, 276, 339].

Higher/fusel alcohols

Higher alcohols, also known as fusel alcohols, can be synthsized via both anabolic and catabolic metabolic pathways. In case they emerge by the anabolic pathway, 2-oxo acids derived from carbohydrate metabolism are decarboxylated to aldehydes. The aldehydes, in turn, are subsequently reduced to the corresponding higher alcohols [17]. On the catabolic pathway, the higher alcohols derive from 2-oxo acids, which, however, originate from amino acids via the Ehrlich pathway (cf. Figure 4) [62]. Higher alcohols such as 2-phenyl ethanol, isobutyl alcohol and isoamyl alcohols positively affect the aroma profile of beer provided they stay below 300 mg/L [250]. They contribute to an alcoholic, solvent-like flavor and a warm mouthfeel [17].

Carbonyl compounds

The group of carbonyl compounds includes aldehydes and ketones.

Aldehydes play a central role in yeast cell metabolism as they are metabolized as part of the anabolic and catabolic pathways to form higher alcohols during fermentation. Furthermore, aldehydes are formed under the thermal influence of wort preparation as a Maillard product and as a result of lipid oxidation. Acetaldehyde, in particular, is mainly produced by yeast during its growth phase as a result

of the sugar metabolism and is an intermediate product of ethanol and acetate formation. Above a threshold of 10–20 mg/L, it can cause green apple and grassy flavors in beer [191, 210].

During wort fermentation, ketones are mainly synthesized as vicinal diketones. Vicinal diketones include diacetyl (2,3-butanedione) and 2,3-pentanedione, which are formed from the precursors 2-acetolactate and 2-acetohydroxybutyrate as well as acetoin [57]. Diacetyl is a by-product of the amino acid anabolism of valine. It is formed from glucose in a four-step reaction during fermentation and can be resumed by the yeast cell during maturation where it is reduced to acetoin and 2,3-butanediol. While 2,3-butanediol is not a flavor-active substance, diacetyl leads to a buttery flavor which, depending on the beer type, can have a negative influence on the flavor quality of the beer in case the threshold value of 0.10–0.15 mg/L is exceeded [17, 170, 210, 260, 266, 277].

In addition to diacetyl, the ketone (*E*)-*β*-damascenone is one of the known carbonyl compounds in beers. During wort boiling, thermal input can cause the formation of Strecker aldehydes, which can lead to the formation of compounds such as (*E*)-*β*-damascenone by acid hydrolysis of glycoside precursors [122, 258, 293, 301]. The formation of this ketone is further associated with beer aging, explaining why (*E*)-*β*-damascenone is partly used as an aging indicator [126, 277, 334]. In this context, the formation of (*E*)-*β*-damascenone is considered to be caused by oxidative cleavage from the carotenoid neoxanthin or from acid hydrolysis of plant secondary metabolites [26, 64, 292, 296]. Another formation pathway may also occur during fermentation by yeast cell metabolism [190]. The ketone is mainly described to induce flavors reminiscent of apple/cooked-apple in beer [126, 168, 258, 286].

Organic and fatty acids

The majority of organic acids are formed during yeast cell metabolism as a by-product of glycolysis and TCA cycle from the catabolism of amino acids and redox reactions. In some cases, organic acids also originate from the wort. In the latter case, the yeast has no influence on the concentration in the beer. The organic acids are partially responsible for the pH drop during fermentation. Fatty acids are particularly interesting due to their involvement in ester synthesis, as described previously [46]. Relevant organic acids/fatty acids in beer are primarily isovaleric, hexanoic, octanoic and decanoic acids, which can create unpleasant flavor characteristics [253, 337].

Sulfuric compounds

The major sulfuric compounds produced by yeast during fermentation are sulfur dioxide (SO_2), hydrogen sulfide and dimethyl sulfide (DMS) [90, 237, 328]. Sulfur dioxide operates as an antioxidant and consequently positively contributes to the flavor stability by increasing the shelf life of the beer. Occasionally, it can also lead to a positive aroma character in bottom-fermented beers [149]. In contrast, hydrogen sulfide belongs to the undesirable flavor-active substances as it causes a rotten-egg smell at low threshold values [249]. DMS can either emerge during wort boiling by thermal degradation of S-methylmethionine or it can originate during fermentation as a result of the reduction of dimethyl sulfoxide (DMSO) by the yeast. In case it exceeds the threshold values, it causes an unpleasant sulfury flavor in the beer [8, 84, 191].

Phenols

Phenolic flavors, which are mainly attributed to the flavor substances 4-VG and 4-vinyl phenol (4-VP) are primarily found in wheat beers and impart characteristic clove- and medicinal-like flavors. However, the flavor input of 4-VP plays a minor role, while the clove-like flavor dominates [12, 288]. 4-VG derives from ferulic acid, a hydroxycinnamic acid mainly found as a cell wall component of malt or raw barley. Ferulic acid is transferred to the wort during mashing. Provided the yeast possesses the enzyme

systems phenylacrylic acid decarboxylase (PAD1) and ferulic acid decarboxylase (FDC1), it can actively decarboxylate the ferulic acid to 4-VG [68–70, 185, 231]. Although 4-VG can also thermally arise from ferulic acid at reaction temperatures exceeding 80 °C, this formation pathway plays a minor role [288]. Furthermore, reduction products of 4-VP and 4-VG are known in the form of ethyl derivatives. *Brettanomyces*/*Dekkera* spp. possess a vinylphenol reductase activity [332]. It is yet unkown whether the same activity exists for the non-*Saccharomyces* yeasts investigated in this thesis. In general, non-*Saccharomyces* yeasts are more frequently considered to be phenolic off-flavor (POF)-positive than *Saccharomyces* yeasts [185, 300, 319]. The term "POF" was originated based on the phenolic flavor perceived as undesirable in bottom-fermented pilsner beers. However, in Belgian and German wheat beers, for example, the phenolic flavors are characteristic and desirable. Accordingly, they are not considered as off-flavors in these types of beers [319, 332].

For further details, Dzialo et al. elaborated figures with flow charts for the formation of the most relevant flavor substances in beer [91], while Meilgaard and Sannino et al. summarized the corresponding organoleptic thresholds of volatile compounds [210, 283]. Meilgaard refers exclusively to beers fermented with traditional *Saccharomyces* brewing yeasts whereas Sannino et. al also consider beers produced by using non-*Saccharomyces* yeasts. Steensels and Verstrepen summarized the bioflavoring potential of non-*Saccharomyces* yeasts for the production of beer and described the contribution of a wide range of flavors. For example, *B. bruxellensis* produces phenolic compounds, ethyl esters and fatty acids in spontaneous beer fermentations, the combination of which creates an aroma reminiscent of clove, barnyard, smoke, humid leather, tropical fruit, and/or spices [314]. Some non-*Saccharomyces* yeast strains also proved to be positive flavor enhancers during the fermentation of brewer's wort when applied as monocultures. Basso et al. summarized the aroma profiles of several beers fermented by yeast species in mono-cultivation. *C. saturnus* and *P. kluyveri* were described to cause banana, pear and fruity flavors. It needs to be noted, however, that the references for *C. saturnus* only refer to wine flavors although Basso et al. designate them to beer flavors. *H. uvarum* and *W. anomalus* led to similar banana, pear and fruity, as well as solvent-like flavors, while *T. delbrueckii* caused versatile fruity flavors reminiscent of banana, pear, apple, strawberry and green apple. Consequently, they offer a great potential for bioflavoring [23]. Building on the findings from various studies on bioflavoring using non-*Saccharomyces* yeasts summarized by Basso et al., there are now further studies on the yeast species investigated in this thesis. Table 3 in the following presents an overview of novel flavor characteristics and their corresponding aroma-active substances in beer, which were formed by means of the individual yeast species during the fermentation of brewer's wort. Although the focus is on mono-cultivations, some examples of co-cultivations are also mentioned.

Table 3. Overview of novel flavor characteristics and their corresponding flavor-active substances in beer fermented by the 20 yeast species listed in Table 1.

Yeast species	**Flavor-active substances and characteristics in beer**
Cyberlindnera fabianii	- relatively high levels of esters in comparison with *S. cerevisiae* leading to a fruity flavor [331] - high levels of esters: ethyl acetate (129.5 mg/L); ethyl 9-hexanodecanoate, ethyl decanoate, ethyl dodecanoate, ethyl hexanoate, ethyl pentadecanoate, propyl decanoate, propyl octanoate, 2-methylpropyl hexanoate, 3-methylbutyl octanoate, 3-methylbutyl pentadecanoate and decanoic acid found in higher

	levels in co-cultivation compared to mono-cultivations [330, 331] - cabbage-like, unpleasant flavor [30]
Cyberlindnera misumaiensis	- high levels of ethyl acetate (65.7 mg/L) leading to an unpleasant solvent-like flavor, isoamyl acetate around flavor threshold (0.90 mg/L) [30]
Cyberlindnera saturnus	No conspicuous flavor substances described in literature during fermentation of brewer's wort for *C. saturnus*, only studies for *C. saturnus* var. *mrakii* have been conducted: - isoamyl acetate (1.60 mg/L) causing a fruity, pleasant flavor [30] - in comparison to *S. cerevisiae* significantly higher levels of acetate esters, in particular isoamyl acetate, ethyl acetate and 2-phenylethyl acetate leading to more distinct fruity, banana-like and floral aroma impression; higher production of ethyl acetate can lead to a glue-like off-flavor [189]
Debaryomyces hansenii	No conspicuous flavor substances described in literature during fermentation of brewer's wort
Hanseniaspora uvarum	- in direct comparison to the flavor produced by a *S. cerevisiae* reference strain almost double concentration of 2-phenyl ethanol and more than twice as much ethyl decanoate [119] - 4-VG detected causing a distinctive clove-like flavor [156] - 2-phenylethyl acetate and ethyl acetate measured [270]
Kazachstania servazzii	- in direct comparison to the flavor produced by a *S. cerevisiae* reference strain increase of fruitiness based on higher levels of 3-methylbutanol, phenyl ethanol, phenylethyl acetate, ethyl decanoate and ethyl caprylate [119] - 2-phenylethyl acetate (7.39 mg/L) inducing pear and apple notes [156]
Kluyveromyces lactis	- almost double the amount of 2-phenyl ethanol and 2.5 times the concentration of ethyl decanoate in direct comparison with the reference yeast *S. cerevisiae* [119] - strawberry fragrance with butter/diacetyl notes on glucose wort plate; acetate esters (especially phenylethyl acetate) leading to rose and floral flavors in synthetic glucose wort media [127]
Kluyveromyces marxianus	- 2-phenylethyl acetate (3.9 mg/L) causing a rose-like aroma, increase in the aldehydes 2-methylbutanal and 2-methylpropanal [156] - 2-phenylethyl acetate detected [119]
Lachancea kluyveri	No conspicuous flavor substances described in literature during fermentation of brewer's wort
Metschnikowia sp.	- Low concentrations of volatile phenols, volatile esters and acids measured [270]
Nakazawaea holstii	No conspicuous flavor substances described in literature during fermentation of brewer's wort

Pichia kluyveri	- Formation of high concentrations of esters, in particular isoamyl acetate, low production of undesirable acids [274] - enhances fruitiness and banana-flavor in beer due to isoamyl acetate, ethyl acetate leading to solvent-like flavor [139]
Saccharomycodes ludwigii	- formation of alcohols and esters depending on the applied yeast strain [103] - three different strains investigated with interestingly high levels of fusel alcohols (approximately 1.37–1.50 mg/L), fatty esters (approximately 0.19–0.44 mg/L) and isoamyl acetate (approximately 0.10–0.14 mg/L) [270] - 2-phenylethyl acetate (6.11 mg/L) responsible for apple notes, increase in aldehydes (3-methylbutanal and phenylacetaldehyde), DMS, methional, 2-methylbutanal and 3-methylbutanal leading to main sensory descriptors cereal, worty, sweet corn and cooked tomato [156] - slight wort off-flavor and diacetyl [43, 241] - diacetyl detected [50] - low concentrations of ethyl acetate (0.65 mg/L), isoamyl acetate (0.1 mg/L), and 4-VG (0.1 mg/L), overall flavor of the beers described as being honey-like [207]
Saccharomycopsis fibuligera	No conspicuous flavor substances described in literature during fermentation of brewer's wort
Schizosaccharomyces pombe	- acetaldehyde responsible for a green and herbal aroma, diacetyl detected above threshold value, esters and higher alcohols achieved higher global values at increasing original gravity of the wort [50] - benzene acetaldehyde leading to a green and honey odor as well as benzaldehyde reminiscent of almond and cherry were detected [335]
Torulaspora delbrueckii	- 4-VG responsible for clove-like flavor [139] - low concentration of esters (0.8 mg/L) and higher alcohols (18 mg/L) were measured, diacetyl detected (0.1 mg/L), beer was described as being worty, bready and slightly fruity [31] - diacetyl concentrations between 0.1–0.3 mg/L detected, POF-negative, notes of honey, pear, citrus, generally fruity flavors perceived [219] - floral/fruity flavors, more pronounced ester character, complexity and intensity, and lower phenolic character compared to *S. cerevisiae* [317] - pleasant and aromatic taste [53] - *β*-caryophyllene and geranyl acetone produced, depending on strain, and significant amounts of long-chained ethyl esters or isoamyl alcohol and ethyl butanoate could be analyzed [322]
Torulaspora microellipsoides	No conspicuous flavor substances described in literature during fermentation of brewer's wort

Wickerhamomyces anomalus	- in direct comparison of the flavor produced by a *S. cerevisiae* reference strain increase of higher alcohols, significant increase in ethyl butyrate and ethyl acetate [55] - higher acidity of beers, high concentration of ethyl esters [66]
Zygosaccharomyces rouxii	- production of high amount of higher alcohols, esters (4.42–71.15 mg/L), acetaldehyde (5.58–8.15 mg/L), diacetyl (0.234–0.851 mg/L) above threshold leading to a buttery flavor [103] - isoamyl alcohol and ethyl acetate above sensory flavor threshold, 2-phenylethyl alcohol higher than levels found in the reference beer fermented with *S. pastorianus* [112] - successive application of *S. cerevisiae* and afterwards *Z. rouxii* for the production of NAB leading to higher concentrations of acetaldehyde, and diacetyl (responsible for worty and buttery off-flavors) than fermentation with mono-cultivation of *S. cerevisiae*; temperature dependence as acetaldehyde, and diacetyl significantly lower at 24 °C than at 12 °C fermentation temperature [304]
Zygotorulaspora florentina	- isoamyl alcohol above sensory threshold of 70 mg/L, 4-VG detected (1.78 mg/L) leading to POF-positive flavor impression, unable to produce fruity acetate and ethyl esters except for ethyl decanoate [139] - increase in isoamyl acetate and *α*-terpineol responsible for floral aroma positively influenced final flavor of beer [55] - 4-VG above flavor threshold could not be perceived in sensory testing as spice/clove-like flavor potentially due to masking effects of other more dominant compounds [244]

In summary, it can be concluded that some yeast species consistently produced positive flavor characteristics during brewer's wort fermentation while others exhibited strain-dependent differences. *C. fabianii*, *K. marxianus*, *S. ludwigii*, *S. pombe*, *T. delbrueckii* and *Z. rouxii* revealed strain-dependent differences. In particular, *C. fabianii* appeared to be much more suitable for co-cultivations with *S. cerevisiae* to produce positive aroma traits. *K. servazzii*, *K. lactis*, *P. kluyveri* and *W. anomalu*s consistently showed promising flavor characteristics in brewer's wort, however, some of the data are still very limited and either only sensory evaluations or instrumental analysis were performed. *H. uvarum*, *Z. florentina*, and *T. delbrueckii* (strain dependent) exhibited POF-positive flavor properties due to 4-VG, which, however, could not always be perceived during sensory evaluations. There is a lack of studies on *C. misumaiensis,* which revealed rather negative aroma characteristics in the one existing study. There is a similarly low data availability on brewing for *Metschnikowia* sp., which led to a rather neutral flavor. For *C. saturnus*, *D. hansenii*, *L. kluyveri*, *N. holstii*, *S. fibuligera*, and *T. microellipsoides*, no studies in brewer's wort exist that focus on flavor formation.

1.4.4 Food safety: Requirements for the safe use of non-*Saccharomyces* yeasts in brewing

Non-*Saccharomyces* yeasts offer an outstanding potential for diversifying the flavor characteristics in beer. Nevertheless, in the first instance, it is necessary to comply with the legal regulations for the safe use of microorganisms. The yeast species selected in this thesis are without exception known from food and/or beverage fermentations, which represents a significant factor for their basic suitability.

However, this does not guarantee that they can be applied without any restrictions for fermentations in brewer's wort. Due to the increasing research activities in this field and the growing interest for industrial and thus also commercial use, there are fundamental requirements that the yeast species must meet to ensure food safety, which are outlined as follows.

In general, there are two organizations dealing with the issue of biosafety for food production. Both follow different principles, looking either at scientific studies or at the historically proven safe use of microorganisms in food production [42]. In the US, this is the US Food and Drug Administration (FDA), which has published a list of yeast species that are suitable for safe use in food and beverage fermentations and have thus been given GRAS (Generally Recognized As Safe) status. This GRAS status refers to the use in specific applications and is primarily based on a historically safe use before 1958 [327].

In the EU, the European Food Safety Authority (EFSA) is responsible for the safe use of microorganisms in food and beverage fermentations. They consider the safe use of microbes independent of the application. Instead, EFSA grants the qualified presumption of safety (QPS) status, based on a risk assessment of biological agents conducted by the Panel of Biological Hazards (BIOHAZ) [92]. This risk assessment includes, for example, an examination of the microbes for the formation and release of biogenic amines, which are considered safe only in moderate amounts. By exceeding 50 mg/kg food they can cause adverse health effects [24, 96, 192]. The EFSA also provides a list of microorganisms that were granted QPS status, which includes the following yeast species: *Candida cylindracea, D. hansenii, H. uvarum, K. lactis, K. marxianus, Komagataella pastoris, C. jadinii, P. angusta, Saccharomyces bayanus, S. cerevisiae, S. pastorianus, S. pombe, W. anomalus, Xanthophyllomyces dendrorhous*. Thus, six out of the 20 yeast species investigated in this thesis are on the QPS list and could be used commercially without safety concerns [267]. In 2019, three further yeast species were proposed for inclusion on the QPS list, namely *Komagataella phaffi, Y. lipolytica* and *Z. rouxii* [166]. Furthermore, it must be taken into account that according to EFSA regulation, any food that was not used to a significant degree before 15 May 1997 must be designated as "novel food" [326].

In 2014, Steensels and Verstrepen drew attention to the challenges of using non-*Saccharomyces* yeasts with regard to the biosafey aspect [314]. Although there has been increased research in this field in the past few years, the knowledge for safe use is still limited to some extent. It is assumed that yeasts traditionally occurring in spontaneous food fermentations can be used without health risks. Still, it must be ensured, depending on the application, that no biogenic amines, methanol (from pectin) or urea, which can react spontaneously with ethanol to form ethyl carbamate, are synthesized [307, 314]. Basso et al. described the yeast species *C. mrakii*, *Brettanomyces anomalus*, *Brettanomyces bruxellensis*, *H. uvarum*, *P. kluyveri*, *T. delbrueckii*, *W. subpelliculosus* and *W. anomalus* as being safe for brewing applications according to Bourdichon et al. [23, 42]. In addition, the clinical importance of the 20 yeast species investigated in this thesis was already addressed in section 1.2.1 and must also be taken into account in any safety consideration. Miguel et al. provide an indepth insight into safety aspects and considerations, which need to be fulfilled for the safe application of non-*Saccharomyces* yeasts in beer brewing. These include the evaluation of pathogenic potential, antifungal resistance, biogenic amines, and potential allergic reactions [222].

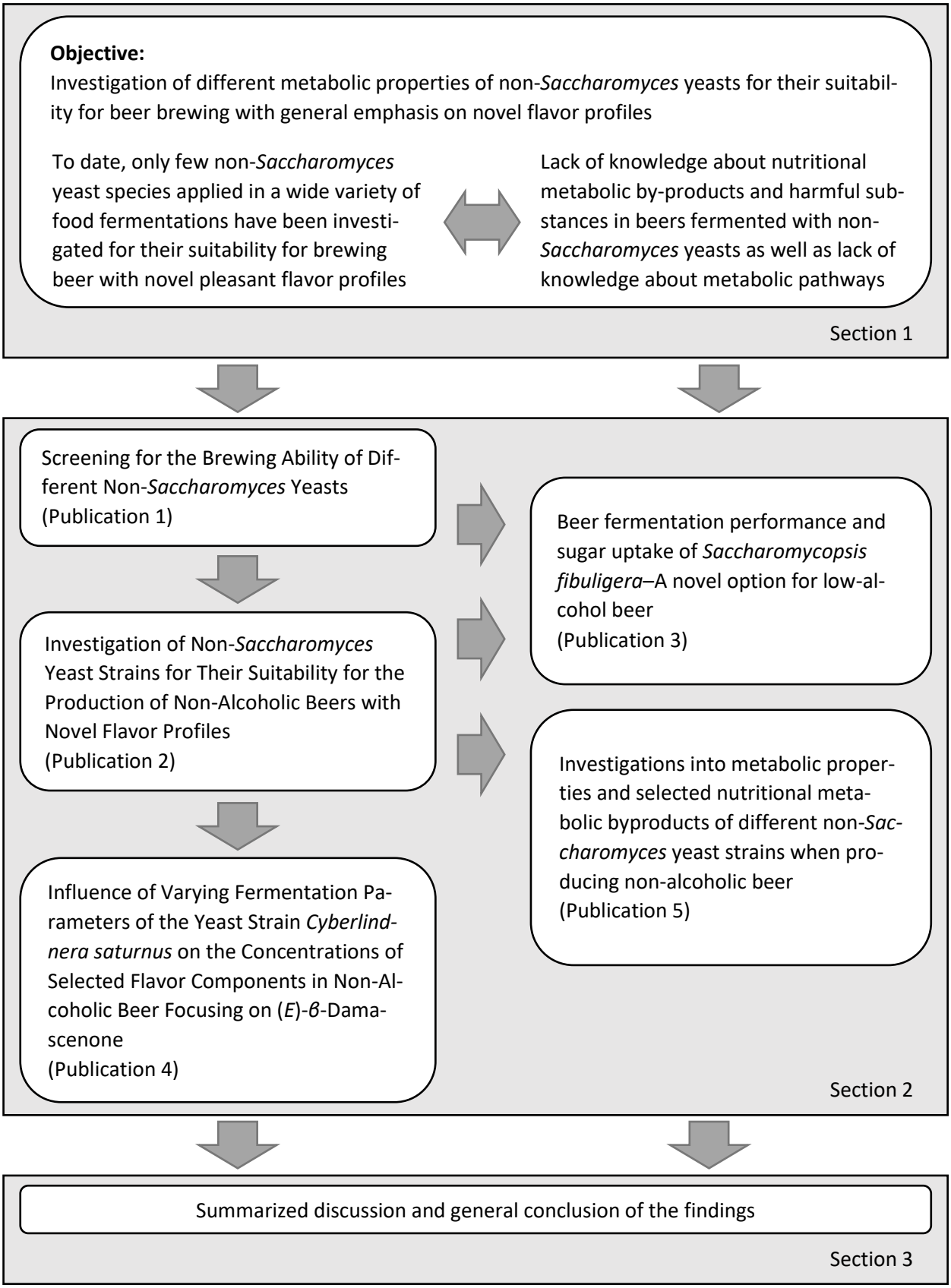
1.4.5 Potential for positive health effects by using non-*Saccharomyces* yeasts

In general, beer is discussed as a beverage with health-promoting ingredients. Positive health effects could be attributed to polyphenols, hop constituents such as flavones and bitter acids, and B vitamins. While ethanol in beer causes negative health effects, the non-alcoholic compounds most likely have a

beneficial influence [18, 200, 255]. They are even discussed as having a preventative effect against various chronic diseases. NAB also contain these bioactive compounds with potential positive health effects and could therefore be a healthy alternative for beer consumers [325]. As described in the previous sections of this introduction, there is great potential for using non-*Saccharomyces* yeasts to produce NAB. Firstly, because a large number of non-*Saccharomyces* yeasts are unable to metabolize maltose (cf. Table 2) and, secondly, because the yeasts show great potential to produce positive flavor compounds during the fermentation of brewer's wort thus leading to a high sensory quality of the beer (cf. Table 3). Basso et al. already suggested product innovation by applying non-*Saccharomyces* yeasts like *C. saturnus* and *T. delbrueckii* for the fermentation of LABs [23]. The health-promoting substances in beer often derive from the raw materials. In addition to minerals such as potassium and magnesium, polyphenols play an important role. These secondary metabolites are introduced into the beer by the hops and barley [81, 223]. B vitamins can be traced back to the raw materials, too. However, yeasts are also capable of synthesizing B vitamins during fermentation. For example, the ability to produce biotin during fermentation was discovered in some sake yeasts [349]. Moreover, *S. cerevisiae* was found to synthesize significant concentrations of folate during the production of yeast extract [153]. There is still a need for research whether it is possible to produce beers with higher B vitamin concentrations by using non-*Saccharomyces* yeasts.

A further field of potential positive health effects by using non-*Saccharomyces* yeasts in food fermentations is their use as probiotics. According to the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), probiotics are live microorganisms that, when administered in sufficient quantities, provide health benefits to the host [137, 311]. Additionally, clinical studies must deliver evidence that the microbes confer health benefits to the host, which apply at a strain-specific level. The definition of the term "probiotic" was often not uniformly understood. While it was repeatedly assumed that the probiotic strain had to be of "human origin", effective probiotic strains were also discovered that were not originally isolated from humans [2, 281, 282]. Nevertheless, probiotic microbes need to have in common that they possess the ability to survive in human gastrointestinal tract conditions, grow at 37 °C and contribute to the health of the host environment by regulating microflora and performing biological functions [100, 311]. In 2015, Gil-Rodriguez et al. still mentioned *S. cerevisiae* var. *boulardii* as the only proven probiotic yeast, which was subsequently used in a probiotic alcohol-free beer [123, 297]. Meanwhile, in addition to *S. cerevisiae* var. *boulardii*, *S. cerevisiae* is also known for its probiotic properties while non-*Saccharomyces* yeasts, such as *K. marxianus*, *K. lactis*, *S. fibuligera*, *S. pombe* and *T. delbreuckii* increasingly appear in the context of probiotic or potentially probiotic yeasts [67, 101, 123, 134, 178, 180, 251]. Nevertheless, the scientific data is often insufficient and there is consequently a high need for research to be able to prove an actual probiotic benefit for selected yeast strains.

2 Results (Thesis publications)



2.1 Publication 1: Screening for the Brewing Ability of Different Non-*Saccharomyces* Yeasts

The suitability of using non-*Saccharomyces* yeasts for beer brewing and the resulting novel flavor profiles of the beers have been demonstrated in selected yeast strains in recent years. There is a large variety of different yeasts, which are applied in various food fermentation processes. However, for most of them there are no studies on the fermentation of all-malt wort and the resulting production of beers. Neither the physiological properties of the yeasts during the fermentation of brewer's wort nor their flavor formation are known.

As a result, a screening method in 96-well plates was developed as part of this publication. Using this method, 110 different non-*Saccharomyces* yeast strains could be phenotypically tested for their fermentation properties in selective media found as components in brewer's wort within a relatively short period of time. The yeast strains screened belonged to the genera *Cyberlindnera*, *Debaryomyces*, *Hanseniaspora*, *Kazachstania*, *Kluyveromyces*, *Lachancea*, *Metschnikowia*, *Nakazawaea*, *Pichia*, *Saccharomycopsis*, *Schizosaccharomyces*, *Torulaspora*, *Wickerhamomyces*, *Zygosaccharomyces* and *Zygotorulaspora*. The different yeast strains were individually added to each main wort carbohydrate to investigate, which of the sugars could be metabolized by the yeasts. It was found that of all the yeasts examined, only 30% could metabolize maltose and 25% maltotriose. Hence, the majority of these yeast strains would have potential to produce NABs or LABs. Furthermore, the effect of varying concentrations of hop *iso*-*α*- and *β*-acids and ethanol on the growth ability of the yeasts was investigated. However, no significant inhibitory effects could be deciphered that would have any relevance in beer brewing. Additionally, phenolic off-flavor (POF) and sensory odor tests of the fermented wort samples were carried out in order to gain initial flavor impressions.

The most promising yeast strains, which showed both physiological suitability and POF-negative results while exhibiting exceptionally pleasant flavor formation in the sensory odor tests, were then subjected to brewing trials in small-scale fermentations. Out of approximately ten of the most promising yeast strains belonging to the species *Saccharomycopsis fibuligera*, *Schizosaccharomyces pombe* and *Zygosaccharomyces rouxii*, five were selected for the brewing trials, including three different *S. fibuligera* strains. Applying the five selected yeast strains, unhopped wort with 12.5 °P original gravity was fermented unpressurized at a temperature of 27 °C and a pitching rate of 30×10^6 cells/mL in a volume of 1,400 mL each. The beer fermented with *S. pombe* revealed the highest fermentation activity, reaching a final ethanol concentration of around 5.7% (*v*/*v*), while the other four beers ranged between 2.6–4.0% (*v*/*v*). The pH drop was sufficient in all beers reaching values between 4.30–4.51. During a sensory evaluation, trained assessors rated the three beers fermented with *S. fibuligera* as being the best and described the profiles as desirably fruity, reminiscent of plum and berry. As part of an additional GC analysis, the esters ethyl butyrate and ethyl acetate were detected above the threshold values for beer, which confirm the sweet, fruity flavor impression. By applying the described screening method and the subsequent brewing trials, it was possible to select non-*Saccharomyces* yeast strains that are suitable for the production of beers with novel flavor profiles.

Authors/Authorship contribution:

Methner, Y.: Conception and design of experiments, performance of experiments, analysis of data using OriginPro 2019 as statistical software, paper writing; **Hutzler, M.**: Organization and provision of a large amount of yeast strains, revision of conception; **Matoulková, D.**: Organization and provision of a large amount of yeast strains; **Jacob, F.**: Revision of manuscript, approval for submission, funding acquisition; **Michel, M.**: Conception and design of experiments, revision of manuscript, approval for submission.

fermentation

MDPI

Article

Screening for the Brewing Ability of Different Non-*Saccharomyces* Yeasts

Yvonne Methner [1], Mathias Hutzler [1,*], Dagmar Matoulková [2], Fritz Jacob [1] and Maximilian Michel [1]

[1] Research Center Weihenstephan for Brewing and Food Quality, Technische Universität München, Alte Akademie 3, 85354 Freising, Germany; yvonne.methner@tum.de (Y.M.); f.jacob@tum.de (F.J.); m.michel@tum.de (M.M.)

[2] Research Institute of Brewing and Malting, Lípová 511/15, 120 00 Praha 2, Czech Republic; matoulkova@beerresearch.cz

* Correspondence: m.hutzler@tum.de; Tel.: +49-8161-71-3100

Received: 30 October 2019; Accepted: 6 December 2019; Published: 12 December 2019

check for updates

Abstract: Non-*Saccharomyces* yeasts have aroused interest in brewing science as an innovative and seminal way of creating new beer flavors. A screening system for potential brewing strains of non-*Saccharomyces* yeasts was set up to investigate the yeast's utilization of wort sugars and to examine the effect of hop acids as well as ethanol on the growth of different yeast strains. Additionally, phenolic off-flavor (POF) and sensory odor tests of fermented wort samples were performed. The promising strains were further investigated for their propagation ability and for following fermentation trials. The produced beers were analyzed for secondary metabolites, ethanol content and judged by trained panelists. Subsequently to the screening, it was discovered that among the 110 screened yeast strains, approx. 10 strains of the species *Saccharomycopsis fibuligera*, *Schizosaccharomyces pombe* and *Zygosaccharomyces rouxii* generate promising fruity flavors during fermentation and were able to metabolize maltose and maltotriose as a prerequisite for the production of alcoholic beers. Consequently, the screening method described in this study makes it possible to investigate a tremendous number of different non-*Saccharomyces* yeasts and to test their brewing ability in a relatively short period of time.

Keywords: yeasts; non-*Saccharomyces* yeasts; microbiology; fermentation; 96-well microtiter plate test; sensory analysis; brewing ability; secondary metabolites/volatile compounds

1. Introduction

Known to most brewers as spoilage yeasts or as co-fermenters in mixed fermentations, non-*Saccharomyces* yeasts have received very little attention since the introduction of what are referred to as high-performance *Saccharomyces* brewing yeasts [1,2]. With the rise in craft brewing and breweries seeking greater individualization, the use of such unconventional yeasts might be a solution [3]. Michel et al. [4] and Basso et al. [5] emphasized the great potential of non-*Saccharomyces* yeasts to develop beers with different alcohol contents and a broad range of flavors. They highlighted the varying abilities of unconventional yeasts to metabolize desirable aroma-active substances such as fruity esters, monoterpenes, higher alcohols, phenols and acids. Moreover, the fermentation ability of brewer's wort carbohydrates was discussed with the resulting alcohol content. Both reviews were, amongst others, focusing on species such as *Torulaspora delbrueckii*, *Dekkera/Brettanomyces* and *Pichia kluyveri*.

A screening system for non-*Saccharomyces* brewing strains was published by Michel et al. in 2016 [6]. A variety of different tests (e.g., wort carbohydrate consumption, hop and ethanol sensitivity, fermentation potential) was introduced to predict the potential of ten *T. delbrueckii* strains to ferment

brewer's wort to a desirable beer. Ravasio et al. also evaluated the fermentation and aroma profile of 60 different non-*Saccharomyces* strains in 2018. The applied yeasts were cultured in a medium based on glucose and the resulting volatile compounds were detected by gas chromatography/mass spectrometry (GC/MS) measurement. Only the promising species were additionally analyzed for their maltose utilization on serial-dilution plate assays [7].

The study presented here includes screening the brewing ability of 110 non-*Saccharomyces* strains with optimized screening conditions as the metabolism of the full range of main wort carbohydrates (glucose, fructose, sucrose, maltose and maltotriose [8]) were taken into account. As maltose and maltotriose represent more than 80% of the total carbohydrates in brewer's wort [9], the ability to ferment these two substances is essential for a fast, complete and predictable fermentation [10]. Melibiose was additionally included into the screening, as a study of Wickerham indicated that bottom-fermenting yeasts are able to metabolize this type of sugar whereas top-fermenting yeasts do not [11]. Furthermore, part of the first screening step looked at the effect of hop iso-α- and β-acids as well as ethanol on the growth of the yeast strains to determine whether there are any existing resistances at certain concentrations that would restrict the production of a conventional beer. As hop acids have antimicrobial properties and β-acids, in particular, were reported to have an even stronger antimicrobial effect than iso-α-acids [12,13], it is necessary to test the yeast's tolerance to these acids. Although a conventional Pils has up to 38 IBU, which is approximately comparable to 38 mg iso-α-acids/L, some IPAs can reach 100 IBU [14]. During fermentation, the increasing ethanol concentration is one of the greatest stress factors of the yeast cells, so there is a need to examine whether the yeast can adapt to the ethanol influence [15]. Additionally, the pH of all nutrient solutions was adjusted to 5.2 to map the pH value of a standard wort, which is between 5.0 to 5.7 [16]. To obtain an initial sensory impression of the individual strains, odor tests were performed by transferring the yeasts into brewer's wort. In addition, a POF test was accomplished to investigate the potential of the different strains to produce 4-vinylphenol, 4-vinylguaiacol or 4-vinylbenzol from precursors [17–19].

The non-*Saccharomyces* yeasts screened in this investigation were of the genera *Cyberlindnera*, *Debaryomyces*, *Hanseniaspora*, *Kazachstania*, *Kluyveromyces*, *Lachancea*, *Metschnikowia*, *Nakazawaea*, *Pichia*, *Saccharomycopsis*, *Schizosaccharomyces*, *Torulaspora*, *Wickerhamomyces*, *Zygosaccharomyces* and *Zygotorulaspora*. They were chosen because of their positive influence on the aroma profile of fermented foods by contributing high amounts of esters, higher alcohols and other volatile flavor compounds [20–23]. As esters are volatile flavor compounds which mainly contribute to the aroma of beer, these strains might also be interesting as contributors to beer aroma [24–26]. According to Verstrepen et al., the esters that mainly convey a fruity sensory impression are ethyl acetate, isoamyl acetate, phenylethyl acetate, ethyl caproate, ethyl caprylate and ethyl decanoate [27]. In addition to esters, higher alcohols such as 2-phenyl ethanol, isobutyl alcohol and isoamyl alcohols also positively affect the aroma profile of beer as long as they stay below 300 mg/L [28]. In contrast, aldehydes and vicinal diketones such as diacetyl or 2,3-pentanedione, which cause an unpleasant buttery flavor, can negatively alter the quality of a beer once their sensory threshold is exceeded [7,29,30]. The organoleptic thresholds of volatile compounds mainly found in beer were summarized by Sannino et al. [31]. In addition, short-chain fatty acids such as isovaleric, hexanoic, octanoic and decanoic acids produced by yeasts during fermentation can create unpleasant, rancid flavor characteristics [25,32].

The aim of this study was to investigate yeast strains for their brewing ability and to test the promising strains in small brewing trials. Yeast strains that cannot metabolize maltose and those that release phenolic off-flavors were excluded from further investigation as the main objective of this study was to discover strains that produce less well known wheat beer flavors and secondary metabolites that create pleasant and novel aroma impressions.

2. Materials and Methods

2.1. Yeast Strains and Nutrient Media

Table 1 shows the yeast strains with their given abbreviations (abbr.) that were investigated in this study. They were grown in YM bouillon (malt extract 0.3%, yeast extract 0.3%, peptone 0.5%, glucose anhydrous 1.0%, double distilled water 97.9%) for 72 h at 20 °C on a WiseShake orbital shaker at an orbital agitation of 80 rpm (Witeg Labortechnik GmbH, Wertheim, Germany) before they could be used for the 96-well plate tests.

Table 1. Yeast strains applied to 96-well plate tests.

Strain Number	Strain Abbr.	Species	Strain Number	Strain Abbr.	Species
YH837A-3D4	CM1	*Cyberlindnera misumaiensis*	YH725B-1F6	M10	*Metschnikowia* sp.
YH824B-1I7	CM2	*Cyberlindnera misumaiensis*	YH729A-1G1	M11	*Metschnikowia* sp.
YH837B-3D5	CM3	*Cyberlindnera misumaiensis*	YH730A-1G2	M12	*Metschnikowia* sp.
YHMH22AA-3H1	CSa1	*Cyberlindnera saturnus*	YH730B-1G3	M13	*Metschnikowia* sp.
WYSC 29	DH1	*Debaryomyces hansenii*	YH737A-1G4	M14	*Metschnikowia* sp.
WYSC 1664	DH3	*Debaryomyces hansenii*	YHMH50A-3B4	NH2	*Nakazawaea holstii*
DSMZ70244	DH4	*Debaryomyces hansenii*	YHMH50-3C5	NH3	*Nakazawaea holstii*
PI BB 151/BLQ 208	DH8	*Debaryomyces hansenii*	YH607A-1D1	NH5	*Nakazawaea holstii*
YH811A-3F4	DH10	*Debaryomyces hansenii*	YHAK1A-3I1	PK1	*Pichia kluyveri*
YH813A-3F5	DH11	*Debaryomyces hansenii*	PI S 4; Lu 36	SF1	*Saccharomycopsis fibuligera*
YH817-3F6	DH12	*Debaryomyces hansenii*	PI S 5; Lu 29/45	SF2	*Saccharomycopsis fibuligera*
YH832A-1I9	DH13	*Debaryomyces hansenii*	PI S 6; Lu 27	SF3	*Saccharomycopsis fibuligera*
YH883A-3E8	DH14	*Debaryomyces hansenii*	PI S 7; Lu 26	SF4	*Saccharomycopsis fibuligera*
YH885A-3E9	DH15	*Debaryomyces hansenii*	PI S 9; Lu 7/41	SF5	*Saccharomycopsis fibuligera*
YH887A-3E10	DH16	*Debaryomyces hansenii*	PI S 10; Lu 35	SF6	*Saccharomycopsis fibuligera*
YH888A-3F1	DH17	*Debaryomyces hansenii*	PI S 12; Lu 32	SF8	*Saccharomycopsis fibuligera*
CBS 5074	H1	*Hanseniaspora uvarum*	PI S 15; Lu 6/48	SF10	*Saccharomycopsis fibuligera*
CBS 2585	H2	*Hanseniaspora uvarum*	PI BB 132	S2	*Schizosaccharomyces pombe*
CBS 314T	H3	*Hanseniaspora uvarum*	DSMBZ 70576	S3	*Schizosaccharomyces pombe*
CBS 1517	H4	*Hanseniaspora uvarum*	BLQ TUM S4	S4	*Schizosaccharomyces pombe*
RIBM A4	H5	*Hanseniaspora uvarum*	TUM G5S	S5	*Schizosaccharomyces pombe*
RIBM A7	H6	*Hanseniaspora uvarum*	TUM G6S	S6	*Schizosaccharomyces pombe*
RIBM A10	H7	*Hanseniaspora uvarum*	TUM G7S	S7	*Schizosaccharomyces pombe*
RIBM A12	H8	*Hanseniaspora uvarum*	TUM G8S	S8	*Schizosaccharomyces pombe*
RIBM A15	H9	*Hanseniaspora uvarum*	TUM G9S	S9	*Schizosaccharomyces pombe*
RIBM A16	H10	*Hanseniaspora uvarum*	TUM G10S	S10	*Schizosaccharomyces pombe*
YHMH56B-3C8	KS2	*Kazachstania servazzii*	TUM G11S	S11	*Schizosaccharomyces pombe*
YHMH47B-3C4	KS3	*Kazachstania servazzii*	YH725A-1F5	T21	*Torulaspora delbrueckii*
BLQ TUM K1	K1	*Kluyveromyces lactis*	YH739A-1G6	T22	*Torulaspora delbrueckii*
TUM G2K	K2	*Kluyveromyces lactis*	YH739B-1G7	T23	*Torulaspora delbrueckii*
TUM G3K	K3	*Kluyveromyces lactis*	YH740B-1H1	T24	*Torulaspora delbrueckii*
TUM G4K	K4	*Kluyveromyces lactis*	YH832D-3D3	T25	*Torulaspora delbrueckii*
TUM G5K	K5	*Kluyveromyces lactis*	YH824A-1I6	T26	*Torulaspora delbrueckii*
TUM G6K	K6	*Kluyveromyces lactis*	YH837D-3D7	T27	*Torulaspora delbrueckii*
TUM G7K	K7	*Kluyveromyces lactis*	YHYF2-1D6	T28	*Torulaspora delbrueckii*
TUM G8K	K8	*Kluyveromyces lactis*	YHMS8-1E2	TM1	*Torulaspora microellipsoides*
TUM G9K	K9	*Kluyveromyces lactis*	YH601A-1C5	WA1	*Wickerhamomyces anomalus*
TUM G1KM	Km 1	*Kluyveromyces marxianus*	YH601C-1C6	WA2	*Wickerhamomyces anomalus*
TUM G2KM	Km 2	*Kluyveromyces marxianus*	YHB23-1D7	WA3	*Wickerhamomyces anomalus*
TUM G3KM	Km 3	*Kluyveromyces marxianus*	YHDC1211B-3H3	WA4	*Wickerhamomyces anomalus*
TUM G4KM	Km 4	*Kluyveromyces marxianus*	TUM WA25	WA25	*Wickerhamomyces anomalus*
TUM G5KM	Km 5	*Kluyveromyces marxianus*	WYSC 137	ZR1	*Zygosaccharomyces rouxii*
TUM G6KM	Km 6	*Kluyveromyces marxianus*	WYSC 84	ZR2	*Zygosaccharomyces rouxii*
TUM G7KM	Km 7	*Kluyveromyces marxianus*	WYSC 82	ZR3	*Zygosaccharomyces rouxii*
TUM G8KM	Km 8	*Kluyveromyces marxianus*	WYSC 83	ZR4	*Zygosaccharomyces rouxii*
TUM G9KM	Km 9	*Kluyveromyces marxianus*	WYSC/G20	ZR5	*Zygosaccharomyces rouxii*
TUM G10KM	Km 10	*Kluyveromyces marxianus*	WYSC/G 1673	ZR6	*Zygosaccharomyces rouxii*
CBS 3082T	LK1	*Lachancea kluyveri*	WYSC/G 1998	ZR7	*Zygosaccharomyces rouxii*
YHMH44C-3C3	M3	*Metschnikowia* sp.	WYSC/G 2005	ZR8	*Zygosaccharomyces rouxii*
YHMS-1D4	M4	*Metschnikowia* sp.	WYSC/G 2091	ZR9	*Zygosaccharomyces rouxii*
YH750A-1H2	M5	*Metschnikowia* sp.	WYSC/G 2093	ZR10	*Zygosaccharomyces rouxii*
YH713B-1E5	M6	*Metschnikowia* sp.	WYSC/G 2142	ZR11	*Zygosaccharomyces rouxii*
YH721A-1F2	M7	*Metschnikowia* sp.	WYSC/G 2274	ZR13	*Zygosaccharomyces rouxii*
YH724A-1F3	M8	*Metschnikowia* sp.	WYSC/G 2325	ZR14	*Zygosaccharomyces rouxii*
YH724B-1F4	M9	*Metschnikowia* sp.	YHMH55A-3B10	ZF1	*Zygotorulaspora florentina*

The individual nutrient solutions were prepared according to the recipes as shown in Table 2, filled up to 1000 mL with deionized water, adjusted to a pH of 5.2 and were sterile filtered with 0.2

µm pore sized Whatman™ sterile filters (Sigma-Aldrich, St. Louis, MO, USA) once the components were completely dissolved. For each nutrient or inhibiting medium, 7 g/L of Difco Yeast Nitrogen Base (BD Biosciences, San Jose, CA, USA) and Y3627 Sigma Yeast Carbon Base (Sigma-Aldrich, St. Louis, MO, USA), according to the Table 2, were used as nitrogen and carbon sources. The individual carbohydrate concentrations were adjusted to approximately those of a standard wort.

Table 2. Utilized nutrients and inhibiting substances and their compositions for the 96-well microtiter plate tests.

Nutrient/Inhibiting Media	Supplier	Concentrations of Nutrient/Inhibiting Media	Yeast Carbon Base
D(+)-Glucose, anhydrous	Carl Roth, Karlsruhe, Germany	10 g/L	0 g/L
D(-)-Fructose BioChemica	AppliChem, Darmstadt, Germany	10 g/L	0 g/L
D(+)-Sucrose ≥ 99.0%	Merck, Darmstadt, Germany	20 g/L	0 g/L
D(+)-Maltose monohydrate 95%	Alfa Aesar, Karlsruhe, Germany	55 g/L	0 g/L
D-Maltotriose 98%	Alfa Aesar, Karlsruhe, Germany	20 g/L	0 g/L
D(+)-Melibiose ≥ 99.0%	Merck, Darmstadt, Germany	10 g/L	0 g/L
Ethanol 1% (*v/v*), undenatured	Serva, Heidelberg, Germany	1% (*v/v*)	23.3 g/L
Ethanol 2% (*v/v*), undenatured	Serva, Heidelberg, Germany	2% (*v/v*)	23.3 g/L
Ethanol 5% (*v/v*), undenatured	Serva, Heidelberg, Germany	5% (*v/v*)	23.3 g/L
Ethanol 8% (*v/v*), undenatured	Serva, Heidelberg, Germany	8% (*v/v*)	23.3 g/L
Ethanol 10% (*v/v*), undenatured	Serva, Heidelberg, Germany	10% (*v/v*)	23.3 g/L
IBU 50 (iso-α-acid), isomerized hop extract 30%	Hopsteiner, Mainburg, Germany	3.3 g/L	23.3 g/L
IBU 100 (iso-α-acid), isomerized hop extract 30%	Hopsteiner, Mainburg, Germany	6.6 g/L	23.3 g/L
β_1: β-acid 100 ppm, beta-rich hop extract 40%	Hopsteiner, Mainburg, Germany	0.1 g/L	23.3 g/L
β_2: β-acid 200 ppm, beta-rich hop extract 40%	Hopsteiner, Mainburg, Germany	0.2 g/L	23.3 g/L
βmix_1: α- + β-stock-mix (50 IBU + 100 ppm β-acids)	Hopsteiner, Mainburg, Germany	3.3 g/L, 0.1 g/L	23.3 g/L
βmix_2: α- + β-stock-mix (100 IBU + 200 ppm β-acids)	Hopsteiner, Mainburg, Germany	6.6 g/L, 0.2 g/L	23.3 g/L

2.2. Yeast Sample Preparation

Yeast cells grown in YM bouillon were washed prior to being applied to the nutrient media in the 96-well plates to eliminate the influence of the previous growth medium. For the washing step, 40 g of each yeast suspension was added to 50 mL Falcon™ centrifuge tubes (Sarstedt, Nümbrecht, Germany). The suspension was centrifuged (centrifuge Z 366 K, HERMLE, Wehingen, Germany) for 10 min at 750 g before the supernatant of nutrient solution was discarded. After resuspending the yeast with sterile water, the washing procedure was repeated twice to ensure that there were no residues of the nutrient medium. To ensure direct comparability of the yeast growth, the cells of each strain were counted using the Cellometer® Vision (Nexcelom Bioscience LLC, Lawrence, MA, USA) and the corresponding yeast amounts were calculated to start the 96-well plate tests with an incubation of 100,000 yeast cells for each well, resulting in 500,000 cfu/mL.

For the 96-well microtiter plate Corning™ Costar™ (Sigma-Aldrich, St. Louis, MO, USA) tests, each plate was prepared under sterile conditions in the sterile bench Uniflow UVUB 1200 (UniEquip, Planegg, Germany) to avoid contamination. The pipetting scheme was structured in such a way that 200 µL of the 17 different nutrient solutions listed in Table 2 were pipetted in each case to four wells. The yeast cells were inoculated in triplicate with a cell count of 100,000 in columns 2 to 4, 6 to 8, and 10 to 12, leaving one blank value for each media in columns 1, 5 and 9. Finally, each plate was sealed with a cover sheet SealPlate® 100-SEAL-PLT (Excel Scientific, Victorville, CA, USA) to protect against contamination. The extinctions of the suspensions of the 96-well microtiter plates were recorded in triplicate using the Photometer Synergy 2™ Multi-Mode Detection Microplate Reader (BioTek®, Winooski, VT, USA), subsequently, at a wavelength of 600 nm and 25 °C. The results were recorded using the BioTek Gen5™ software. Before each measurement, the plate was automatically

shaken for 30 seconds by the device to ensure that the suspensions were thoroughly mixed. As the inoculated plates were incubated in a tempered room at 28 °C, they were kept in a styrofoam box to avoid condensate forming on the cover sheets. Every 24 h, the measurement of the extinction was repeated for four consecutive days. This measurement method over 72 hours allowed the complex screening of a multitude of strains in a manageable time.

2.3. Phenolic Off-Flavor Test (POF Test)

Three stock solutions were prepared for the POF test. Therefore, 1 g of each trans-ferulic and trans-cinnamic acid was diluted in 20 mL of 96% (*v/v*) ethanol while 0.2 g p-coumaric acid was added to 20 mL of 96% (*v/v*) ethanol. The ferulic and cinnamic acids were both dosed at 1% into 45–50 °C tempered YM agar (malt extract 0.3%, yeast extract 0.3%, peptone 0.5%, glucose anhydrous 1.0%, agar 2.0%, double distilled water 95.9%) while the coumaric acid was added at 0.2%. Immediately thereafter, the POF agar plates were poured out under sterile conditions and allowed to cool. The yeast strains to be examined were then removed from wort agar slopes with an inoculation loop and spread on three plates, each containing the different acids. A positive control with the strain LeoBavaricus-TUM 68® (Research Center Weihenstephan for Brewing and Food Quality, Freising, Germany) and a negative control with the strain Frisinga-TUM 34/70® (Research Center Weihenstephan for Brewing and Food Quality, Freising, Germany) were also prepared. After an incubation time of three days at 28 °C, the POF plates were evaluated.

2.4. Sensory Odor Test

In order to perform the sensory odor test, unhopped wort was first prepared from unhopped liquid Bavarian Pilsner malt extract (extract anhydrous 72–79%, Weyermann®, Bamberg, Germany) by re-diluting the extract with hot water to 7 °P and 12 °P wort. Since standard alcoholic beers generally have an original gravity of about 12 °P, whereas maltose- and maltotriose-negative yeasts require an original gravity of about 7 °P to avoid exceeding the limit of 0.5% (*v/v*) alcohol in the final beer, the original gravity was adjusted accordingly in the experiments. The use of malt extract ensured a standardized wort quality for all the trials to be able to compare the different yeast strains. In each case, 75 mL of wort was filled into sterile flasks and cooled down to room temperature at 20 °C. The different yeast strains were inoculated with a sterile loop from wort agar slopes into each of the 7 °P and 12 °P wort batches and incubated for 72 h. Thereafter, the individual samples were evaluated by a sensory panel of ten panelists, describing the odor impressions as well as rating them as positive, negative or neutral.

2.5. Yeast Propagation and Wort Analysis

The promising yeast strains not showing phenolic off-flavors but desired results from the sensory odor test and with the ability to at least ferment glucose, fructose and maltose to produce a beer with a reduced or even a standard alcohol content of approx. 5% (*v/v*) were investigated for their propagation ability. If found to grow in high cell numbers, they were pitched from wort agar slopes under sterile conditions into a 500 mL flask containing 250 mL of unhopped brewer's wort (12.5 °P, pH 5.4) diluted from Bavarian Pilsner malt extract (Weyermann®, Bamberg, Germany). The malt extract was chosen as the composition is always the same and the results are highly comparable. The composition of the wort used for the yeast propagation and fermentation trials is shown in Table 3.

After 72 h of propagation at 20 °C and orbital shaking at 80 rpm on a WiseShake orbital shaker (Witeg Labortechnik GmbH, Wertheim, Germany), the yeast suspensions were transferred to sterile 2500 mL flasks filled with 2000 mL unhopped wort and propagated for a further 72 h. Following the propagation, the cell count was performed using the Cellometer® Vision (Nexcelom Bioscience LLC, Lawrence, MA, USA).

Table 3. Sugar composition of the wort used for the fermentation trials.

Sugar Composition in g/L	
Fructose	2.8
Glucose	11.9
Sucrose	1.5
Maltose	58.2
Maltotriose	12.7

2.6. Fermentation Trials

The pitching rate was chosen at 30×10^6 cells/mL ($\pm\sigma = 3 \times 10^6$ cells/mL) as many non-*Saccharomyces* cells are much smaller than usual brewer's yeast cells and therefore, show differing fermentation speeds [33]. The respective amounts of propagated yeast suspensions were inoculated in triplicate into 1400 mL of sterilized wort (wort attributes can be viewed in Table 3) in 2000 mL sterile Duran glass bottles (Schott AG, Mainz, Germany) with glass fermentation blocks on top. The samples were stored at 27 °C for the main fermentation. The fermentation progress was checked every 24 h by recording the weight loss (mainly due to escaping carbon dioxide) of the samples. The fermentation was considered to be completed either after 336 h or as soon as the samples had lost 50 g. Starting at 1400 mL of 12.5 °P wort, the weight loss was set at 50 g, since over 60% of the fermentable extract is spent once this limit is reached. This is based on the assumption of Balling that during fermentation, an average of 2.0665 g of extract is converted to 1 g of alcohol, 0.9565 g of carbon dioxide and 0.11 g of yeast [34]. Following the main fermentation, the samples were sealed with sterile screw caps and the young beers were ripened for another 168 h at 27 °C to continue fermentation of the remaining extract while the carbon dioxide produced during the secondary fermentation could be enriched in the beers under pressure. After maturation, the young beers were stored in the sealed bottles at 2 °C for a further 168 h.

2.7. Analysis of Produced Beers

The final beer samples from five selected promising strains were analyzed for the parameters shown in Table 4.

Table 4. Examinations of the final beers after MEBAK [1].

Analysis	MEBAK No.	Device
Sugar composition	MEBAK II 3.2.2.1.2	HPLC UltiMate 3000 (Thermo Fisher Scientific, Waltham, MA, USA)
Original wort, ethanol content, extract content	MEBAK WBBM 2.9.6.3	Bending vibration and NIR spectroscopy, Alcolyzer Plus with DMA 5000 X sample 122 (Anton-Paar GmbH, Ostfildern, Germany)
pH value	MEBAK WBBM 2.13	pH meter with pH electrode, ProfiLine pH3210 pH meter (Xylem Inc., New York, NY, USA)
Fatty esters, 2-Phenylethanol	MEBAK WBBM 2.23.6	GC-FID Clarus 580 (Perkin Elmer, Waltham, MA, USA), Column: 50m 0.32mm Phenomenex FFAP, 0.25 µm
Acetaldehyde, Ethylacetate, higher alcohols (n-Propanol, i-Butanol, Amylalcohols)	MEBAK WBBM 2.21.1	GC-FID Clarus 580, Turbo Matrix 40, Head Space (Perkin Elmer, Waltham, MA, USA), Column: INNOWAX cross-linked polyethylene glycol, 60m × 0.32mm 0.5 µm
Diacetyl, 2,3-Pentandione	MEBAK WBBM 2.21.5.1	GC-FID Clarus 580, Turbo Matrix 40, Head Space (Perkin Elmer, Waltham, MA, USA), Column: INNOWAX cross-linked polyethylene glycol, 60m × 0.32mm 0.5 µm

[1] MEBAK® (2012), Editor: Dr. F. Jacob: The MEBAK collection of brewing analysis methods: Wort, beer and beer-based beverages. Collection of methods of the Mitteleuropäischen Brauchtechnischen Analysenkommission. Self-published by MEBAK.

Additionally, glycerol was analyzed using the glycerol UV-test by Boehringer Mannheim/R-Biopharm, Germany, according to the manufacturer's instructions.

2.8. Sensory Evaluation

Finally, the beer samples were profiled at 20 °C room temperature by a sensory panel of ten DLG (Deutsche Landwirtschafts-Gesellschaft e.V.)-certified assessors to determine the main flavor components and the acceptance of the individual beers. Based on the sensory profiling, a sensory analysis was developed to obtain a consistent rating which was not differentiated into smell and taste.

3. Results

Primarily, yeast species were selected that were already used in food fermentations and are known for their aroma contribution, as described in the introduction. The heat map displayed in Figure 1 summarizes the results of the 96-well microtiter plate tests and gives an overview of the brewing ability of the selected 110 yeast strains from 18 yeast species with regard to the key parameters of wort sugar utilization, the ability to grow in hopped wort and ethanol resistance. Additionally, POF and odor tests were taken into consideration as the aim of this study was to identify yeast strains that are able to produce pleasant novel flavors during the wort fermentation, however, excluding POF positive yeast strains. Although non-alcoholic beers are as promising as those with an average alcohol content, this study first focused on the yeasts strains which are able to metabolize maltose to produce a standard or at least an alcohol-reduced beer.

As shown in Figure 1 and to enable a simple evaluation, the carbohydrate utilization, ethanol and hop resistance were evaluated according to whether there is growth or no growth. The limit above which growth can be detected was set at an extinction of 0.4 as distinct growth can be determined from this value. Values equal to or above 0.4 are displayed in green in the heat map, while values smaller than 0.4 are marked in red. The POF test and odor test provide initial information about the aroma profile the yeasts can produce during the fermentation of wort. The alc./non alc. column summarizes the sugar utilization of the individual yeasts and is therefore an indicator of whether the yeast is suitable for the production of an alcohol-free beer or beer with an average alcohol content. The yeast's suitability for beer fermentation outlines all the described aspects and already represents a selection of the screened yeasts for further fermentation trials.

3.1. Overall Results

According to the results in Figure 1, the hop as well as ethanol resistance of the screened yeasts is generally high enough to be considered for the production of a standard beer. Neither a concentration of iso-α-acids of up to 100 ppm, a concentration of β-acids of up to 200 ppm, nor a mixture of both acids inhibit the growth of most yeasts. Only 3% of the total screened yeasts, namely two yeast strains of the species *D. hansenii* (DH13, DH14) and one *T. delbrueckii* strain (T22) do not grow at a β-acid concentration of 200 ppm in the medium. DH14 was the only yeast significantly inhibited even at an iso-α-acid concentration of 100 ppm, so that no more growth was recorded. Moreover, the growth of individual yeast strains was restricted as soon as higher concentrations of iso-α- (100 IBU) and β-acids (200 ppm) in the form of βmix_2 were used, which affected approx. 7% of the yeasts, including one *L. kluyveri* strain (LK1), two yeasts of the species *Metschnikowia* (M8, M13), one strain of the yeast *Z. rouxii* (ZR6) and one strain of the species *D. hansenii* (DH16) besides the above-mentioned strains DH13, DH16 and T22. In addition, the βmix_1 containing 50 IBU iso-α-acids and 100 ppm β-acids inhibited growth in nearly 4% of the yeasts. While only 3% of them were inhibited at a concentration of 200 ppm β-acids, this shows that the combination of both hop acids seems to lead to an enhanced inhibitory effect which suggests that both hop acids have an antimicrobial effect. However, since a standard beer does not contain more than 50 ppm iso-α-acids and the β-acids only become relevant after an additional cold hopping, all yeasts have a sufficient hop tolerance for standard beer brewing. In contrast to iso-α-acids, β-acids are poorly soluble in beer. They are not isomerized during wort boiling and are not conveyed into beer [35].

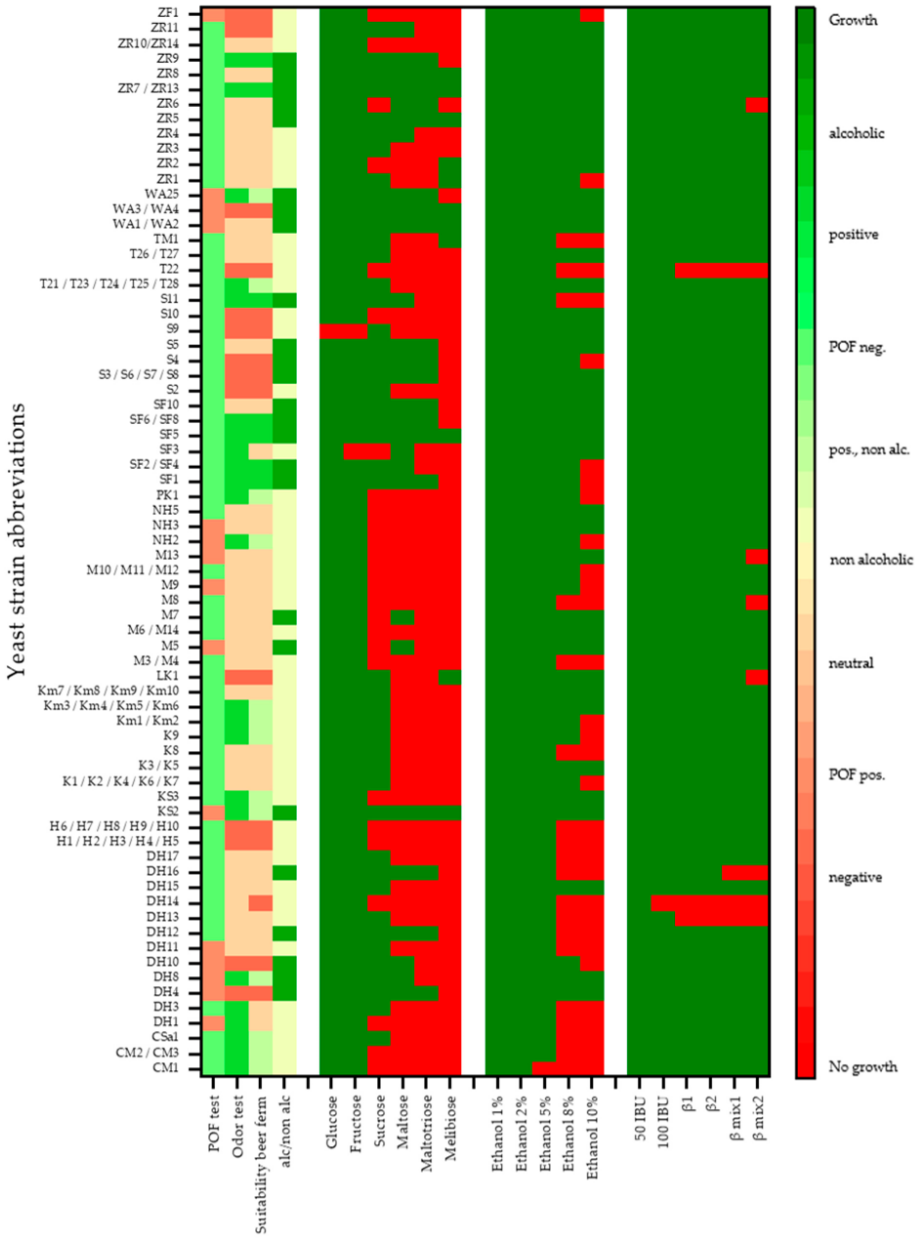


Figure 1. Heat map indicating carbohydrate utilization, ethanol and hop resistance (iso-α-acids 50 IBU/100 IBU = 50 and 100 international bitterness units; β_1/β_2 = β-acids in different concentrations; $\beta mix_1/\beta mix_2$ = mix from iso-α-acids und β-acids in different concentrations) as well as POF and odor test results of the yeast strains screened in the 96-well plate test (ferm. = fermentation; alc. = alcoholic; neg. = negative; pos. = positive).

With regard to the ethanol tolerance, approx. 25% of the screened yeasts show no growth at an ethanol concentration of 8% (*v/v*), especially yeasts of the species *Cyberlindnera*, *D. hansenii* and *H. uvarum*. For only one of the 110 screened yeasts, namely one strain of the species *C. misumaiensis* (CM1), growth was significantly inhibited at an ethanol concentration of 5% (*v/v*). Consequently, apart from strain CM1, all the screened yeasts have a sufficient ethanol tolerance to produce a standard beer with 5% (*v/v*) ethanol concentration. However, since the yeast CM1 can only be used to produce non-alcoholic beers or possibly mixed fermentations because of its carbohydrate utilization (only glucose and fructose), its lower ethanol tolerance is negligible.

Sugar utilization is variable; however, it is striking that all the yeast strains, with the exception of *S. pombe* S9, can metabolize glucose. *S. pombe* S9 does not ferment fructose either, similarly to the strain *S. fibuligera* SF3. Since there are literature sources that state that *S. pombe* can metabolize glucose and *S. fibuligera* utilizes fructose [36–38], the lack of growth in this case can be considered to be an outlier. In terms of the disaccharides sucrose, maltose and melibiose, the ability to ferment these carbohydrate sources decreases. Yet, while 65% of the yeasts still metabolize sucrose, maltose can only be utilized by approx. 30% of the yeasts, whereas melibiose is utilized by 14%. The trisaccharide maltotriose is metabolized by merely 25% of the yeast strains. Therefore, only about one third of the screened yeasts can be considered for the production of alcoholic beers and these belong to the species *D. hansenii*, *K. servazzii*, *S. fibuligera*, *S. pombe*, *W. anomalus*, *Z. rouxii* and *Metschnikowia*. In order to further limit the selection of suitable yeasts for the production of beers with novel flavor properties, all yeasts with POF-positive results or neutral or negative odor outcomes, such as *D. hansenii*, *K. servazzii*, *Metschnikowia* and *W. anomalus*, were excluded.

As a result, six strains of the species *S. fibuligera* (SF1, SF2, SF4, SF5, SF6, SF8), one *S. pombe* strain (S11) and three strains of the species *Z. rouxii* (ZR7, ZR9, ZR13) were found to be POF-negative and also had a positive odor impression. In order to ensure a manageable experimental framework in this study, half of the promising yeast strains were further tested for the following fermentation trials, namely SF2, SF4, SF8, S11 und ZR9. Although several other yeasts species, such as *C. misumaiensis*, *C. saturnus*, *K. marxianus* and *T. delbrueckii*, revealed pleasant sensory odor impressions and POF-negative outcomes, they were not further considered for this study as they can be considered for the production of non-alcoholic beers.

3.2. *Exemplary Evaluation of Carbohydrate Utilisation, Ethanol and Hop Resistance*

The diagrams (Figures 2–4) represent the extinction curves at 600 nm of carbohydrate utilization, ethanol and hop tolerance using the yeast *T. delbrueckii* T21 as an example over a measurement period of 72 h and an incubation temperature of 28 °C. The yeast *T. delbrueckii* T21 was selected as it is maltose-negative and therefore, represents about 70% of the screened yeasts. The extinction threshold of whether the yeast has grown in the nutrient media was marked at 0.4 in the diagrams. Values above an extinction of 0.4 indicate significant growth, while values below 0.4 indicate that the yeast has not grown in the corresponding nutrient solution.

3.2.1. Carbohydrate Utilization

Figure 2 depicts the increase of the extinctions of the yeast strain T21 in different sugar solutions over 72 h. After 24 h incubation at 28 °C, it can be observed that the wells containing glucose, fructose and sucrose solutions clearly exceed the growth threshold of 0.4 so they already show distinct turbidity. Taking the standard deviation into account, the curves for glucose and sucrose are similar, whereas the yeast's metabolism of fructose is slightly lower. Although the extinction curve for maltose increases slightly within the first 24 h, it then stagnates at an extinction of approx. 0.2 and does not show a significant growth accordingly. The values for the wort sugars maltotriose and melibiose hardly increase. Therefore, the sugars maltose, maltotriose and melibiose cannot be utilized by the yeast T21.

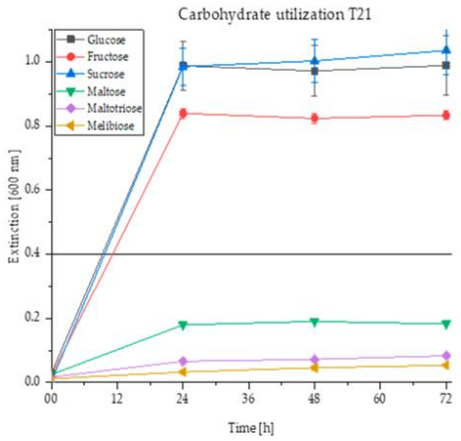


Figure 2. Yeast growth of the strain *T. delbrueckii* T21 measured at an extinction of 600 nm in nutrient solutions with the carbohydrate additives glucose, fructose, sucrose, maltose, maltotriose and melibiose in the 96-well microtiter plates at 28 °C for four days and the respective standard deviations per measurement day (displayed as error bars). The growth threshold is set at an extinction of 0.4.

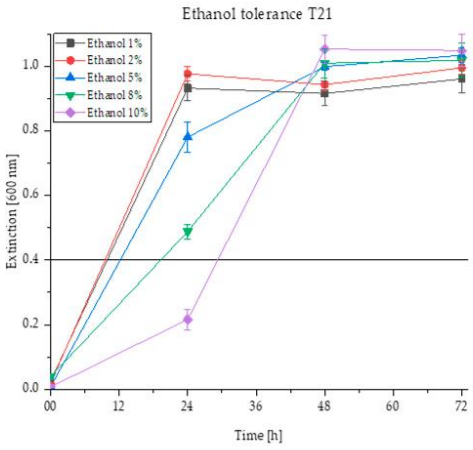


Figure 3. Ethanol tolerance of the strain *T. delbrueckii* T21 shown as growth at an extinction of 600 nm in nutrient solutions with different ethanol concentrations of 1% (*v/v*), 2% (*v/v*), 5% (*v/v*), 8% (*v/v*) and 10% (*v/v*) in the 96-well microtiter plates at 28 °C for four days and the respective standard deviations per measurement day (displayed as error bars). The growth threshold is set at an extinction of 0.4.

While the growth of the yeast T21 in Figure 2 stagnates after the first measuring day, the growth of other yeasts could be partially detected first after 48 or 72 h of incubation by reaching the growth threshold of 0.4. All the yeast strains presented in the heat map in Figure 1 and evaluated as "non-alcoholic" show similar curves at least for maltose and maltotriose, as demonstrated in Figure 2 remaining below the extinction threshold of 0.4.

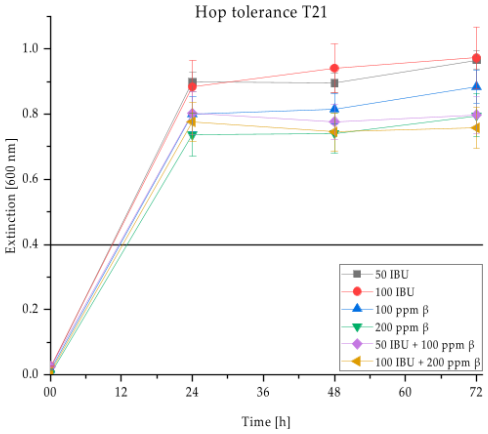


Figure 4. Hop tolerance of the strain *T. delbrueckii* T21 shown as growth at an extinction of 600 nm in nutrient solutions with different hop acid additives 50 and 100 IBU, 100 and 200 ppm β-acids, 50 IBU + 100 ppm β-acids and 100 IBU + 200 ppm β-acids in the 96-well microtiter plates at 28 °C for four days and the respective standard deviations per measurement day (displayed as error bars). The growth threshold is set at an extinction of 0.4.

3.2.2. Ethanol Tolerance

From Figure 3, in which the ethanol tolerance of the yeast strain T21 was investigated, it can be seen that the extinction curves for all concentrations of 1 to 10% (*v/v*) ethanol far exceed the threshold of 0.4. It is noticeable that the yeast growth within the first 24 h differs depending on the ethanol concentration. The higher the ethanol concentration in the nutrient solution, the slower the growth. This is shown by the decreasing turbidity at increasing ethanol concentrations. Nevertheless, there is significant growth at an ethanol concentration of up to 8% (*v/v*) after 24 h, while the growth in the 10% (*v/v*) solution remains below the 0.4 extinction threshold. During the second measuring day, all extinction values converge again and the yeast T21 also shows a significant growth at 10% (*v/v*) after 48 h of incubation. Taking the standard deviations into account, the extinction values all remain similar at approx. 1.0 after 48 h.

3.2.3. Hop Tolerance

With regard to the yeast's hop tolerance, Figure 4 demonstrates that the yeast T21 grows within 24 h of incubation at 28 °C in nutrient solutions with different hop acid additives as the curves already exceed the extinction limit of 0.4 during 24 h of incubation. While the curves of all nutrient solutions look similar and the yeast stops growing after 24 h in all cases, it can nevertheless be observed that the growth of the yeast T21 is the strongest at 50 and 100 IBU iso-α-acids. As soon as β-acids were added into the nutrient solutions, the extinctions and the corresponding growth of the yeast are slightly lower.

3.3. Results of the Sensory Odor Test

In addition to the general evaluation of the odor tests, which are divided into the categories "positive", "neutral" and "negative" within the heat map (cf. Figure 1), a descriptive evaluation was performed by ten panelists. The odor descriptions are listed in Table 5.

Table 5. Evaluation of the descriptive sensory odor impressions of the screened yeast strains after fermentation in 7 °P wort as well as 12 °P wort.

Strain Abbr.	Evaluation Odor 7 °P	Evaluation Odor 12 °P	Strain Abbr.	Evaluation Odor 7 °P	Evaluation Odor 12 °P
CM1	isoamyl acetate	isoamyl acetate	M10	neutral, yeasty	yeasty
CM2	isoamyl acetate	honey, sweet	M11	neutral, yeasty	yeasty
CM3	fruity	yeasty	M12	neutral, yeasty	yeasty
CSa1	isoamyl acetate	isoamyl acetate	M13	sour, yeasty	yeasty
DH1	yeasty, lychee	fruity, banana	M14	neutral, yeasty	neutral, yeasty
DH3	fruity, pineapple	yeasty, strong	NH2	fruity	overripe banana
DH4	clove-like, acidic	clove-like, acidic, earthy	NH3	pale, fruity	yeasty, fruity
DH8	fruity	rice wine, fruity	NH5	yeasty, diacetyl	fruity
DH10	cardboard	cardboard, fruity	PK1	yeasty, isoamyl acetate	yeasty, isoamyl acetate
DH11	neutral	yeasty, fruity	SF1	fruity	fruity, plum
DH12	neutral	neutral	SF2	fruity	fruity, plum
DH13	neutral	yeasty, diacetyl	SF3	fruity	fruity, plum, honey
DH14	yeasty, wort-like	bready, caramel	SF4	fruity	fruity, plum, honey
DH15	fruity, sulfurous	neutral, earthy	SF5	fruity	fruity, phenolic
DH16	DMS	cardboard, DMS	SF6	fruity	fruity, honey
DH17	earthy, cooked	diacetyl	SF8	fruity	fruity, honey, plum
H1	fruity, acidic	acidic	SF10	fruity	fruity, musty
H2	fruity, acidic	acidic	S2	sulfurous, pea soup	sulfurous, pea soup
H3	fruity, acidic	acidic	S3	fruity, sulfurous	sulfurous
H4	fruity, acidic	acidic	S4	fruity, sulfurous	sulfurous
H5	neutral	acidic	S5	malty, wort-like, sweet	sulfurous, sweet
H6	neutral	acidic	S6	sulfurous	sulfurous, pea soup
H7	neutral	acidic	S7	sulfurous	sulfurous, pea soup
H8	sweet-sourish	acidic	S8	sulfurous	sulfurous
H9	sweet-sourish	acidic	S9	sulfurous	sulfurous
H10	sweet-sourish	acidic	S10	fruity, sulfurous	fruity, sulfurous
KS2	yeasty	yeasty, fruity	S11	fruity	fruity, honey
KS3	fruity, flowery	cooked vegetables	T21	neutral	fruity
K1	fruity, sulfurous	fruity	T22	sour, cheesy	cheesy
K2	fruity	fruity	T23	neutral	fruity
K3	fruity	fruity	T24	neutral	fruity
K4	fruity, pungent	fruity, pungent	T25	neutral	fruity, apple, berry
K5	fruity	fruity	T26	neutral	fruity, mirabelle plum
K6	fruity	fruity	T27	neutral	yeasty
K7	fruity	fruity	T28	neutral, flowery	fruity, mirabelle plum
K8	fruity, smoky	fruity, smoky	TM1	yeasty, sweet	yeasty, acidic
K9	fruity	fruity, apple	WA1	wheat beer	phenolic, wheat beer
Km 1	fruity	fruity	WA2	vinous	phenolic, vinous
Km 2	fruity	fruity	WA3	musty	rotten eggs, phenolic
Km 3	fruity	fruity	WA4	sherry	phenolic, vinous
Km 4	fruity	fruity	WA25	fruity, pungent	fruity, banana
Km 5	fruity, sweet	fruity, sweet	ZR1	diacetyl	diacetyl
Km 6	fruity	fruity	ZR2	fruity, pineapple	fruity
Km 7	fruity, sulfurous	fruity, sulfurous	ZR3	fruity	fruity
Km 8	fruity, musty	fruity, musty	ZR4	neutral, yeasty	yeasty
Km 9	fruity, musty	fruity, musty	ZR5	fruity, acidic	fruity, musty
Km 10	fruity	fruity	ZR6	fruity, honey	fruity, honey
LK1	neutral, acidic	slightly fruity, acidic	ZR7	fruity, sweet	fruity, sweet
M3	ethyl acetate	yeasty	ZR8	fruity	fruity, tea-like
M4	neutral, yeasty	neutral, yeasty	ZR9	fruity, citrus	fruity
M5	neutral, yeasty	neutral, yeasty	ZR10	sweet-sourish, fruity	sweet-sourish, fruity
M6	neutral, yeasty	neutral, yeasty	ZR11	fruity, smoky	fruity, smoky
M7	neutral, yeasty	neutral, yeasty	ZR13	fruity, honey, sweet	fruity, honey, sweet
M8	neutral	sour	ZR14	fruity, sweet	fruity
M9	neutral, yeasty	neutral, yeasty	ZF1	fruity	fruity, pear, musty

In general, the results in Table 5 show that the sensory differences of the fermented wort samples do not differ between 7 °P and 12 °P in most cases and that a great variety of the yeasts produce fruity flavors. Only the wort samples fermented with *Metschnikowia* sp. (M3 to M14) and *T. microellipsoides* (TM1) were found to be mainly neutral with regard to their flavor perception while e.g. *Z. florentina* (ZF1) causes a fruity, but also an unpleasant musty smell. Although *K. lactis* (K1 to K9) and *S. pombe* (S2 to S11) partly produce disagreeable sulfurous and musty flavors, they also reveal fruity odor impressions. The wort fermented with *L. kluyveri* (LK1) and *H. uvarum* (H1 to H10) shows neutral to fruity flavors as well, however, the samples are unacceptable due to their acidic smell. The yeasts *D. hansenii* (DH1 to DH17) and *W. anomalus* (WA1 to WA4, WA25) turned out to be the strains with the greatest diversity. While all screened *W. anomalus* strains are POF positive, this varies between the investigated *D. hansenii* strains. The olfactory impression of the *W. anomalus* samples clearly reflects

the POF character, although their acceptance varies strongly. The wort samples fermented with *D. hansenii* range from neutral to sulfurous to an exotic fruity and POF character and differ widely in their sensory acceptance. The samples fermented with *N. holstii* (NH2 to NH5) and *Z. rouxii* (ZR1 to ZR14) are partly found to be positive due to their fruity properties, yet some strains produce dominating POF and diacetyl odors. Generally, *Cyberlindnera* (CM1 to CM3, CSa1), *P. kluyveri* (PK1), *K. servazzii* (KS2, KS3), *K. marxianus* (Km1 to Km10), *S. fibuligera* (SF1 to SF10) and *T. delbrueckii* (T21 to T28) produce the purest fruity flavors and are perceived as the most pleasing. *S. fibuligera* is characterized by its ability to generate particularly plum-like aromas, while *Cyberlindera* and *P. kluyveri* reveal noticeable amounts of isoamyl acetate.

3.4. Fermentation Trials

As previously mentioned in Chapter 3.1, the promising yeast strains *S. fibuligera* SF2, SF4, SF8, *S. pombe* S11 and *Z. rouxii* ZR9 were further tested in brewing trials.

3.4.1. Fermentation Process

In Figure 5, the fermentation process of the five selected yeast strains is illustrated by a total weight loss in grams of the fermentation samples due to the extract degradation of the yeast strains over a period of 336 h at a fermentation temperature of 27 °C. The yeast strain S11 shows by far the highest weight loss over this period and the extract degradation by this yeast occurs faster compared to the other yeasts. After 240 h (10 days), a weight loss of more than 50 g of the original pitching quantity of 1400 mL wort is reached. With an average original wort content of 12.5 °P, more than 60% of the extract is therefore fermented and the secondary fermentation can be started. The fermentation process of the other four yeasts shows that they ferment much slower than the strain S11 and have a significantly lower fermentation activity. None of the four yeast strains reached the specified weight loss of 50 g within 14 days. While ZR9 and SF4 show a similar fermentation activity, followed by SF2, and even continue to ferment slowly during the second week, SF8 degraded the least extract and did not even reach a weight loss of 20 g after 336 h. However, it should be noted that the yeasts of the species *S. fibuligera* formed distinct lumps during their growth phase, which means that counting the cells results in a significantly increased standard deviation compared with conventional yeasts. Accordingly, the number of 30×10^6 cells/mL could possibly be imprecise, which may have led to a lower cell number of the samples fermented with SF8.

3.4.2. Wort Sugar Utilization

Figure 6 and Table 6 provide a more detailed insight of the fermentation activity of the five selected yeast strains.

Table 6. Original wort [%], apparent attenuation [%], ethanol content [% *v/v*], pH and glycerol [g/L] values in the final beers fermented with the yeast strains *S. fibuligera* SF2, SF4, SF8, *S. pombe* S11 and *Z. rouxii* ZR9.

Strain Abbr	Original Wort [%]	Apparent Attenuation [%]	Ethanol Content [% *v/v*]	pH Value	Glycerol [g/L]
SF2	12.73 ±σ = 0.07	39.53 ±σ = 0.33	2.65 ±σ = 0.03	4.37 ±σ = 0.01	0.24 ±σ = 0.00
SF4	12.65 ±σ = 0.11	46.53 ±σ = 2.05	3.10 ±σ = 0.14	4.30 ±σ = 0.03	0.24 ±σ = 0.01
SF8	12.78 ±σ = 0.07	22.57 ±σ = 1.11	1.52 ±σ = 0.08	4.51 ±σ = 0.04	0.33 ±σ = 0.01
S11	12.54 ±σ = 0.06	86.43 ±σ = 1.79	5.73 ±σ = 0.13	4.39 ±σ = 0.01	0.43 ±σ = 0.04
ZR9	12.86 ±σ = 0.06	58.07 ±σ = 4.20	3.95 ±σ = 0.30	4.39 ±σ = 0.05	0.47 ±σ = 0.01

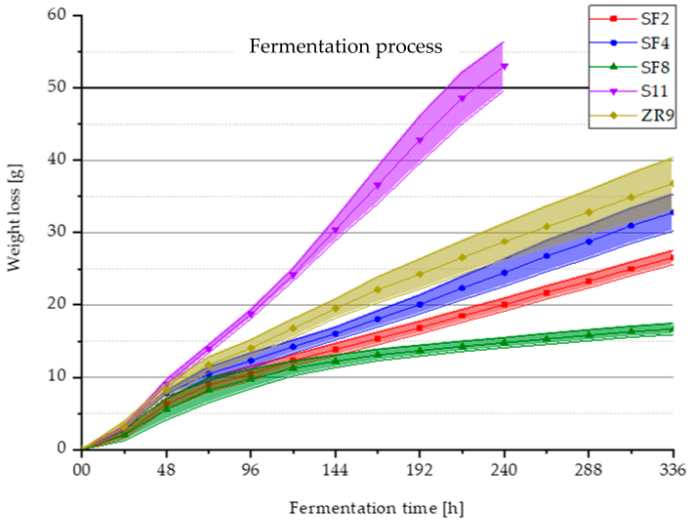


Figure 5. Mean values (n = 3) with standard deviations of the fermentation samples' total weight loss in grams of the yeast strains *S. fibuligera* SF2, SF4, SF8, *S. pombe* S11 and *Z. rouxii* ZR9 during the fermentation process over a fermentation time period of 336 hours (14 days) at 27 °C.

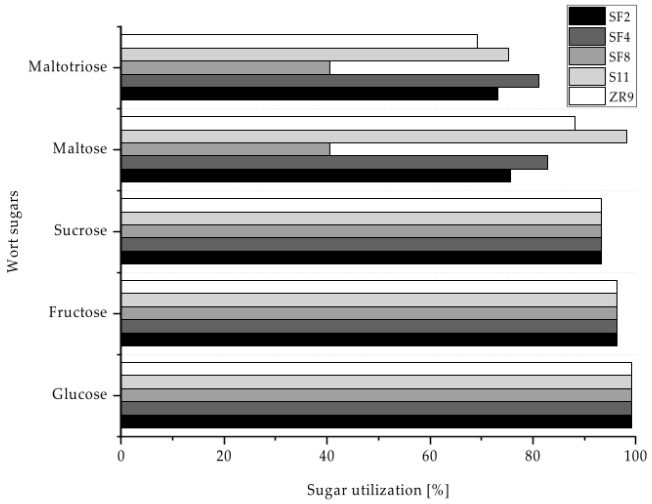


Figure 6. Wort sugar utilization in % of yeast strains *S. fibuligera* SF2, SF4, SF8, *S. pombe* S11 and *Z. rouxii* ZR9 during fermentation from wort to beers.

All experimental yeasts almost completely metabolize glucose, fructose and sucrose. The yeast strain S11 can metabolize over 98% maltose and almost 80% maltotriose, which explains the high degree of fermentation and the beer's final ethanol concentration of around 5.7% (*v/v*). This is followed by yeasts ZR9 and SF4, which ferment about 80% of each maltose and maltotriose on average, followed by SF2, which still ferments over 70% maltose and maltotriose. Consequently, the ethanol content of the beers is between 2.6 and 4% (*v/v*). The strain SF8 shows the weakest fermentation performance

and only metabolizes half the maltose and maltotriose compared with SF4, which results in lower fermentation and an ethanol concentration of only 1.5% (*v/v*) in the final beer. As a result of the 96-well plate screening, it was expected that S11, SF2 and SF4 would not metabolize maltotriose and therefore, SF8 and ZR9 had a significantly higher sugar utilization. In fact, SF8 and ZR9 were the worst metabolizers of maltotriose in comparison with the other three yeast strains (cf. Figure 1). However, as maltotriose cannot be fully utilized by the yeasts, this may have led to deviations in the screening. Still, all yeasts without exception can cause a pH drop to between 4.3 and 4.5. The glycerol content of the final beers was also measured. All beers have low glycerol levels between 0.24 and 0.47 g/L, while the average value in beer lies between 1 and 3 g/L with a threshold of 10 g/L [39]. Therefore, glycerol does not noticeably influence the mouthfeel of the beers.

3.4.3. Volatile Compounds in Final Beers

In the final beers, esters, organic acids, higher alcohols, acetaldehyde and ketones were analyzed by GC measurement. In order to ensure comparability between the individual compounds, the analytical results are expressed as a percentage and are listed in Figure 7.

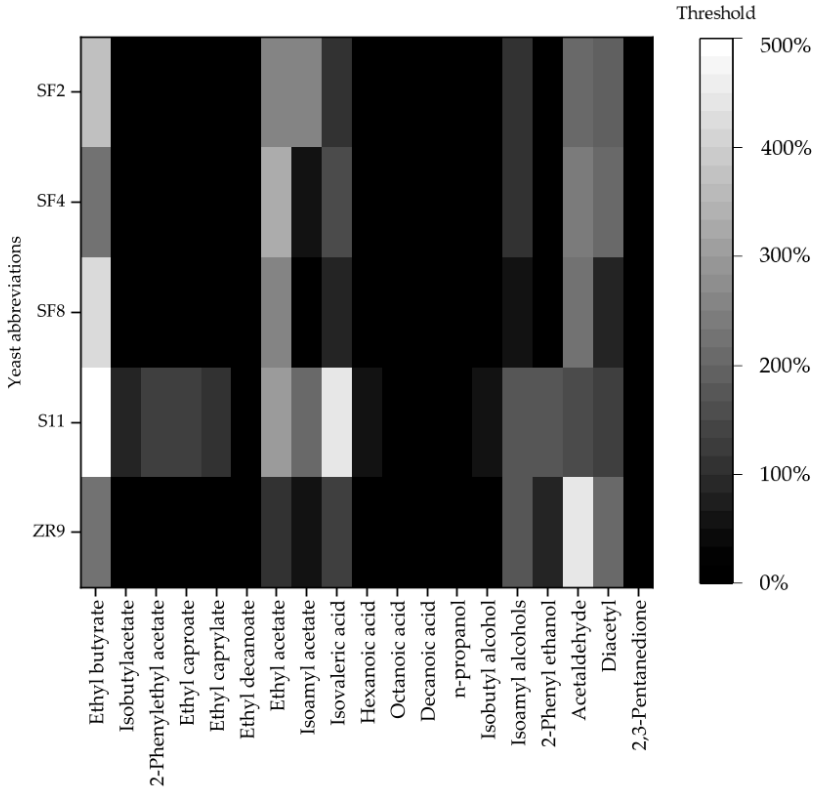


Figure 7. Heat map of volatile compounds in final beers produced by the yeast strains *S. fibuligera* SF2, SF4, SF8, *S. pombe* S11 and *Z. rouxii* ZR9 during fermentation of brewer's wort at 27 °C and detected by headspace gas chromatography measurement. The 100% value represents the threshold of volatile compounds in beer, which is based on relative values from Meilgaard and Sannino et al. [29,31].

By evaluating the different esters measured in the beers, Figure 7 shows that all the yeasts produce ethyl butyrate in concentrations above the threshold. Except for the beer fermented with ZR9, the other beers contain considerable amounts of ethyl acetate. Isoamyl acetate is above the threshold in the beers produced with SF2 and S11. While the organic acids are only significantly above the threshold in the form of isovaleric acid in the sample fermented with S11, higher alcohols are not significantly increased. In contrast, all five yeast strains are responsible for the acetaldehyde values being above the threshold. While 2,3-pentanedione has no influence as a ketone, diacetyl is increased in the beers fermented with SF2, SF4 and ZR9.

3.4.4. Aroma Profiles of Final Beers

The tasting results in Figure 8 give an overview of the variety of aromas produced by the different yeast strains. Evaluation levels range from 0 to 10, where 0 is considered to be the weakest and 10 the strongest intensity for the flavor impressions "fruity", "plum", "grape", "red wine", "honey", "floral", "clove", "sulfurous", "wheat beer" and "strawberry".

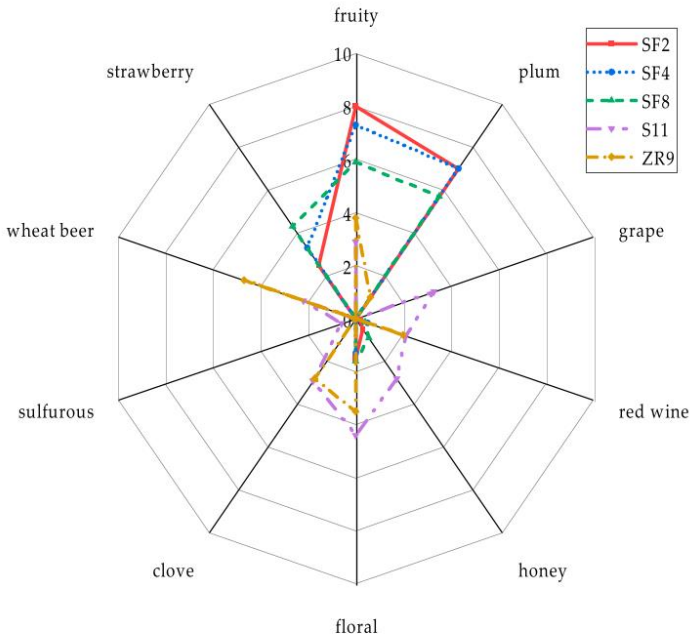


Figure 8. Aroma profiles of the beers fermented with the yeast strains *S. fibuligera* SF2, SF4, SF8, *S. pombe* S11 and *Z. rouxii* ZR9.

By comparing the aroma profiles of the beers from the five different yeast strains, it is obvious that the yeasts SF2, SF4 and SF8 have a similar profile with distinct fruity notes that reach towards plum and strawberry. Only the flavor intensity of the strain SF8 is weaker. Additionally, some slightly floral aromas are noticeable. The aroma profile of the yeast strain S11 goes more towards the direction of wheat beer. Although there are fruity notes that are described as grape, the aroma spectrum is quite broad. Dominating floral impressions are supported by clove, honey and red wine aromas. The beer fermented with ZR9 exhibits a remarkable clove-like wheat beer character as well as floral, fruity and less intense red wine notes. Finally, the acceptance of the five beers was compared. The beer fermented with the yeast SF2 scores best with 8 out of 10 points, closely followed by the beer produced with SF4

(7.8 points). The strain SF8 with 7.4 points and S11 with 7.3 points are in the midfield, while beer from yeast ZR9 with 6.7 points is rated the worst in sensory terms.

Although the tasting samples were spot checks, the results largely reflect the flavor results from the GC analyses. The three screened *S. fibuligera* strains produce significant ester amounts of ethyl butyrate and ethyl acetate, which lead to sweet and fruity aroma impressions in taste and smell. SF2 further releases isoamyl acetate which underlines the pleasant fruity character [31]. Despite diacetyl being present in the beers fermented with SF2 and SF4, a buttery off-flavor was not noticed [40]. As both beers are rated highest, diacetyl cannot be considered to be an off-flavor in this case. Strawberry and plum flavors are not obviously apparent in the GC results, so further analyses may be necessary. The yeast strain S11 generally forms high amounts of ester compounds with a total concentration of 86 mg/L, which even exceeds the usual value of 70 mg/L for top-fermented beers, and leads to the fruity aroma character [41]. The significant amount of isoamyl acetate in conjunction with isobutyl acetate causes the wheat beer impression, which is supported by the increased amount of isoamyl alcohols. The synergistic effect of isoamyl acetate and isobutyl acetate was already published by Meilgaard in 1975 [29]. The floral aroma may be attributed to the threshold of 2-phenyl ethanol being exceeded. Although 2-phenylethyl acetate stays below the threshold, the concentration is still relatively high and can be traced back to the honey-like flavor. The overall increased values of esters, acids and higher alcohols trigger the perceptible red wine aroma. It was expected that the high amount of isovaleric acid would produce a rancid, cheesy aroma [29], however, this was not criticized by the sensory panel. The beer fermented with the yeast strain ZR9 has a remarkable wheat beer character and is most likely caused by the increased values of isoamyl alcohols, whereas the fruity perception can be mainly attributed to ethyl butyrate. Despite the fact that acetaldehyde and diacetyl are above the threshold, there is no remarkable flavor influence. Only the citrus note from the sensory odor test (cf. Table 5) may originate from acetaldehyde. In addition, the odor impressions of the sensory odor tests presented in Table 5 correspond basically to the results of the GC analysis and the tastings of the sensory panel. In some cases, the beers contain aromas which are not directly attributed to a single precise volatile compound, however, the flavor is a result of the synergistic interaction of different substances [25].

4. Discussion

The results described in this study reveal that the applied screening method provides a useful first overview to investigate a great number of yeast strains for their brewing ability in a short period of time. Still, it should be remarked that extinction curves may not have increased or slightly decreased over the measurement period. The reason for this is the yeast growth within the single wells. A few yeast strains tended to sediment on the walls of the wells, causing decreased turbidity at the bottom and in the liquid of the wells. Although the well plate was shaken by the photometer prior to measurement, the cells stayed in place. Other yeasts formed small clumps during their growth phase, for example, *S. fibuligera*, which also caused non-homogeneous distribution in the wells. As a result, the light from the photometer was directed through a medium with apparently less turbidity resulting in a lower extinction value, which could lead to measurement inaccuracies.

The screening of the utilization of different wort carbohydrates was a main criterion for the later selection of fermentation trials. Although there are reliable sources on the fermentation ability of glucose, sucrose and maltose by various yeast species published by Kurtzman [36], it often varies within the species as to the kind of carbohydrates they utilize. For instance, within the species *S. pombe*, *T. delbrueckii*, *W. anomalus*, *Z. rouxii* and *K. lactis*, the sugar utilization differs significantly between the single yeast strains [36]. With the exception of *W. anomalus*, the same observations were made during the screening. While *W. anomalus* strains consistently metabolized all carbohydrates used in the test, this varied within the other strains mentioned. However, it should be noted that only five *W. anomalus* strains were screened.

Furthermore, it was of interest to investigate the yeasts' assimilation of fructose, maltotriose and melibiose. Since non-*Saccharomyces* yeasts are classified as top-fermenting yeasts, it is to be expected

that they cannot metabolize melibiose based on literature [11]. However, since the comparison with regard to the melibiose utilization of Wickerham is only made between *S. cerevisiae* as a top-fermenting yeast and *S. pastorianus* as a bottom-fermenting yeast, it is assumed that individual top-fermenting yeast strains also partly utilize melibiose. Whether melibiose is metabolized depends on the presence of the enzyme alpha galactosidase in the yeast cell. This enzyme catalyzes the hydrolysis from melibiose to galactose and glucose [42]. The screening reveals that *W. anomalus* species, in particular, ferment melibiose. As a result of this study, some strains of the species *Z. rouxii*, *S. fibuligera*, *K. servazzii*, *L. kluyveri* and *T. microellipsoides* also have the ability to assimilate melibiose. Nevertheless, only 14% of the 110 screened yeasts were able to utilize this kind of sugar.

Some of the screened yeast species, such as *P. kluyveri*, are already known from beer fermentations for which there is already a patent for the production of low-alcohol or alcohol-free beer [43]. *S. pombe* was first isolated from an ancient beer called "pombe" by Lindner in 1893 [44] and is still one of the main aroma-contributing species today in many mixed beer fermentations of traditional African beers [45]. *T. delbrueckii* was first assumed to be a suitable yeast strain for beer brewing by King and Dickinson in 2000 [46] and *W. anomalus*, *Z. rouxii* as well as *Z. florentina* were investigated in relation to wort fermentations. The yeasts have a common ability to release a high concentration of volatile flavor metabolites such as esters by fermenting brewer's wort, which results in fruity and floral sensory impressions [47,48]. The fruity and floral flavors can be confirmed in this study for the yeast strains *S. pombe* (S11) and *Z. rouxii* (ZR9). The strain S11 releases even greater amounts of esters than typical wheat beer yeast strains do. In addition, further *T. delbrueckii* strains with promising flavor impressions were discovered following the investigations of Michel et al. [6], whereby none of the screened strains could utilize maltose. According to several existing studies, *W. anomalus* releases significant amounts of ethyl butyrate and ethyl acetate leading to fruity flavor impressions. *P. kluyveri* and *Z. florentina* produce considerable amounts of pleasant esters, such as isoamyl acetate, during the fermentation process of all-malt wort, which can be attributed to the fruity banana-like sensory perception [5,7,43,47]. While the *W. anomalus* (WA1 to WA4, WA25) strains investigated in this study revealed a broad range of different positive to negative sensory odor impressions, *P. kluyveri* (PK1) actually produced remarkable amounts of pleasant isoamyl acetate. In contrast, the *Z. florentina* strain (ZF1) caused a musty smell next to the fruity flavor impression. However, since only a single yeast strain was tested, it does not represent the entire species. As per previous investigations, *Z. rouxii* strains release higher amounts of esters causing fruity, floral and solvent-like aromas. However, its diacetyl content is described to be above the threshold which negatively influences the sensory perception [48]. The analysis of the strain ZR9 confirms that the yeast produces an increased amount of diacetyl. Although this could not be tasted in the final beer, one *Z. rouxii* (ZR1) strain was found to be responsible for noticeable amounts of diacetyl in the sensory odor test. The floral and fruity aroma components are also confirmed by the sensory panelists and the fruity compounds, in particular, were detected during the GC measurement for the strain ZR9. A solvent-like aroma could not be observed explicitly. Nevertheless, the beer fermented with ZR9 had a distinct wheat beer character and a significant amount of ethyl acetate was analyzed. Even though ZR9 and S11 were described as wheat beer types by the panelists and were described as clove-like, they were evaluated as POF-negative. As part of the POF test, the yeasts obviously did not convert the ferulic acid to 4-vinylguaiacol, which would have led to a clove-like odor. Despite the POF-negative results, it can be assumed that the clove-like flavor either came from eugenol, which was not analyzed in this study [49], or that the complex interaction of the flavors in the beer triggered the clove-like wheat beer character. Neither strains of the species *Z. rouxii* nor *S. pombe* are known to convert ferulic acid to 4-VG in the literature, which is confirmed by the POF tests.

Most of the investigated yeast species i.e. *C. saturnus*, *C. misumaiensis*, *D. hansenii*, *H. uvarum*, *L. kluyveri*, *Metschnikowia* sp., *N. holstii*, *P. kluyveri*, *S. fibuligera*, *S. pombe*, *T. delbrueckii*, *W. anomalus* are known for their positive fruity aroma impact caused by releasing volatile flavor compounds during wine fermentation [50–54] and *C. misumaiensis*, *H. uvarum* and *W. anomalus* also during cider fermentation [20,55–57]. Various studies state that *H. uvarum*, *S. pombe*, *T. delbrueckii* and *W. anomalus*

improve the aromatic quality and the flavor complexity of wine [58–60]. The release of pleasant fruity aroma components during wine production by the yeasts can only be partially transferred to the fermentation of brewer's wort as the *H. uvarum* and *L. kluyveri* strains screened in this study released rather undesired flavors. While *C. misumaiensis*, *C. saturnus*, *P. kluyveri*, *S. fibuligera* and *T. delbrueckii* almost all developed positive aroma impressions in wort (cf. Table 5), the aroma impression of *D. hansenii*, *N. holstii*, *S. pombe* and *W. anomalus* varied within the species. Therefore, a direct transfer from wine to beer aromas cannot be assumed and differences could be based on the different fermentation medium (e.g. due to a varying carbohydrate composition) and fermentation conditions. Nevertheless, the diversity of flavors within one species is so extensive that individual strains do not necessarily provide information on the entire species, a fact proven by the species *S. pombe* in this study. Only strain S11 shows a positive aroma impression in the odor test, while the nine other strains mainly released negative flavor components (cf. Figure 1, Table 5). Therefore, although general statements can be made about the aroma profile of one yeast species, individual yeast strains may deviate from the norm and produce exceptionally positive or negative aromas.

C. saturnus in particular is known for its remarkably high synthesis of isoamyl acetate during wine fermentation which, as previously mentioned, causes a banana-like flavor and ethyl acetate, which leads to a fruity flavor [5,61–63]. Although the yeast strain CSa1 was not explicitly analyzed for isoamyl acetate, the olfactory tests clearly revealed that perceptible amounts of this compound were produced. In terms of the fermentation of rice wine with *S. fibuligera*, it can be highlighted that recent studies revealed the distribution of higher alcohols and acetate esters [64]. In fact, the investigated strains of the species *S. fibuligera* (SF2, SF4 and SF8) mainly produced large amounts of ethyl acetate, and SF2 additionally produced isoamyl acetate esters from brewer's wort. Larger amounts of higher alcohols were only released in the form of isoamyl alcohols, which did not exceed the threshold of 65 mg/L (cf. Figure 7) [29].

Although *C. misumaiensis*, *L. kluyveri* and *N. holstii* are associated with the fermentation of wine or apple products [53,55,56] they have not yet been investigated in literature for their flavor characteristics. This study reveals that it might be promising to investigate specific yeasts of the species *C. misumaiensis* for their volatile flavor compounds as in some cases they produced sweet, honey-like aromas and consistently pleasant fruity flavors with isoamyl acetate to some extent (cf. Figure 1, Table 5). Referring to wort fermentation, this yeast species could be used for the production of non-alcoholic beers.

Originally related to the production of milk products, *K. lactis* and *K. marxianus* were also screened for their brewing ability as the literature points out that both yeasts have the ability to produce aroma compounds that give fruity, floral and honey-like aromas [65–67]. While the sensory odor test showed that the yeasts of the species *K. lactis* produced slightly fruity aromas, some of the yeasts of the species *K. marxianus* stood out with remarkably pleasant aroma impressions (cf. Figure 1, Table 5). These yeasts could also be considered for the production of alcohol-free beers.

5. Conclusions

In general, it can be summarized that the yeast strains of the species *S. fibuligera* are fairly interesting for the fermentation of brewer's wort to produce an alcoholic beer. The strains SF2, SF4 and SF8 release, without exception, desirable fruity flavors reminiscent of plum and berry. Esters such as ethyl butyrate and ethyl acetate result in sweet and fruity aroma impressions. Additionally, significant amounts of isoamyl acetate were analyzed in the beer fermented with SF2, which underlined the pleasant fruity character. In contrast to the yeast strains *S. pombe* (S11) and *Z. rouxii* (ZR9), no wheat beer aroma was perceptible in the beers produced with the *S. fibuligera* yeast strains. Although the POF test results of S11 and ZR9 were negative, both yeasts still produced flavor compounds that gave a phenolic, clove-like character.

Author Contributions: M.M. and Y.M. conceived and designed the experiments; Y.M. performed the experiments and analyzed the data using OriginPro 2019 as statistical software; M.H. and D.M. organized and provided a

large amount of yeast strains and M.H. revised the conception; Y.M. wrote the paper; M.M. and F.J. revised the manuscript and agreed the submission.

Funding: This research was funded by the Wifö (Wissenschaftsförderung der Deutschen Brauwirtschaft e.V., Berlin, Germany) in the project AiF 20658 N and by the Ministry of Agriculture of the Czech Republic (RO1918).

Acknowledgments: The isomerized hop extract 30% (iso-α-acid) and the beta-rich hop extract 40% were kindly provided by Hopsteiner, Mainburg, Germany.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Piškur, J.; Rozpędowska, E.; Polakova, S.; Merico, A.; Compagno, C. How did *Saccharomyces* evolve to become a good brewer? *Trends Genet.* **2006**, *22*, 183–186. [CrossRef] [PubMed]
2. Claussen, N.H. Eine Methode zur Anwendung von Hansens Reinzuchtsystem bei der Herstellung von englischen gelagerten Biersorten. *Wochenschr. Brau* **1904**, *1904*, 370–383.
3. De Estela-Escalante, W.; Rosales-Mendoza, S.; Moscosa-Santillán, M.; González-Ramírez, J.E. Evaluation of the fermentative potential of *Candida zemplinina* yeasts for craft beer fermentation. *J. Inst. Brew.* **2016**, *122*, 530–535. [CrossRef]
4. Michel, M.; Meier-Dörnberg, T.; Jacob, F.; Methner, F.-J.; Wagner, R.S.; Hutzler, M. Pure non-*Saccharomyces* starter cultures for beer fermentation with a focus on secondary metabolites and practical applications. *J. Inst. Brew.* **2016**, *122*, 569–587. [CrossRef]
5. Basso, R.F.; Alcarde, A.R.; Portugal, C.B. Could non-*Saccharomyces* yeasts contribute on innovative brewing fermentations? *Food Res. Int.* **2016**, *86*, 112–120. [CrossRef]
6. Michel, M.; Meier-Dörnberg, T.; Zarnkow, M.; Jacob, F.; Hutzler, M. Screening for the Brewing Ability of Non-Saccharomyces Yeast and Optimization of Fermentation Performance of One Torulaspora Delbrueckii Strain Found Suitable for Beer Production. In Proceedings of the World Brewing Congress, Denver, CO, USA, 13–17 August 2016.
7. Ravasio, D.; Carlin, S.; Boekhout, T.; Groenewald, M.; Vrhovsek, U.; Walther, A.; Wendland, J. Adding Flavor to Beverages with Non-Conventional Yeasts. *Fermentation* **2018**, *4*, 15. [CrossRef]
8. Narziß, L.; Back, W.; Miedaner, H.; Lustig, S. Untersuchung zur Beeinflussung der Geschmacksstabilität durch Variation technologischer Parameter bei der Bierherstellung. *Monatsschrift für Brauwissenschaft* **1999**, *1999*, 192–206.
9. Stewart, G.G. The Horace Brown Medal Lecture: Forty Years of Brewing Research. *J. Inst. Brew.* **2009**, *115*, 3–29. [CrossRef]
10. Vidgren, V.; Multanen, J.-P.; Ruohonen, L.; Londesborough, J. The temperature dependence of maltose transport in ale and lager strains of brewer's yeast. *FEMS Yeast Res.* **2010**, *10*, 402–411. [CrossRef]
11. Wickerham, L.J. A simple technique for the detection of melibiose-fermenting yeasts1. *J. Bacteriol.* **1943**, *46*, 501–505.
12. Srinivasan, V.; Goldberg, D.; Haas, G.J. Contributions to the Antimicrobial Spectrum of Hop Constituents. *Econ. Bot.* **2004**, *58*, S230–S238. [CrossRef]
13. Hazelwood, L.A.; Walsh, M.C.; Pronk, J.T.; Daran, J.-M. Involvement of vacuolar sequestration and active transport in tolerance of *Saccharomyces cerevisiae* to hop iso-alpha-acids. *Appl. Environ. Microbiol.* **2010**, *76*, 318–328. [CrossRef] [PubMed]
14. Bamforth, C.W. Wort Composition and Beer Quality. In *Brewing Yeast Fermentation Performance*, 2nd ed.; Smart, K., Ed.; Blackwell Science: Oxford, UK; Malden, MA, USA, 2003; pp. 75–85. ISBN 9780470696040.
15. Ding, J.; Huang, X.; Zhang, L.; Zhao, N.; Yang, D.; Zhang, K. Tolerance and stress response to ethanol in the yeast *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **2009**, *85*, 253. [CrossRef]
16. Narziss, L.; Back, W.; Gastl, M.; Zarnkow, M. *Abriss der Bierbrauerei*, 8th ed.; John Wiley & Sons Incorporated: Newark, NJ, USA, 2017; ISBN 9783527340361.
17. Shinohara, T.; Kubodera, S.; Yanagida, F. Distribution of phenolic yeasts and production of phenolic off-flavors in wine fermentation. *J. Biosci. Bioeng.* **2000**, *90*, 90–97. [CrossRef]
18. Montanari, L.; Perretti, G.; Natella, F.; Guidi, A.; Fantozzi, P. Organic and Phenolic Acids in Beer. *LWT* **1999**, *32*, 535–539. [CrossRef]

19. Schwarz, K.J.; Stübner, R.; Methner, F.-J. Formation of styrene dependent on fermentation management during wheat beer production. *Food Chem.* **2012**, *134*, 2121–2125. [CrossRef]
20. De Arruda Moura Pietrowski, G.; dos Santos, C.M.E.; Sauer, E.; Wosiacki, G.; Nogueira, A. Influence of fermentation with *Hanseniaspora* sp. yeast on the volatile profile of fermented apple. *J. Agric. Food Chem.* **2012**, *60*, 9815–9821. [CrossRef]
21. Benito, S.; Palomero, F.; Morata, A.; Calderón, F.; Suárez-Lepe, J.A. New applications for *Schizosaccharomyces pombe* in the alcoholic fermentation of red wines. *Int. J. Food Sci. Technol.* **2012**, *47*, 2101–2108. [CrossRef]
22. Amaya-Delgado, L.; Herrera-López, E.J.; Arrizon, J.; Arellano-Plaza, M.; Gschaedler, A. Performance evaluation of *Pichia kluyveri*, *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* in industrial tequila fermentation. *World J. Microbiol. Biotechnol.* **2013**, *29*, 875–881. [CrossRef]
23. Steensels, J.; Verstrepen, K.J. Taming wild yeast: Potential of conventional and nonconventional yeasts in industrial fermentations. *Annu. Rev. Microbiol.* **2014**, *68*, 61–80. [CrossRef]
24. Hiralal, L.; Olaniran, A.O.; Pillay, B. Aroma-active ester profile of ale beer produced under different fermentation and nutritional conditions. *J. Biosci. Bioeng.* **2014**, *117*, 57–64. [CrossRef] [PubMed]
25. Verstrepen, K.J.; Derdelinckx, G.; Delvaux, F.R. Esters in beer-part 1: The fermentation process: More than ethanol formation. *Cerevisia* **2003**, *28*, 41–49.
26. Vanderhaegen, B.; Neven, H.; Coghe, S.; Verstrepen, K.J.; Derdelinckx, G.; Verachtert, H. Bioflavoring and beer refermentation. *Appl. Microbiol. Biotechnol.* **2003**, *62*, 140–150. [CrossRef]
27. Verstrepen, K.J.; Derdelinckx, G.; Dufour, J.-P.; Winderickx, J.; Thevelein, J.M.; Pretorius, I.S.; Delvaux, F.R. Flavor-active esters: Adding fruitiness to beer. *J. Biosci. Bioeng.* **2003**, *96*, 110–118. [CrossRef]
28. Olaniran, A.O.; Hiralal, L.; Mokoena, M.P.; Pillay, B. Flavour-active volatile compounds in beer: Production, regulation and control. *J. Inst. Brew.* **2017**, *123*, 13–23. [CrossRef]
29. Meilgaard, M.C. Flavor Chemistry of Beer: Part II: Flavor and Threshold of 239 Aroma Volatiles. *MBAA Tech. Tech. Quart. Master. Brew. Assoc. Am.* **1975**, *12*, 151–168.
30. Pires, E.J.; Teixeira, J.A.; Brányik, T.; Vicente, A.A. Yeast: The soul of beer's aroma—A review of flavour-active esters and higher alcohols produced by the brewing yeast. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 1937–1949. [CrossRef]
31. Sannino, C.; Mezzasoma, A.; Buzzini, P.; Turchetti, B. Non-conventional Yeasts for Producing Alternative Beers. In *Non-conventional Yeasts: From Basic Research to Application*; Sibirny, A., Ed.; Springer International Publishing: Cham, Switzerland, 2019; pp. 361–388. ISBN 978-3-030-21109-7.
32. Olšovská, J.; Vrzal, T.; Štěrba, K.; Slabý, M.; Kubizniaková, P.; Čejka, P. The chemical profiling of fatty acids during the brewing process. *J. Sci. Food Agric.* **2019**, *99*, 1772–1779. [CrossRef]
33. Wahyono, A.; Kang, W.-W.; Park, H.-D. Characterization and application of *Torulaspora delbrueckii* JK08 and *Pichia anomala* JK04 as baker's yeasts. *J. Food Nutr. Res.* **2015**, *54*.
34. Esslinger, H.M. *Handbook of Brewing: Processes, Technology, Markets*; John Wiley & Sons: Hoboken, NJ, USA, 2009; ISBN 3527623493.
35. Sakamoto, K.; Konings, W.N. Beer spoilage bacteria and hop resistance. *Int. J. Food Microbiol.* **2003**, *89*, 105–124. [CrossRef]
36. Kurtzman, C.P. (Ed.) *The Yeasts. A taxonomic study*, 5th ed.; Elsevier: Amsterdam, The Netherland, 2011; ISBN 9780123847072.
37. Owens, J.D. *Indigenous Fermented Foods of Southeast Asia*; CRC Press: Boca Raton, FL, USA, 2014; ISBN 0429105916.
38. Lee, S.M.; Jung, J.H.; Seo, J.-A.; Kim, Y.-S. Bioformation of Volatile and Nonvolatile Metabolites by *Saccharomycopsis fibuligera* KJJ81 Cultivated under Different Conditions-Carbon Sources and Cultivation Times. *Molecules* **2018**, *23*, 2762. [CrossRef] [PubMed]
39. Zhao, X.; Procopio, S.; Becker, T. Flavor impacts of glycerol in the processing of yeast fermented beverages: A review. *J. Food Sci. Technol.* **2015**, *52*, 7588–7598. [CrossRef] [PubMed]
40. Duong, C.T.; Strack, L.; Futschik, M.; Katou, Y.; Nakao, Y.; Fujimura, T.; Shirahige, K.; Kodama, Y.; Nevoigt, E. Identification of Sc-type ILV6 as a target to reduce diacetyl formation in lager brewers' yeast. *Metab. Eng.* **2011**, *13*, 638–647. [CrossRef] [PubMed]
41. Herrmann, M. *Entstehung und Beeinflussung qualitätsbestimmender Aromastoffe bei der Herstellung von Weißbier*; Technische Universität München: Munich, Germany, 2005.

42. Boulton, C.; Quain, D. *Brewing Yeast and Fermentation*; John Wiley & Sons: Hoboken, NJ, USA, 2008; ISBN 0470999403.
43. Saerens, S.; Swiegers, J.H. Production of Low-Alcohol or Alcohol-Free beer with Pichia Kluyveri Yeast Strains. Google Patents EP2964742A2, 2016.
44. Lindner, P. *Schizosaccharomyces pombe* n. sp., ein neuer Gährungserreger. *Wochenschrift für Brauerei* **1893**, 1298–1300.
45. Sefa-Dedeh, S.; Sanni, A.I.; Tetteh, G.; Sakyi-Dawson, E. Yeasts in the traditional brewing of pito in Ghana. *World J. Microbiol. Biotechnol.* **1999**, *15*, 593–597. [CrossRef]
46. King, A.; Richard Dickinson, J. Biotransformation of monoterpene alcohols by *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Kluyveromyces lactis*. *Yeast* **2000**, *16*, 499–506. [CrossRef]
47. Canonico, L.; Galli, E.; Ciani, E.; Comitini, F.; Ciani, M. Exploitation of Three Non-Conventional Yeast Species in the Brewing Process. *Microorganisms* **2019**, *7*, 11. [CrossRef]
48. De Francesco, G.; Turchetti, B.; Sileoni, V.; Marconi, O.; Perretti, G. Screening of new strains of *Saccharomycodes ludwigii* and *Zygosaccharomyces rouxii* to produce low-alcohol beer. *J. Inst. Brew.* **2015**, *121*, 113–121. [CrossRef]
49. Meilgaard, M.C.; Reid, D.S.; Wyborski, K.A. Reference standards for beer flavor terminology system. *J. Am. Soc. Brew. Chem.* **1982**, *40*, 119–128. [CrossRef]
50. Andorrá, I.; Berradre, M.; Mas, A.; Esteve-Zarzoso, B.; Guillamón, J.M. Effect of mixed culture fermentations on yeast populations and aroma profile. *LWT* **2012**, *49*, 8–13. [CrossRef]
51. Varela, C.; Barker, A.; Tran, T.; Borneman, A.; Curtin, C. Sensory profile and volatile aroma composition of reduced alcohol Merlot wines fermented with *Metschnikowia pulcherrima* and *Saccharomyces uvarum*. *Int. J. Food Microbiol.* **2017**, *252*, 1–9. [CrossRef] [PubMed]
52. Anfang, N.; Brajkovich, M.; Goddard, M.R. Co-fermentation with *Pichia kluyveri* increases varietal thiol concentrations in Sauvignon Blanc. *Aust. J. Grape Wine Res.* **2009**, *15*, 1–8. [CrossRef]
53. Díaz, C.; Molina, A.M.; Nähring, J.; Fischer, R. Characterization and dynamic behavior of wild yeast during spontaneous wine fermentation in steel tanks and amphorae. *Biomed Res. Int.* **2013**, *2013*, 540465. [CrossRef] [PubMed]
54. Varela, C.; Borneman, A.R. Yeasts found in vineyards and wineries. *Yeast* **2017**, *34*, 111–128. [CrossRef]
55. Hutzler, M.; Riedl, R.; Koob, J.; Jacob, F. Fermentation and spoilage yeasts and their relevance for the beverage industry-a review. *BrewingScience-Monatsschrift Für Brauwissenschaft* **2012**, *65*, 33–50.
56. Coton, E.; Coton, M.; Levert, D.; Casaregola, S.; Sohier, D. Yeast ecology in French cider and black olive natural fermentations. *Int. J. Food Microbiol.* **2006**, *108*, 130–135. [CrossRef]
57. Ye, M.; Yue, T.; Yuan, Y. Effects of sequential mixed cultures of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* on apple cider fermentation. *FEMS Yeast Res.* **2014**, *14*, 873–882. [CrossRef]
58. Cañas, P.M.I.; García-Romero, E.; Manso, J.M.H.; Fernández-González, M. Influence of sequential inoculation of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* in the quality of red wines. *Eur. Food Res. Technol.* **2014**, *239*, 279–286. [CrossRef]
59. De Benedictis, M.; Bleve, G.; Grieco, F.; Tristezza, M.; Tufariello, M. An optimized procedure for the enological selection of non-*Saccharomyces* starter cultures. *Antonie Van Leeuwenhoek* **2011**, *99*, 189–200. [CrossRef]
60. Loira, I.; Morata, A.; Comuzzo, P.; Callejo, M.J.; González, C.; Calderón, F.; Suárez-Lepe, J.A. Use of *Schizosaccharomyces pombe* and *Torulaspora delbrueckii* strains in mixed and sequential fermentations to improve red wine sensory quality. *Food Res. Int.* **2015**, *76*, 325–333. [CrossRef]
61. Lee, P.-R.; Ong, Y.-L.; Yu, B.; Curran, P.; Liu, S.-Q. Evolution of volatile compounds in papaya wine fermented with three *Williopsis saturnus* yeasts. *Int. J. Food Sci. Technol.* **2010**, *45*, 2032–2041. [CrossRef]
62. Yilmaztekin, M.; Erten, H.; Cabaroglu, T. Production of Isoamyl Acetate from Sugar Beet Molasses by *Williopsis saturnus* var. *saturnus*. *J. Inst. Brew.* **2008**, *114*, 34–38. [CrossRef]
63. Erten, H.; Tanguler, H. Influence of *Williopsis saturnus* yeasts in combination with *Saccharomyces cerevisiae* on wine fermentation. *Lett. Appl. Microbiol.* **2010**, *50*, 474–479. [CrossRef]
64. Son, E.Y.; Lee, S.M.; Kim, M.; Seo, J.-A.; Kim, Y.-S. Comparison of volatile and non-volatile metabolites in rice wine fermented by Koji inoculated with *Saccharomycopsis fibuligera* and *Aspergillus oryzae*. *Food Res. Int.* **2018**, *109*, 596–605. [CrossRef]
65. Medeiros, A.B.P.; Pandey, A.; Christen, P.; Fontoura, P.S.G.; de Freitas, R.J.S.; Soccol, C.R. Aroma compounds produced by *Kluyveromyces marxianus* in solid state fermentation on a packed bed column bioreactor. *World J. Microbiol. Biotechnol.* **2001**, *17*, 767–771. [CrossRef]

66. Fabre, C.E.; Duviau, V.J.; Blanc, P.J.; Goma, G. Identification of volatile flavour compounds obtained in culture of *Kluyveromyces marxianus*. *Biotechnol. Lett.* **1995**, *17*, 1207–1212. [CrossRef]
67. Jiang, J. Identification of flavour volatile compounds produced by *Kluyveromyces lactis*. *Biotechnol. Tech.* **1993**, *7*, 863–866. [CrossRef]

2.2 Publication 2: Investigation of Non-*Saccharomyces* Yeast Strains for Their Suitability for the Production of Non-Alcoholic Beers with Novel Flavor Profiles

Publication 1 revealed that around 70% of the examined non-*Saccharomyces* yeast strains were maltose-negative, while even around 75% could not metabolize maltotriose. Nevertheless, some of these yeast strains showed exceptionally pleasant flavor properties in the sensory odor test in brewer's wort. Therefore, they offered a great potential for the production of NABs. In this publication, 15 different yeast strains of the genera *Cyberlindnera*, *Kazachstania*, *Kluyveromyces*, *Lachancea*, *Pichia*, *Saccharomycopsis*, *Schizosaccharomyces* and *Torulaspora* were examined for their suitability to produce NABs or LABs with novel, pleasant flavor properties. The selection of eleven yeast strains resulted directly from publication 1, while two *Cyberlindnera fabianii* and two *Cyberlindnera saturnus* yeast strains were additionally included due to the strikingly positive flavor results of the *Cyberlindnera* genera.

In 1,800 mL small-scale fermentations, the 15 yeast strains were fermented unpressurized alongside the commercially used reference yeast strain *Saccharomycodes ludwigii* TUM SL17 in 7 °P unhopped wort at 20 °C for 144 h with a pitching rate of 15×10^6 cells/mL. All yeast strains, as expected, showed weak fermentation processes, which resulted in ethanol concentrations between 0.26–0.55% (*v*/*v*). As the measured pH values between 4.55–5.08 were relatively high, an additional wort acidification was recommended. In addition, the beers were analyzed using instrumental analytics to determine sugar utilization and the formation of volatile compounds. Moreover, sensory tests were carried out by trained panellists to evaluate the beer flavors. Despite the weak fermentation performances, some yeast strains showed maltose and maltotriose utilization above 5% in the HPLC sugar analysis. Consequently, additional aerobic fermentation trials were carried out in selective carbohydrate media. These revealed that *Saccharomycopsis fibuligera* Lu27, the two *C. fabianii* yeast strains 5640 and 5650 as well as *Pichia kluyveri* PK1 and *Lachancea kluyveri* 3082 could metabolize maltose and/or maltotriose. Apart from high isoamyl acetate concentrations (3.1-7.5 mg/L), especially in the beers fermented with *C. saturnus*, hardly any other flavor-active substances could be measured above the threshold values for beer. Still, the tastings demonstrated that almost all beers exhibited pleasant fruity flavors without wort off-flavors. Three yeast strains of the species *C. saturnus* (247, 4549 and CSa1) stood out particularly positively in the sensory evaluation, impressing tasters with cool mint sweets, pear and partly red berry (C. sat 247) flavors. The beers fermented with *S. fibuligera* Lu27 and *K. marxianus* 653 also showed pleasant flavor profiles reminiscent of red berries, honey, stone fruit and apple. Based on the results, the threshold values of the flavor-active substances for NABs fermented with non-*Saccharomyces* yeasts need to be adjusted and the GC/MS analysis should be expanded. Nevertheless, it was shown in the context of this publication that the 15 non-*Saccharomyces* yeasts examined were suitable for the production of NABs or LABs with desirable novel flavor properties and that there is great potential in this field of research.

Authors/Authorship contribution:

Methner, Y.: Literature research, conception and design of experiments, performance of experiments, analysis of data using OriginPro 2020 as statistical software, paper writing; **Hutzler, M.**: Organization and provision of a large amount of yeast strains, revision of conception, critical revision of manuscript; **Zarnkow, M.**: Critical revision of manuscript; **Prowald, A.**: Performance of HPLC with UV/Vis analyses, critical revision of manuscript; **Endres, F.**: Data evaluation and graphical representation of HPLC with UV/Vis analyses, critical revision of manuscript; **Jacob, F.**: Supervision of project, approval for submission, funding acquisition.

Journal of the American Society of Brewing Chemists
The Science of Beer

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/ujbc20

Investigation of Non-*Saccharomyces* Yeast Strains for Their Suitability for the Production of Non-Alcoholic Beers with Novel Flavor Profiles

Yvonne Methner, Mathias Hutzler, Martin Zarnkow, Alexandra Prowald, Frank Endres & Fritz Jacob

To cite this article: Yvonne Methner, Mathias Hutzler, Martin Zarnkow, Alexandra Prowald, Frank Endres & Fritz Jacob (2022): Investigation of Non-*Saccharomyces* Yeast Strains for Their Suitability for the Production of Non-Alcoholic Beers with Novel Flavor Profiles, Journal of the American Society of Brewing Chemists, DOI: 10.1080/03610470.2021.2012747

To link to this article: https://doi.org/10.1080/03610470.2021.2012747

JOURNAL OF THE AMERICAN SOCIETY OF BREWING CHEMISTS
https://doi.org/10.1080/03610470.2021.2012747

Investigation of Non-*Saccharomyces* Yeast Strains for Their Suitability for the Production of Non-Alcoholic Beers with Novel Flavor Profiles

Yvonne Methner[a], Mathias Hutzler[a], Martin Zarnkow[a], Alexandra Prowald[b], Frank Endres[b] and Fritz Jacob[a]

[a]Research Center Weihenstephan for Brewing and Food Quality, Technical University of Munich, Freising, Germany; [b]Institute of Electrochemistry and Brewery, Clausthal University of Technology, Clausthal-Zellerfeld, Germany

ABSTRACT

Non-alcoholic beers have seen steady growth in recent years and are attracting customer interest. At the same time, consumer demand for non-alcoholic beers is rising. While wort-like flavors are often criticized in beer and also regarded as off-flavors, there is growing demand for beer flavor diversification. The latest research results prove that certain non-*Saccharomyces* yeast species can produce distinctive fruity flavor profiles during wort fermentation. In this study, 15 different yeast strains were evaluated for their suitability to produce novel flavor profiles during fermentation in wort from malt extract while being tested for their ability to produce less than 0.5% (*v/v*) ethanol. The yeast strains were therefore analyzed for their sugar utilization and were compared with the maltose-negative reference yeast strain *Saccharomycodes ludwigii* TUM SL17. Following the fermentation experiments, the beers were analyzed for secondary metabolites and ethanol content before being tasted by trained assessors. The results reveal that all 15 yeasts are suitable for the production of non-alcoholic beers based on their sugar utilization. Particularly promising flavors were produced by three yeast strains of the species *Cyberlindnera saturnus*, which were reminiscent of cool mint sweets, pear, and banana. Two further yeasts of the species *Kluyveromyces marxianus* and *Saccharomycopsis fibuligera* also produced a wide range of pleasant fruity flavors. Although the secondary metabolites were mostly below the odor thresholds based on regular alcoholic beers, this study reveals that the thresholds in non-alcoholic beers can be classified as significantly lower.

KEYWORDS

Non-*Saccharomyces* yeasts; fermentation; non-alcoholic beers; wort sugar utilization; flavor profiles; secondary metabolites

Introduction

The global market share for non-alcoholic beers has grown steadily in recent years and is expected to grow further in the years ahead. Alcohol is increasingly the subject of critical debate from both a health and social perspective, which is causing consumers to turn more and more to non-alcoholic beers.[1,2]

A simple technological tool of producing non-alcoholic beers is the use of low-fermentation yeasts that are maltose- and maltrotriose-negative, however, they also need sufficient hop tolerance. A study by Methner et al. revealed that most yeasts from food fermentations have a high tolerance to hops. Out of a total of 110 yeasts examined, without exception, all grew at iso-α-acids up to 50 IBU.[3] Maltose and maltotriose account for over 80% of the total carbohydrates in brewer's wort so there is a significant reduction in alcohol unless the yeasts are able to metabolize these two wort sugars.[4] Maltose- and maltotriose-negative yeasts are therefore suitable for producing non-alcoholic beers. Whenever these two sugars are not metabolized by the yeasts to alcohol and carbon dioxide, they remain unmodified in the wort and provide residual sweetness as well as body.[5] Due to the relatively small amount of the wort sugars fructose, glucose, and sucrose, the maltose-negative yeasts have only limited carbohydrate sources available for their metabolism. Once they are utilized, the yeast ceases its activity. For this reason, in this study, yeast strains were examined that had already been described in literature as maltose-negative or at least potentially maltose-negative during fermentation. The fact that maltose is not utilized by the yeasts is due to a lack of permease in the yeast cell membrane, which would transport maltose into the cell interior via active transport.[6] This process technology for non-alcoholic beers is already known and was first practiced with the yeast *Saccharomycodes ludwigii*. As early as 1933, a patent by Haehn and Glaubitz described the production of low-alcohol beer using this yeast species,[7] which was extended by an additional patent in 2009 by Kunz et al.[8]

In the meantime, there is an increasing demand for flavor diversification with a focus on fruity beers without any wort flavor or other taste deficiencies as described by Blanco et al.[9] Some studies have already investigated the flavor properties of various yeasts during the fermentation of wort

CONTACT Mathias Hutzler m.hutzler@tum.de
Supplemental data for this article is available online at https://doi.org/10.1080/03610470.2021.2012747.

to produce non-alcoholic beers. In 2016, Liu et al. investigated a *Cyberlindnera* (*Williopsis*) *saturnus* var. *mrakii* yeast strain for its suitability to produce a particularly fruity non-alcoholic beer and found that this resulted in desirable fruity and floral flavors, which were significantly higher in direct comparison to a beer produced with a conventional *Saccharomyces cerevisiae*.[10] The same aim was pursued by Bellut et al. with several different *Cyberlindnera* yeast strains such as *C. misumaiensis*, *C. fabianii*, *C. subsufficiens* and *C. mrakii*. They classified a *C. subsufficiens* in particular as most suitable.[11] A *C. fabianii* yeast strain turned out to be applicable for reducing alcohol content and increasing flavor complexity as a co-culture alongside a *S. cerevisiae*.[12] In addition to the *Cyberlindnera* species, several studies have revealed *Torulaspora delbrueckii* to be a potential yeast strain for producing non-alcoholic beers with fruity aroma characteristics.[13–16] Moreover, several studies proved that not only *S. ludwigii*, but also *Pichia kluyveri* and *Kluyveromyces lactis* enhanced desirable beer flavors by wort fermentations while being suitable for the production of non-alcoholic beers.[15,17,18] Recent study results revealed that by using *Schizosaccharomyces pombe*, low alcohol beers can be produced despite the ability of this yeast to metabolize maltose.[19] *Kazachstania servazzii* was also investigated for the fermentation of wort with the aim of producing a non-alcoholic beer, which was then analyzed for volatile components.[20]

Based on the previous research results, 15 different non-*Saccharomyces* yeast strains were systematically investigated for their suitability in the production of non-alcoholic beers. Yeasts strains were considered from the genera *Cyberlindnera*, *Kazachstania*, *Kluyveromyces*, *Pichia, Schizosaccharomyces*, and *Torulaspora,* which have already been studied in connection with the production of non-alcoholic beers. Additionally, a yeast of the genera *Lachancea* as well as a yeast strain of the genera *Saccharomycopsis* were examined for the first time for their suitability in the production of non-alcoholic beers. To date, the yeast strain *Saccharomycopsis fibuligera* has been studied for the production of alcoholic beers and showed desirable aroma properties. Some strains showed weak fermentation activity in brewer's wort so its use in the non-alcoholic sector could also be promising. The same study indicated that a *Lachancea kluyveri* yeast strain was maltose-negative and thus could have potential.[3] Since *Saccharomycodes ludwigii* is well known for the production of non-alcoholic beers and has been investigated in numerous studies in this context by Meier-Dörnberg et al.,[21] De Francesco et al.,[22] Pahl,[23] Parise et al.,[24] Gutiérrez et al.,[17] Callejo et al., [25] and Vaštík et al.,[19] this yeast was used as a reference in this study.

In addition to continuous fermentation control that included measuring the final ethanol contents as well as pH values of the beers, the parameters of sugar utilization and secondary metabolites were analyzed. The sugar compositions of the beers were determined by two different high-performance liquid chromatography (HPLC) methods, while additional growth tests were performed in selective media. Analysis of the fermentation by-products, various esters, organic acids, higher alcohols, ketones, acetaldehyde, dimethyl sulfide (DMS), and 4-vinylguaiacol (4-VG) was performed by gas chromatography/mass spectrometry (GC/MS). Moreover, sensory analyses were carried out that included profiling as well as testing for off-flavors.

The aim of this study was to find suitable non-*Saccharomyces* yeast strains to produce non-alcoholic beers with distinguished flavor properties but without wort flavor. Wort-like off-flavors [20,26,27] were not analyzed by GC/MS, however, they were considered in the sensory evaluations of the beers. Yeasts were selected based on the fact that they did not produce phenolic off-flavors. The target alcohol content of the beers was a maximum of 0.5% (*v*/*v*) according to the German regulation for non-alcoholic beers.[28]

Experimental

Yeast strains

The 16 yeast strains investigated in this study are listed in Table 1 with corresponding abbreviations. Except for the yeast strain *Cyberlindnera saturnus* CSa1, which comes from a private collection, all other yeast strains can be purchased commercially. The collection site, from which the yeast strains were obtained, is indicated in the footnote of Table 1.

Table 1. Yeast species and strain numbers with corresponding abbreviations used in this study.

Yeast strain number	Yeast strain abbreviation	Yeast species
CBS 5640	C. fab 5640	*Cyberlindnera fabianii*[a]
CBS 5650	C. fab 5650	*Cyberlindnera fabianii*[a]
TUM 238	C. mis 238	*Cyberlindnera misumaiensis*[b]
YH837A-3D4	C. mis CM1	*Cyberlindnera misumaiensis*[b]
TUM 247	C. sat 247	*Cyberlindnera saturnus*[b]
CBS 4549	C. sat 4549	*Cyberlindnera saturnus*[a]
YHMH22AA-3H1	C. sat CSa1	*Cyberlindnera saturnus*[c]
YHMH47B-3C4	K. ser 3C4	*Kazachstania servazzii*[b]
TUM G9K	K. lac G9K	*Kluyveromyces lactis*[b]
TUM 653	K. mar 653	*Kluyveromyces marxianus*[b]
CBS 3082T	L. klu 3082	*Lachancea kluyveri*[a]
YHAK1A-3I1	P. klu PK1	*Pichia kluyveri*[b]
TUM SL17	S. lud SL17	*Saccharomycodes ludwigii*[b]
PI S 6; Lu27	S. fib Lu27	*Saccharomycopsis fibuligera*[b]
TUM G10S	S. pom G10S	*Schizosaccharomyces pombe*[b]
YH824A-1I6	T. del 1I6	*Torulaspora delbrueckii*[b]

[a]Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; [b] Research Center Weihenstephan (BLQ), Freising, Germany; [c] Private collection

Table 2. Analytical methods of the wort and the non-alcoholic beers after MEBAK[a] and Donhauser et al.[b]

Analysis	Method	Device
Sugar composition (glucose, fructose, sucrose, maltose, maltotriose)	Donhauser et al.[b] LS-HPLC 002_2	HPLC UltiMate 3000 (Thermo Fisher Scientific, Waltham, MA, U.S.A.)
Original wort, ethanol content, apparent attenuation	MEBAK[a] WBBM 2.9.6.3	Bending vibration and NIR spectroscopy, Alcolyzer Plus with DMA 5000 X sample 122 (Anton-Paar GmbH, Ostfildern, Germany)
pH value	MEBAK[a] WBBM 2.13	pH meter with pH electrode, ProfiLine pH3210 pH meter (Xylem Inc., New York, NY, U.S.A.)
Dimethyl sulfide	MEBAK[a] WBBM 2.23.1	GC-FID Clarus 580 (Perkin Elmer, Waltham, MA, U.S.A.), Column: 50 m 0.32 mm Phenomenex FFAP, 0.25 µm
Fatty acid esters, fatty acids, 2-phenylethanol	MEBAK[a] WBBM 2.23.6	
Acetaldehyde, ethyl acetate, isoamyl acetate, higher alcohols (n-propanol, i-Butanol, amyl alcohols)	MEBAK[a] WBBM 2.21.1	GC-FID Clarus 580, Turbo Matrix 40, Head Space (Perkin Elmer, Waltham, MA, U.S.A.), Column: INNOWAX cross-linked polyethylene glycol, 60 m × 0.32 mm 0.5 µm
4-Vinylguaiacol	MEBAK[a] WBBM 2.21.3.3	
Diacetyl, 2,3-pentanedione	MEBAK[a] WBBM 2.21.5.1	

[a]MEBAK® (2012), Editor: Dr. F. Jacob: The MEBAK collection of brewing analysis methods: Wort, beer, and beer-based beverages. Collection of methods of the Mitteleuropäischen Brauchtechnischen Analysenkommission. Self-published by MEBAK.
[b]Donhauser, S.; Wagner, D. (1990): Zucker- und Endvergärungsgradbestimmung mittels der HPLC, 9:306-309. Monatsschrift für Brauwissenschaft.

Yeast propagation and fermentation

The selected yeast strains from Table 1 were inoculated from wort slope agars under sterile conditions into 500 mL flasks each containing 250 mL of unhopped wort (7.0°P, pH 5.5) that was made from malt extract (Weyermann®, Bamberg, Germany). Since the composition of malt extract is always the same, the results are highly comparable. The wort was analyzed (extract content, pH, and sugar composition) according to the methods in Table 2.

After 72 h of propagation at 20 °C on a WiseShake orbital shaker (Witeg Labortechnik GmbH, Wertheim, Germany) at 80 rpm, the yeast suspensions were transferred to sterile 2500 mL flasks filled with 1800 mL of comparable unhopped wort and propagated for an additional 72 h. After propagation, the cell counts were determined using the Cellometer® Vision (Nexcelom Bioscience LLC, Lawrence, MA, U.S.A.).

The 16 different yeast strains listed in Table 1 were tested in fermentation trials. For the fermentation experiments, a maltose-negative yeast strain known to produce non-alcoholic beers was included as a reference: *Saccharomycodes ludwigii* TUM SL17. In small-scale fermentations performed in triplicate, 1800 mL unhopped wort (7.0°P, pH 5.5) in 2000 mL sterile Duran glass bottles (Schott AG, Mainz, Germany) was pitched, each at 15×10^6 cells/mL ($\pm\sigma = 1 \times 10^6$ cells/mL) and locked with glass fermentation locks on top. To produce the wort, malt extract (Weyermann®, Bamberg, Germany) was blended in a wort boiler in two batches to the defined original wort, boiled for 5 min and then cooled to the pitching temperature of 20 °C using a plate cooler. For each batch, 50 L wort was pumped into a cylindroconical tank before 1800 mL of each was filled into the 2000 mL bottles for the fermentation trials. Propagation yeasts were centrifuged (Roto Super 40, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) in sterilized 500 mL PPCO centrifuge bottles (Nalgene, Thermo Fisher Scientific, Waltham, MA, U.S.A.) at 750 × g for 5 min and the supernatant was discarded before pitching. The samples were incubated at a 20 °C fermentation temperature. The fermentation time of 144 h was chosen based on previous experiments with the reference yeast S. lud SL17. For this yeast strain, based on well-founded preliminary trials, a recommendation exists for the production of non-alcoholic beers, which suggests an original wort of 7°P and a fermentation temperature of 15-20 °C in order to achieve an ethanol content < 0.5% (*v/v*) in the final beer.[21,30] Since it is known that the reference yeast strain completely converts glucose and fructose as well as sucrose after a fermentation period of 144 h at a temperature of 20 °C and an original wort of 7°P, this experimental design was transferred to the 15 investigated yeast strains. The main fermentation proceeded for 144 h and fermentation progress was checked every 24 h by recording the weight loss. Fermentation progress can be determined by weight loss, which is based on Balling's assumption that during fermentation an average of 2.0665 g of extract is converted into 1 g of alcohol, 0.9565 g of carbon dioxide, and 0.11 g of yeast.[31] Subsequently, the samples were then placed into cold storage at 2 °C for another 96 h before performing the analytical analyses shown in Table 2 and the sensory evaluation.

Analytical methods

Table 2 lists the analyses of the initial wort as well as of the non-alcoholic beers. All beers were prepared in triplicate with each sample analyzed by the methods listed in Table 2. Subsequent statistical analyses were performed using OriginPro 2020 statistical software.

The apparent attenuation is calculated using a formula for the degree of fermentation and the original wort of the sample, which is described in detail in Bleyer et al.[32]

Sugar utilization test

In order to assess whether some of the investigated yeast strains were actually able to metabolize maltose or maltotriose, additional sugar utilization tests were performed based on the method of Nikulin et al.[33] For this purpose, two synthetic YP-media were prepared from 1.0% yeast extract (Sigma-Aldrich, St. Louis, MO, U.S.A.), 2.0% peptone from casein, pancreatic digest (Sigma-Aldrich, St. Louis, MO, U.S.A.), either 1.0% D(+)-maltose monohydrate 95% (Merck, Darmstadt, Germany) or 1.0% D-maltotriose 98% (Thermo Fisher Scientific, Waltham, MA, U.S.A), and 96.7% double distilled water. The yeast strains were transferred from pure

slope agar cultures into 1 mL sterile physiological sodium chloride solutions before they were counted using the Cellometer® Vision (Nexcelom Bioscience LLC, Lawrence, MA, U.S.A.). The required volumes for 0.1×10^6 cells/mL were calculated and inoculated under sterile conditions into 25 mL of YP-medium into 50 mL flasks each and were sealed with cotton plugs. The samples were incubated at 20 °C on a WiseShake orbital shaker (Witeg Labortechnik GmbH, Wertheim, Germany) at 80 rpm. Every 24 h, absorbances were measured in triplicate using an UV-Vis photometer (Evolution 300, Thermo Fisher Scientific, Madison, WI, U.S.A.), set to an optical density (OD) of 600 nm, until there was no further increase in OD. Although the coefficient of variation of the applied HPLC analysis is 13% for maltose and 15% for maltotriose, the limits for the tests in selective media were set significantly lower at 5% to achieve reliable results. In cases where the yeasts were able to utilize more than 5% of the maltose present in the beer by HPLC sugar analysis (cf Table 2) based on the mean value of the triple determination, their growth was tested in YP-medium + 1% maltose. In addition, all yeasts that could metabolize more than 5% maltotriose based on the mean value of the triple determination were exposed to YP-medium + 1% maltotriose.

To check the measured values for maltose and maltotriose due to the relatively high coefficients of variation, glucose, maltose, and maltotriose of a selected beer were randomly checked using a further HPLC method. To be able to safely exclude measurement inaccuracies, the sugars were chemically modified in order to make them subsequently visible in the HPLC with a UV/Vis detector. Chemical modification for visualization in HPLC using UV/Vis detector proceeded as shown in Figure 1.

The reaction procedure followed Honda et al.[34] Fructose was modified with only one molecule, while sucrose did not react with the chromophore. The modified sugars showed strong absorbance at 245 nm. The solutions were then analyzed using an RP18 column in combination with a mixture of 20% (*v/v*) acetonitrile and 80% (*v/v*) 0.15 M ammonium acetate-acetic acid buffer (pH = 5.0). The compound 1H-benzotriazole was added as an internal standard. For the measurements, the 7°P initial wort and the three beers of the selected yeast strain were determined in triplicate. A mean chromatogram was calculated from each of the three wort and the nine beer measurements.

Sensory evaluation

The beer samples, which were tempered to 12 °C, were profiled at 20 °C room temperature by a sensory panel of ten DLG (Deutsche Landwirtschafts-Gesellschaft e.V., Frankfurt, Germany)-certified assessors to determine the main flavor components of the individual beers. To exclude external interferences during the tastings, they were held in an appropriately neutral, white-colored room with individual tasting chambers. The trained panelists were asked to describe both the odor and the taste of the beers. As part of the evaluations, the specified odor and taste attributes were summarized. The first part of the evaluations recorded how many tasters described the respective beers as generally fruity. If significant fruitiness could be perceived in the beers, the individual fruit attributes could be broken down and statistically verified by percentage. The evaluation by percentage indicated how many tasters named the corresponding fruit attribute. The significance level was analyzed for the paired sensory testing of the attribute "fruity" according to MEBAK sensory analysis 3.1.1. and DIN EN ISO 5495:2007 with a significance level $\alpha = 0.05$. Based on these sensory methods, a significant result was achieved in cases where at least nine of the ten tasters had matching results (significant results are marked with* in the results section). The result was classified as non-significant in the cases where eight or fewer tasters delivered a matching result. In addition, a sensory test was carried out according to the DLG evaluation scheme, which comprises a rating scale from zero to five. While zero is considered the lowest score and represents insufficient product quality, a score of five points fully meets the quality expectations of the product and matches the quality description very well.[35] The odor, purity of taste, and body of the beers were evaluated, while the quality of bitterness as well as the carbonation were neglected. For the tasting, the samples were assigned randomized three-digit numbers and 50 mL of each sample was served in brown 200 mL tasting glasses.

To establish possible correlation between the specific volatile compounds measured analytically and the individual flavor characteristics described by the tasters, a principal component analysis (PCA) was performed using OriginPro 2020 statistical software.

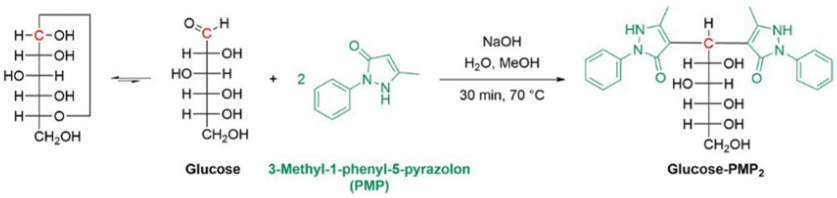


Figure 1. Chemical modification of glucose for HPLC visualization using UV/Vis detector. (Endres, F.; Prowald, A.; Fittschen, U. E. A.; Hampel, S.; Oppermann, S.; Jacob, F.; Zarnkow, M.; Hutzler, M.; Methner, Y.; Laus, A. Vergleichendes isothermes Maischen bei 72 °C, BRAUWELT 2021, 9-10, 223-227.)

Results and discussion

Fermentation

The weight loss of the individual samples was recorded every 24 h to monitor the extract degradation by the 16 investigated yeast strains during the fermentation process. Figure 2 shows the fermentation progress of the yeast strains with the corresponding standard deviations including the reference yeast strain *S. ludwigii* TUM SL17 (marked in red). The weight loss is plotted over a total fermentation period of 144 h at a fermentation temperature of 20 °C. The individual fermentation curves are available in Supplemental File 1.

After the first 24 h of fermentation, all 16 yeasts showed a weak weight loss between 0.1 and 0.6 g. The reference yeast, S. lud SL17, exhibited a weight loss of about 0.5 g and thus fermented relatively strongly in comparison to the other yeast strains. Two yeast strains fermented slightly faster within the first 24 h: *L. kluyveri* 3082 as well as *T. delbrueckii* 1I6. The weakest fermentation activity with only approx. 0.1 g of weight loss was formed by the yeast strains *P. kluyveri* PK1 and *C. misumaiensis* CM1. The fermentation activity of the reference yeast decreased with increasing fermentation time and the other yeasts behaved similarly. The yeast strains L. klu 3082 and T. del 1I6 remained the strongest until fermentation was stopped by cooling down to 2 °C after 144 h. At 2.2 to 2.5 g, these strains lost slightly more weight than the reference yeast with about 2 g on day six. The *Pichia* yeast strain remained the weakest yeast within the six days of fermentation and only lost 0.9 g of weight, while the yeast strain C. mis CM1 lost slightly more weight with just over 1 g.

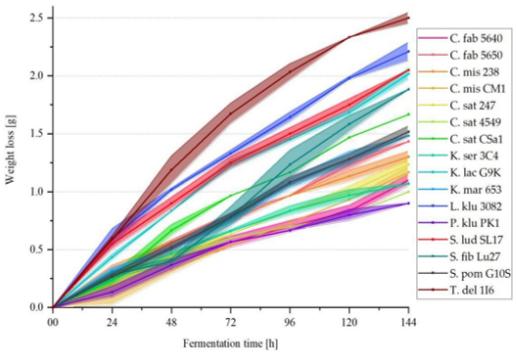


Figure 2. Mean values (n = 3) with standard deviations of the fermentation samples' total weight loss in grams of the investigated yeast strains during the fermentation process over a fermentation period of 144 h (6 days) at 20 °C.

Table 3. Regression equations and corresponding coefficients of determination R^2.

Yeast strain abbreviation	Regression equation	Coefficient of determination R^2
C. fab 5640	y = 0.0074 x	99.1%
C. fab 5650	y = 0.0102 x	99.7%
C. mis 238	y = 0.0096 x	97.9%
C. mis CM1	y = 0.0078 x	98.9%
C. sat 247	y = 0.0085 x	99.7%
C. sat 4549	y = 0.0072 x	97.4%
C. sat CSa1	y = 0.0121 x	98.9%
K. ser 3C4	y = 0.0081 x	97.0%
K. lac G9K	y = 0.0147 x	97.7%
K. mar 653	y = 0.0106 x	99.5%
L. klu 3082	y = 0.0166 x	96.1%
P. klu PK1	y = 0.0067 x	97.9%
S. lud SL17	y = 0.0149 x	96.4%
S. fib Lu27	y = 0.0126 x	97.7%
S. pom G10S	y = 0.0108 x	99.8%
T. del 1I6	y = 0.0196 x	94.8%

For each fermentation curve, a linear regression line with a corresponding coefficient of determination was generated for the mean values of weight losses. The regression equations and coefficients of determination are listed in Table 3. The coefficients of determination range between 94.8 and 99.8%, which indicates a low variation. The gradients of the regression lines can be related to the ethanol contents, which are presented in Table 4. The general trend is that the lower the gradient, the lower the ethanol content in the finished beer.

In terms of ethanol content, it is noticeable in Table 4 that the beers ranged between 0.26% and 0.55% (*v/v*). With an original wort of 7°P, a fermentation temperature of 20 °C, and a fermentation time of 144 h, all of the investigated yeasts are fundamentally suitable for producing non-alcoholic beers. The beers produced using the yeast strains *L. kluyveri* 3028 and T. *delbrueckii* 1I6 had values of 0.54 and 0.55% (*v/v*) making them slightly above the legally permissible ethanol content of 0.50% (*v/v*) according to the German regulation.[28] They showed the strongest fermentation activity in the course of fermentation. By slightly decreasing the original wort, shortening the fermentation time, or even reducing the fermentation temperature, it could be possible to reduce the fermentation activity and the associated ethanol content of the two beers. The low apparent attenuations listed in Table 4 result in relatively high amounts of residual sugars in the beers, which can potentially be metabolized by other yeasts or bacteria. Pasteurization is therefore necessary to ensure microbiological safety and at the same time to counteract a subsequent increase in alcohol content. Since the wort was produced in two batches, the original wort varies by around 0.1°P. While yeasts C. fab 5640, C. fab 5650, C. mis 238, C. mis CM1, C. sat 247, C. sat 4549, C. sat CSa1 and P. klu PK1 were pitched in the first wort batch, the second batch was used for the other yeasts.

The pH values of the beers in Table 4 range between 4.60 and 5.08. Only the yeast strains *S. fibuligera* Lu27, *K. marxianus* 653, *C. misumaiensis* 238 and *C. misumaiensis* CM1 were able to induce a sufficient pH drop. Apart from these four yeast strains, the remaining ones stayed at pH values greater than 4.62, so that an additional drop in the pH to between 4.2 and 4.6 using sour wort would positively impact the taste and produce a safer product from a microbiological point of view.[29]

Table 4. Mean values and standard deviations (n = 3) of original wort (°P), apparent attenuation (%), ethanol content (% *v/v*) and pH value in the beers fermented by the 16 selected yeast strains.

Yeast strain abbreviation	Original wort (°P)	Apparent attenuation (%)	Ethanol content (% *v/v*)	pH value
C. fab 5640[a]	7.05 ± 0.00	9.07 ± 0.21	0.33 ± 0.00	4.83 ± 0.00
C. fab 5650[a]	7.02 ± 0.01	12.00 ± 0.00	0.43 ± 0.00	4.79 ± 0.01
C. mis 238[a]	7.03 ± 0.00	8.93 ± 0.12	0.32 ± 0.01	4.55 ± 0.00
C. mis CM1[a]	7.03 ± 0.02	8.60 ± 0.08	0.31 ± 0.00	4.62 ± 0.00
C. sat 247[a]	7.02 ± 0.01	10.00 ± 0.00	0.36 ± 0.00	4.76 ± 0.01
C. sat 4549[a]	7.03 ± 0.00	7.73 ± 0.05	0.28 ± 0.00	4.84 ± 0.00
C. sat CSa1[a]	7.03 ± 0.01	12.77 ± 0.05	0.46 ± 0.00	4.83 ± 0.00
K. ser 3C4[b]	6.94 ± 0.01	9.23 ± 0.09	0.33 ± 0.00	4.88 ± 0.01
K. lac G9K[b]	6.85 ± 0.09	13.97 ± 0.09	0.49 ± 0.00	5.08 ± 0.01
K. mar 653[b]	6.88 ± 0.00	10.53 ± 0.12	0.37 ± 0.00	4.60 ± 0.00
L. klu 3082[b]	6.92 ± 0.00	15.73 ± 0.05	0.55 ± 0.00	4.69 ± 0.00
P. klu PK1[a]	7.02 ± 0.01	7.33 ± 0.17	0.26 ± 0.01	4.82 ± 0.00
S. lud SL17[b]	6.95 ± 0.05	14.02 ± 0.10	0.50 ± 0.00	4.92 ± 0.04
S. fib Lu27[b]	6.91 ± 0.01	13.20 ± 0.29	0.47 ± 0.01	4.60 ± 0.01
S. pom G10S[b]	6.90 ± 0.00	11.30 ± 0.16	0.40 ± 0.00	4.77 ± 0.00
T. del 1I6[b]	6.89 ± 0.00	15.50 ± 0.00	0.54 ± 0.00	4.78 ± 0.01

[a]Wort batch 1; [b] Wort batch 2

Table 5. Sugar composition of the wort used for fermentation trials.

Sugar composition of wort in g/L	
Fructose	0.6
Glucose	4.4
Sucrose	1.6
Maltose	31.7
Maltotriose	7.3

Sugar utilization

The sugar composition of the main wort carbohydrates of fructose, glucose, sucrose, maltose, and maltotriose [36] for the fermentation experiments are illustrated in Table 5, which gives the mean of the two wort batches.

Both the wort sugars (cf Table 5) and the sugar compositions of the beers were analyzed to draw conclusions about the sugar utilization of the individual yeast strains presented in Figure 3. To minimize error when calculating the individual sugar utilizations, the average wort sugar composition of the respective wort batch was used rather than that from Table 5. Supplemental File 2 provides the sugar utilizations for each wort batch.

The reference yeast strain *S. ludwigii* SL17 (marked in red) as well as *K. lactis* G9K were the only yeast strains to metabolize 100% of the glucose, fructose, and sucrose present in the wort, while maltose and maltotriose were not utilized. This was to be expected for the reference yeast *S. ludwigii*.[37] *K. lactis* is known to variably metabolize glucose, sucrose, and maltose.[38] Accordingly, the specific yeast strain *K. lactis* G9K has the ability to utilize glucose and sucrose but presumably it has no permease activity.

Although *T. delbrueckii* 1I6 and *L. kluyveri* 3082 lost more weight in the fermentation control (cf Figure 2) and produced slightly higher amounts of ethanol, they seemed to only fully utilize glucose and sucrose, but not fructose. However, since the fructose concentration in wort is very low at 0.6 g/L (cf Table 5) and the measurement inaccuracy with the applied HPLC method is relatively high for fructose with a coefficient of variation of about 20%, the fructose values should be considered only as an indication of whether fructose could generally be metabolized. Therefore, it is to be assumed that all yeasts examined have the ability to utilize fructose. In addition, the fructose content may initially appear higher when sucrose is hydrolyzed to its monosaccharides of glucose and fructose. Since *S. cerevisiae* is known for displaying a discrepancy in glucose and fructose utilization during fermentation,[39] this behavior could be transferred to further yeast species leading to a relatively higher concentration of fructose. It is to be assumed that fructose would have been completely metabolized if the fermentation period was longer. Additionally, in a previous study by Methner et al., 8 of the 16 yeast strains were investigated in selective medium and completely metabolized fructose.[3] These include yeast strains such as T. del 1I6, which according to Figure 3 metabolized only 33% of the fructose from the wort, K. ser 3C4 and L. klu 3082, which both utilized 48%, or C. Sat CSa1, which was at 60% utilization. This reinforces the assumption that fructose would have been fully utilized if the fermentation period was extended. The yeast strains that have not been investigated to date could also be tested in a fructose selective medium in future work in order to be able to make a reliable statement on fructose utilization. Similarly to *T. delbrueckii* 1I6 and *L. kluyveri* 3082, *C. fabianii* 5640 and *C. saturnus* CSa1 also metabolized 100% of the glucose and sucrose. Comparing the results of sugar utilization with existing literature, it can be confirmed that *T. delbrueckii*,[40] *L. kluyveri*,[41] *C. fabianii*, and *C. saturnus* [42] metabolize glucose and sucrose. Although there are *T. delbrueckii* yeast strains that can variably utilize sucrose, the studied strain 1I6 could completely convert sucrose.

Although *C. fabianii* 5650, *C. saturnus* 4549, *K. marxianus* 653, and *S. pombe* had converted 100% of sucrose, only more than 75% of glucose was consumed. *C. saturnus* 247 was the only yeast strain showing weaker sugar utilization, as about 90% glucose and 60% sucrose were utilized. However, as fermentation was stopped after just six days, it is assumed that glucose and sucrose would also have been completely metabolized by the yeast strains given a slightly longer fermentation period. Another reason for the incomplete utilization of sucrose could be the pH value, whose optimum for the enzyme invertase is not clearly defined according to the literature.[42–44] If wort acidification was applied, this could perhaps result in faster sucrose

degradation. Additionally, the partially high standard deviations (relative standard deviations up to approx. 10%) need to be taken into account. According to the existing literature, *C. saturnus,*[42] *K. marxianus,*[38] and *S. pombe* [46] are known for their ability to ferment glucose and sucrose.

As described in Kurtzman,[45,47,48] the yeast strains *K. servazzii* 3C4, *P. kluyveri* PK1, *C. misumaiensis* 238, and CM1 were indeed unable to cleave sucrose into its monosaccharides, while they completely converted available glucose from the wort. Thus, a lack of extracellular invertase could be the reason why sucrose cannot be metabolized.[49]

Based on the results (cf Figure 3), 75% of the yeasts investigated, namely *C. saturnus,*[42] *C. misumaiensis,*[47] *K. servazzii,*[48] *K. marxianus,*[38] *L. kluyveri,*[41] *P. kluyveri,*[45] and the reference *S. ludwigii* [37] do not possess the property to utilize maltose, which is confirmed by the literature. The ability of *K. lactis,*[38] *S. pombe,*[46] and *T. delbrueckii* [40] to metabolize maltose may vary. Only *C. fabianii* [47] metabolizes maltose slowly and weakly, while *S. fibuligera* [50] can metabolize maltose, albeit slowly. This can be attributed to an extracellular glucoamylase.[50–53] Whether there is also a permease activity in addition to the extracellular glucoamylase activity would have to be investigated. However, the yeast strain has the potential for producing non-alcoholic beers. This is evident from a previous study, which showed that some *S. fibuligera* yeast strains fermented much more weakly at 27 °C and an original gravity of 12.5°P than a conventional brewing yeast such as *Saccharomyces cerevisiae*.[3] In this study, reducing the temperature to 20 °C, and the original wort to 7°P, as well as the fermentation time to 144 h, resulted in an alcohol content < 0.5% (*v/v*) and thus a weak sugar utilization. Although *S. fibuligera* is the only yeast strain investigated besides *C. fabianii,* which is known to convert maltose as well as glucose and sucrose, S. fib Lu27 actually does not prove to be more fermentative. On average, only about 70% glucose, 60% sucrose, and 10% maltose were utilized (cf Figure 3). When comparing the maltose utilization with the other yeasts, some show a maltose utilization that is almost equal. Since the coefficient of variation for maltose is 13% by the applied HPLC method,

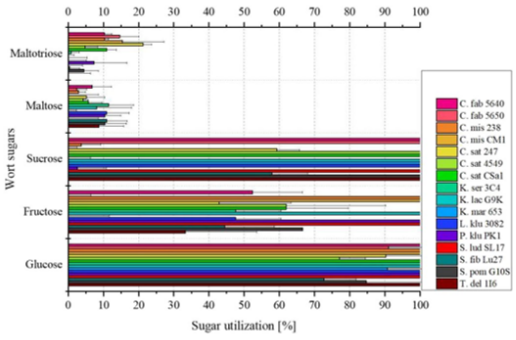


Figure 3. Mean values (n = 3) with standard deviations of wort sugar utilization in % of the 16 investigated yeast strains during fermentation from worts to non-alcoholic beers.

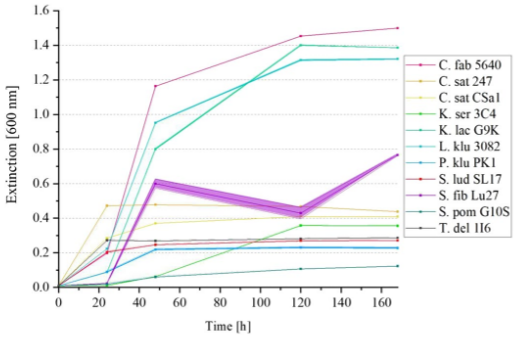


Figure 4. Mean values (n = 3) with standard deviations of yeast growth at 20 °C of ten yeast strains metabolizing more than 5% maltose in the HPLC analysis according to Table 2 (*C. fabianii* 5640, *C. saturnus* 247 and CSa1, *K. servazzii* 3C4, *K. lactis* G9K, *L. kluyveri* 3082, *P. kluyveri* PK1, *S. fibuligera* Lu27, *S. pombe* G10S, *T. delbrueckii* 116) measured at an extinction of 600 nm in YP-media with 1% maltose over 168 h (7 days). *S. ludwigii* SL17 was included as reference yeast strain.

measurement inaccuracies must also be taken into consideration here. Based on previous knowledge of the production of non-alcoholic beers using the reference yeast strain S. lud SL17, it is to be assumed for the investigated maltose-negative yeast strains that glucose and fructose, and optionally also sucrose, would have been utilized within the 144 h fermentation time at a temperature of 20 °C. The results show, however, that individual yeast strains certainly have different optima, which should be investigated within the scope of further research.

In general, the results of the HPLC analyses illustrate (Figure 3) that maltose and maltotriose can either not be metabolized at all or only to a small extent under the applied fermentation parameters in wort by the different yeast strains. Whether the yeasts from this study can actually metabolize maltose or maltotriose was investigated in further experiments in selective media. As described in the Experimental chapter, yeast strains utilizing, on average, more than 5% of maltose or maltotriose present in the beer were additionally incubated in YP-medium + 1% maltose or YP-medium + 1% maltotriose.

Figure 4 depicts the increases in the extinctions at an optical density of 600 nm over 168 h of ten different yeast strains in YP-media with 1% added maltose. The yeast strain *S. ludwigii* SL17 (marked in red) was additionally included as a maltose-negative reference yeast strain.

It can be observed that the extinctions increase most strongly within the first 24 to 48 h. Yeast cell growth mainly takes place during this period as the utilizable sugars from the medium are taken up by the yeast cells. In addition to 10 g/L maltose, traces of glucose, fructose, and sucrose were present in the YP-medium. These remained below concentrations of 0.1 g/L and thus below the detection limit of HPLC analysis. Nevertheless, the amounts were sufficient to ensure a slight growth of the maltose-negative yeasts

and to trigger a slight increase in the absorbance. Therefore, at absorbances < 0.5, there is only slight growth due to the presence of glucose, fructose, and sucrose in the culture medium. Those yeasts that were selectively able to utilize maltose had absorbances > 0.5 within the medium due to their strong growth. Figure 4 shows that the yeast strains *C. fabianii* 5640, *K. lactis* G9K, and *L. kluyveri* 3082 reached extinctions above 1.0 after 72 h and were thus able to metabolize maltose. *S. fibuligera* Lu27 also metabolized maltose. Due to its unusual morphology in the selective medium, which manifested itself in a macroscopically spherical growth (small gelatinous yeast balls), the absorbance could only be measured to a limited extent by photometer. This led to measurement inaccuracies because of an inhomogeneous distribution of the yeast spheres. Even an addition of 10 mM EDTA could not break up these cell formations. HPLC sugar analysis of the selective media after 168 h showed that indeed 100% of the maltose present was metabolized by the four yeasts mentioned, while maltose was still fully detectable in the samples of the remaining six yeast strains (values not shown). The results confirm that *C. fabianii* and *S. fibuligera* are able to metabolize maltose, which is consistent with the literature.[47,50] *K. lactis* 3082 can also partially utilize maltose. This yeast species is known to be Kluyver effect positive on maltose.[50,54] Although Lachance et al. [41] described that *L. kluyveri* is maltose negative during fermentation, the Kluyver effect needs to be considered as well since the samples were not incubated under completely anaerobic conditions.[55] For sterile sampling every 24 h, the samples were opened so that oxygen could enter the flasks. However, further studies would be required to establish why *C. fabianii* and *S. fibuligera* metabolize maltose rapidly only when exposed to a selective medium containing maltose. It is known that these two yeast strains metabolize maltose during fermentation. However, it is not possible to provide a conclusive reason why they metabolize a similarly low level of maltose as actual maltose-negative yeast strains in a brewing wort at 7°P and a fermentation temperature of 20 °C within 144 h.

Figure 5 depicts the increased extinctions at an optical density of 600 nm over 168 h of seven different yeast strains in YP-media with 1% added maltotriose. Again, the maltrotriose-negative *S. ludwigii* SL17 (marked in red) was added as the reference yeast strain.

Analogous to the analysis in YP-medium + 1% maltose, the addition of 1% maltotriose at an absorbance < 0.5 indicates that the yeasts are maltotriose-negative. From the seven yeasts analyzed in total, the two *C. fabianii* yeast strains 5640 and 5650 were the only two to show significant growth after 168 h. They had an absorbance of around 1.4 measured at 600 nm in the photometer and are thus maltotriose-positive. Although the yeast strain *C. fabianii* 5650 was not examined in the selective medium with an additional 1% maltose, based on these findings it can be concluded that the yeast can metabolize both maltose and maltotriose. While the maltotriose utilization of yeast strain C. fab 5640 takes longer in direct comparison to the maltose utilization (cf Figure 4), sugar analysis of the samples by HPLC measurement after

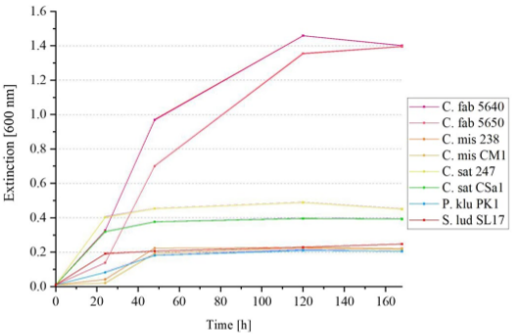


Figure 5. Mean values (n = 3) with standard deviations of yeast growth at 20 °C of seven strains (*C. fabianii* 5640 and 5650, *C. misumaiensis* 238 and CM1, *C. saturnus* 247 and CSa1, *P. kluyveri* PK1) metabolizing more than 5% maltotriose in the HPLC analysis according to Table 2 and measured at an extinction of 600 nm in YP-media with 1% maltotriose for 168 h (7 days). *S. ludwigii* SL17 was included as a reference yeast strain.

168 h showed that the maltotriose was completely metabolized (values not shown). Here, too, further investigations are needed to clarify why maltotriose can be completely metabolized in the selective medium after around 120 h, whereas it is hardly utilized by the yeasts in brewer's wort.

In general, the results show that the HPLC method for sugar analysis provides good indication values. However, additional analyses with greater accuracy are needed for accurate results. To safely exclude measurement inaccuracies, the sugars of a selected non-alcoholic beer produced by the yeast strain *C. saturnus* 247 were additionally chemically modified to make them visible in the HPLC with a UV/Vis detector. Figure 6 depicts the chromatogram with the modified sugars of the 7°P wort and the mean chromatogram of the three beers fermented with *C. saturnus* 247. The two chromatograms – wort (red) and beer (blue) – clearly show that maltose, maltotriose, and also maltotetraose remain unaffected. Glucose was utilized to 90.6%, which is consistent with the measured value from the previous HPLC analysis. Accordingly, the previous HPLC analyses of sugar utilization using the method according to Table 2 are actually measurement inaccuracies. This can be applied to all other investigated yeast strains with a maltose utilization of up to 13% and maltotriose utilization of up to 15%.

Above maltotetraose, higher sugars occurred only in traces, so that it can be assumed that the malt extract used was very well saccharified.

Secondary metabolites

As part of the analysis of secondary metabolites by GC/MS measurement of the 16 non-alcoholic beers, the following common volatiles in beer were quantified: esters, organic acids, higher alcohols, ketones, acetaldehyde, DMS, and 4-VG. In the heat map in Figure 7, the volatile compounds are shown as relative values. The 100% value represents the

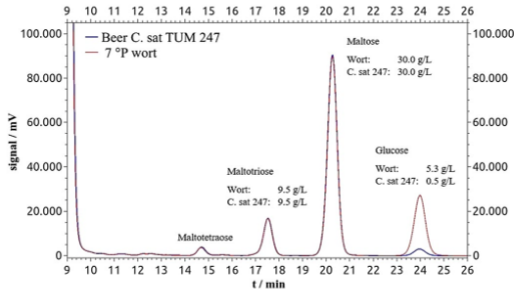


Figure 6. Chromatograms (HPLC) of the modified sugars glucose, maltose, maltotriose and maltotetraose of the 7°P wort and beer fermented with the yeast strain *C. saturnus* 247. The sugar molecules contain one molecule of 3-methyl-1-phenyl-5-pyrazolone (fructose) and two molecules of 3-methyl-1-phenyl-5-pyrazolone (glucose, maltose, maltotriose and maltotetraose).

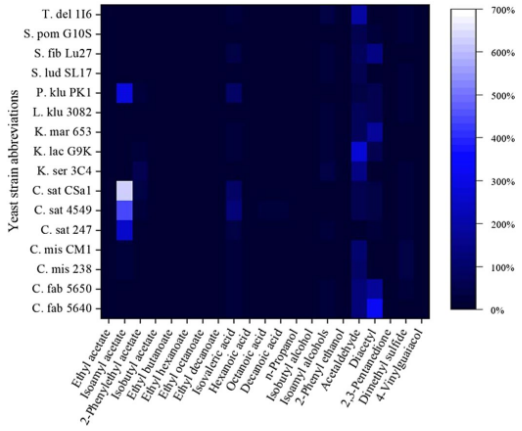


Figure 7. Heat map of volatile compounds in final non-alcoholic beers produced by the 16 investigated yeast strains during fermentation of brewer's wort at 20 °C for 144 h and detected by headspace gas chromatography measurement. The 100% value represents the threshold of volatile compounds in beer, which is based on relative values from Meilgaard, Sannino et al., and Schieberle.[56–58]

odor threshold of the respective substance in beer based on literature sources from Meilgaard, Sannino et al., and Schieberle.[56–58] If the values are above 100%, the volatile compounds should have a perceptible influence on the beer flavor. The complete table with values from triplicates can be found in Supplemental File 3. It should be noted that the flavor threshold values refer to regular alcoholic beers, so they should be considered as indicator values only. It is to be expected that some threshold values of non-alcoholic beers are fundamentally lower for selected volatiles, as both ethanol and certain volatile compounds have masking effe cts.[27,59]

Table 6. Number of sensory assessors (n = 10) identifying the beers produced with the 16 different non-*Saccharomyces* yeast strains as "fruity".

Yeast strain abbreviation	Number of assessors identifying the beer as "fruity"
C. fab 5640	10*
C. fab 5650	10*
C. mis 238	10*
C. mis CM1	9*
C. sat 247	10*
C. sat 4549	9*
C. sat CSa1	10*
K. ser 3C4	8
K. lac G9K	9*
K. mar 653	9*
L. klu 3082	9*
P. klu PK1	8
S. lud SL17	8
S. fib Lu27	9*
S. pom G10S	9*
T. del 1I6	7

*Significant fruitiness (α = 0.05) according to MEBAK sensory analysis 3.1.1. and DIN EN ISO 5495:2007

By taking a closer look at the fermentation by-products, it is evident that only a few volatiles were above the odor threshold. The brighter the fields in the heat map, the more the odor threshold was exceeded. The levels of isoamyl acetate in the non-alcoholic beers produced using *C. saturnus* yeast strains were strikingly high between 3.1 and 7.5 mg/L. In particular, the isoamyl acetate value of the yeast strain C. sat CSa1 stood out with a concentration of 7.5 mg/L, which is 6.25 times the threshold value of 1.2 mg/L. The *P. kluyveri* yeast strain PK1 was also capable of producing approx. 3.5 mg/L isoamyl acetate. This substance leads to desirable fruity flavors reminiscent of banana, pear, and apple, although the flavor can also be solvent-like.[56,60]

A less desirable impact on the beer flavor is described for volatiles such as acetaldehyde, diacetyl, and isovaleric acid. Aldehydes are known to cause worty off-flavors in non-alcoholic beers. The specific aldehydes for unpleasant worty off-flavors in non-alcoholic beers such as methional, 2-methylbutanal, and 3-methylbutanal were not analyzed in this study.[9,61] However, acetaldehyde in particular is known for its fruity, green apple, grassy flavor at a threshold above 10 mg/L. Therefore, it does not necessarily lead to an undesirable flavor in beer. The same is true for diacetyl, which causes buttery flavors at thresholds above 0.10 to 0.15 mg/L.[56,59] Isovaleric acid, on the other hand, is associated with negative flavor characteristics, as it is responsible for sweaty-cheesy to rancid flavors when exceeding the threshold of 1.5 mg/L.[56,61–64] Isovaleric acid was found in a single beer from the *C. saturnus* strain 4549 slightly above the odor threshold at a concentration of 1.77 mg/L. Diacetyl was above the threshold of 0.15 mg/L in four non-alcoholic beers. The values of the two beers from the *C. fabianii* yeast strains were significantly high at 0.48 mg/L (C. fab 5640) and 0.25 mg/L (C. fab 5650). The *K. marxianus* and *S. fibuligera* yeast strains also produced diacetyl quantities above the odor threshold at values of 0.26 mg/L (K. mar 653) and 0.21 mg/L (S. fib Lu27). Acetaldehyde was detected above the threshold in six beers. The highest value, which was more than 2.5 times the threshold value, was reached by

K. lactis G9K. With values ranging from about 10.0 to 14.5 mg/L, beers produced using *C. fabianii* 5640 and 5650, *C. misumaiensis* CM1, and *K. servazzii* 3C4 also exceeded the acetaldehyde threshold. The *T. delbrueckii* yeast strain 1I6 even produced about 19.5 mg/L acetaldehyde. *Saccharomyces* yeasts are known to degrade both diacetyl and acetaldehyde during the maturation phase.[65] Whether this also applies to the non-*Saccharomyces* yeast strains in this study would have to be verified, provided that a stronger diacetyl and acetaldehyde degradation is targeted. Since the cold storage of the non-alcoholic beers lasted for only 72 h after the six-day fermentation, the maturation phase was very short.

Although no further secondary metabolites were above the literature-based flavor thresholds, it is important to note that these thresholds refer to regular beers with alcohol. As shown in the heat map (cf Figure 7), the higher alcohols were well below the thresholds without exception, as expected for non-alcoholic beers. The organic acids hardly had any influence. Therefore, it can be assumed that the total esters already have a perceptible influence on the overall flavor of the respective beers at lower concentrations. According to this assumption, 2-phenylethyl acetate seems to have a noticeable influence on the overall flavor although the substance was measured only in smaller quantities below the odor threshold. In particular, the yeast *K. servazzii* 3C4 produced about 2.2 mg/L of 2-phenylethyl acetate, approaching the known threshold of 3.8 mg/L.[56] The three investigated *C. saturnus* yeast strains, as well as the *K. lactis* G9K, the *P. kluyveri* PK1, and the *K. marxianus* 653, were also able to produce between 0.35 and 1.30 mg/L of the fruity ester, which is linked to floral, honey, and apple flavor impressions.[66,67] The following sensory evaluations were to investigate this assumption in more detail.

The compound 4-VG, with its clove-like flavor,[68] was either not produced by any of the yeasts or only in concentrations below the limit of detection, and DMS was only produced at low concentrations below the odor threshold.

Sensory evaluation

First, all beers were evaluated to determine whether they were identified as fruity by the tasters. For this purpose, the paired test method according to MEBAK sensory analysis 3.1.1. and DIN EN ISO 5495:2007 was applied with a significance level $\alpha = 0.05$. To achieve a significantly fruity result, nine of the ten tasters had to identify the beer as generally fruity. The results are depicted in Table 6.

From the total of 16 beers, twelve beers were identified by the tasters as significantly fruity. These included, without exception, all seven beers from the *Cyberlindnera* yeast strains, the two beers produced with the *Kluyveromyces* yeast strains, and the three beers from L. klu 3082, S. fib Lu27, and S. pom G10S. Although four beers were classified as non-significantly fruity using the paired test method, 80% of the tasters nevertheless identified a fruity flavor in three beers (K. ser 3C4, P. klu PK1, S. lud SL17), while only one beer, namely the beer produced from T. del 1I6, was

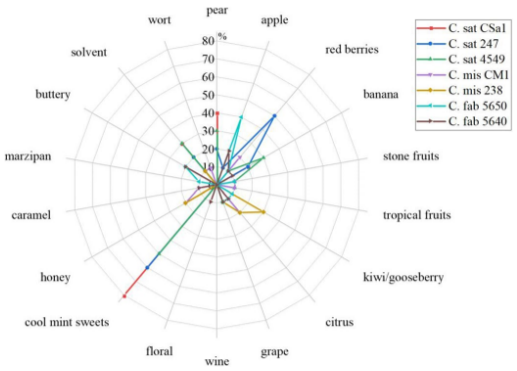


Figure 8. Aroma profiles of the beers fermented with the seven different *Cyberlindnera* yeast strains *C. saturnus* CSa1, 247, 4549, *C. misumaiensis* CM1, 238 and *C. fabianii* 5650, 5640.

described as fruity by just 70% of the tasters. The results reveal that the four beers that were evaluated as non-significantly fruity had a more weakly pronounced fruit character. The individual attributes for all beers are shown in the spiderwebs in Figure 8 and Figure 9.

Due to the large number of yeast strains studied, the tastings of the beers were carried out on two different days. First, all beers produced with the seven *Cyberlindnera* yeast strains were evaluated. The flavor profiles are depicted as spiderwebs in Figure 8. The individual spiderwebs can be found in Supplemental File 4.

The dominant cool mint sweets flavor is striking and was perceived by 50-80% of the tasters for all three *C. saturnus* yeast strains. Between 20 and 40% of the tasters additionally detected pear and banana flavors, as well as a solvent-like aroma. The tasters noted a clear difference between the three beers in 247, as 50% of the tasters perceived a red berry flavor, which was detected by only 10% of the tasters in 4549 and by none in CSa1. With the exception of the red berry flavor, the other attributes can be traced back to the high concentrations of isoamyl acetate, which were well above the odor threshold in the beers (cf Figure 7). To establish which secondary metabolites cause the red berry flavor would require an analysis of all volatile components, which was not conducted as part of these investigations.

No distinct aromas could be identified in the two beers of the *C. misumaiensis* yeast strains. The beer produced with C. mis 238 was most strongly reminiscent of kiwi and gooseberries with slight hints of citrus and honey, whereas the C. mis CM1 beer was perceived as non-specifically fruity with a slight honey-caramel character. Although acetaldehyde was above the threshold of 10 mg/L in the case of CM1, the assessors did not mention any green or apple-like impression. The fact that non-specific fruity flavors were perceived could be explained by the low concentrations of fruit esters. The conceivably lower threshold values could result in the total esters contributing to a slightly fruity character. Accordingly, these two yeast strains would be

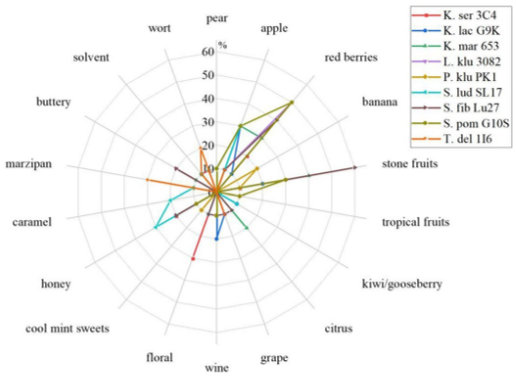


Figure 9. Aroma profiles of the beers fermented with the reference yeast strain *S. ludwigii* SL17 and the investigated yeast strains *K. servazzii* 3C4, *K. lactis* G9K, *K. marxianus* 653, *L. kluyveri* 3082, *P. kluyveri* PK1, *S. fibuligera* Lu27, *S. pombe* G10S, and *T. delbrueckii* 1I6.

suitable for producing more neutral non-alcoholic beers with subtle fruity flavors.

The two *C. fabianii* yeast strains appeared to produce similar flavors. Apple flavors and buttery aromas dominated, which can be attributed to acetaldehyde and diacetyl (cf Figure 7). Nevertheless, these two beers were also perceived as non-specifically fruity, which would suggest that here, too, the sum of the esters has a fruity influence on the overall flavor character of the beers.

In Figure 9, the flavor profiles of the further nine beers are depicted. The individual spiderwebs can be found in Supplemental File 4.

While the beer from *K. servazzii* 3C4 was perceived as floral and honey-like, possibly due to a lower threshold of 2-phenylethyl acetate, red berries were additionally detected by 20% of the tasters. Although acetaldehyde was analyzed in concentrations above the threshold, none of the assessors detected an apple-like flavor. It should be noted here that only 80% of tasters identified the beer as generally fruity, so it is classified as non-significant (cf Table 6). In contrast, an apple flavor was perceptible in the beer from *K. lactis* G9K, which could be due to acetaldehyde as well as 2-phenylethyl acetate. Otherwise, the beer was rated as rather non-specifically but significantly fruity (cf Table 6).

The beers made from *K. marxianus* 653 as well as *S. fibuligera* Lu27 were both described as slightly buttery, which can be attributed to the diacetyl value being above the odor threshold. Both beers were reminiscent of red berry, honey, stone fruit, and apple flavors. However, the apple flavor was more evident with *K. marxianus* 653 (possibly due to the presence of 2-phenylethyl acetate), while Lu27 had more red berry and especially stone fruit flavors. The stone fruit aroma showed a clear expression of plum (data not shown).

Although no flavor components above the odor thresholds were measured in the beers from *S. pombe* G10S, *L. kluyveri* 3082 or in the reference yeast strain *S. ludwigii* SL17 in the GC/MS analysis (cf Figure 7), the three beers exhibited distinct flavor characteristics in the sensory evaluation. Consistent with descriptions from the literature, the beer S. lud SL17 was described as having honey, apple, and also caramel-like flavors.[69,70] Even though the threshold value of 2-phenylethanol was not exceeded, only the beer produced from the *S. ludwigii* showed this flavor substance with honey and floral notes at a concentration of 6.6 mg/L. This could explain why the beer was perceived as less fruity than most of the other evaluated beers. The profiling of the beers S. pom G10S and L. klu 3082 showed strong similarities. Half of the tasters described red berry flavors for both beers, and 30% of the tasters additionally identified stone fruit flavors. An apple flavor was additionally described, although it was more prominent in the S. pom G10S beer. One difference between the two beers was in the honey-buttery flavors exhibited by the beer made from *L. kluyveri* 3082. The diacetyl value was measured at 0.08 mg/L and was thus below the threshold value of 0.1 mg/L. However, due to the generally low concentration of secondary metabolites, a buttery flavor could possibly be perceived by tasters who were sensitive to diacetyl.

The two beers from *P. kluyveri* PK1 and *T. delbrueckii* 1I6 were rated as relatively neutral. This explains why the two beers were not perceived as significantly fruity. Only a few tasters recognized the isoamyl acetate analyzed in the P. klu PK1 beer (3.43 mg/L) as banana, cool mint sweets, and solvent. The beer T. del 1I6 was also described as neutral, however, the tasters detected marzipan and wort-like flavors. These are probably due to aldehydes present in the malt extract or wort, which were enhanced by boiling. Many yeasts are capable of degrading the aldehydes during fermentation, which *T. delbrueckii* 1I6 obviously did not. Although acetaldehyde was analyzed at twice the threshold level, only 10% of the tasters indicated an apple-like aroma. Accordingly, the wort-like flavors seemed to predominate.

Comparable to the beer fermented with the *C. saturnus* yeast strain 247, *L. kluyveri* 3082, *S. pombe* G10S, and *S. fibuligera* Lu27 in particular showed red berry flavors. As mentioned above, further volatile components would have to be analyzed to detect the substances responsible for this kind of flavor. Additionally, there may also be a synergistic effect from different flavor compounds. Furthermore, some beers exhibited a honey flavor that could not be clearly attributed to the analyzed secondary metabolites. It is possible that the honey aroma was caused by phenylacetaldehyde, which was not quantified in this study.[71]

Looking at the biplot in Figure 10 with the principal components PC 1 and PC 2 as a result of principal component analysis, it can be seen that only 55% of the variation in the data is captured by the first two principal components. Therefore, only slightly more than half of the available information is included in the biplot. Accordingly, PC 3 and PC 4 were also included in the analysis to account for over three-quarters (77.09%) of the data. The full data can be found in Supplemental File 5.

Considering the loading plot in Figure 10, the three beers produced with the *Cyberlindnera saturnus* yeast strains as well as the P. klu PK1 have a positive correlation with each

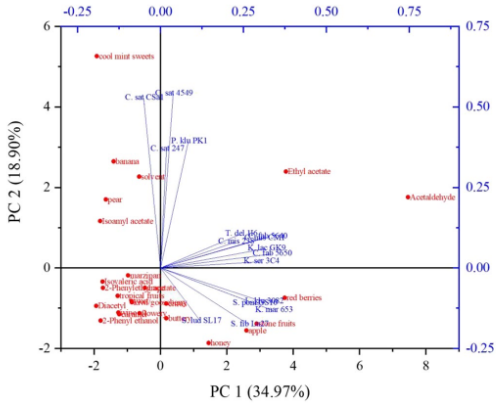


Figure 10. Principal Component Analysis (PCA) biplot with two principal components PC 1 and PC 2 showing the correlation of the beers produced with the 16 investigated yeast strains and the specific volatile compounds measured analytically as well as the individual flavor characteristics described by the tasters.

other. They are directly correlated with PC 2, which results from the flavor impressions cool mint sweets, banana, pear, and solvent perceived by the tasters. While isoamyl acetate correlates with the aforementioned flavor impressions, the correlation with ethyl acetate, which has a solvent-like aroma, is lower according to the PCA plot. Based on PC 1, the PCA plot shows that the beers produced with the yeast strains K. mar 653, L. klu 3082, S. pom GS10, and S. fib Lu27 exhibit red berry, stone fruit, and apple flavors, while the beer from the reference yeast strain S. lud SL17 shows next to apple-like more a honey-like flavor. When PC 1 and PC 3 are correlated with each other in the principal component analysis (cf Supplemental File 5), this correlation can be confirmed. However, the loading plot of PC 1 and PC 3 shows that there is only a positive correlation between K. mar 653, L. klu 3082, S. pom GS10, and S. fib Lu27, but not with S. lud SL17. By depicting PC 4, a correlation can be seen between the beers from the yeast strains C. mis 238 and C. mis CM1, with the flavor impressions kiwi/gooseberry as well as citrus. Apart from the distinct correlation of isoamyl acetate with the flavor impressions of cool mint sweets, banana, and pear, no further correlations between analyzed volatile components and tasted flavors can be statistically verified. This illustrates that further volatile components need to be analyzed in order to be able to explain the flavor characteristics perceived by the tasters and that even synergistic effects of certain volatiles may occur.

Table 7 shows the total DLG score according to the DLG evaluation scheme.[17] Since the beers were unhopped and fermentation was unpressurized, the quality of bitterness and carbonation ratings were excluded from the evaluation. The DLG evaluation scheme is primarily intended to detect sensory product faults. A score of 4.0 designates the product as good, with 5.0 being the top rating.

All beers were rated between 4.0 and 5.0, indicating a consistently good product quality. In a comparison of the

Table 7. Total DLG score according to the DLG evaluation scheme excluding the quality of bitterness as well as carbonation of the 15 investigated yeast strains and the reference yeast strain *S. ludwigii* SL17.

Yeast strain abbreviation	Total DLG score
C. fab 5640	4.16
C. fab 5650	4.44
C. mis 238	4.14
C. mis CM1	4.34
C. sat 247	4.54
C. sat 4549	4.54
C. sat CSa1	4.44
K. ser 3C4	4.16
K. lac G9K	4.00
K. mar 653	4.38
L. klu 3082	4.10
P. klu PK1	4.02
S. lud SL17	4.16
S. fib Lu27	4.32
S. pom G10S	4.22
T. del 1I6	4.08

16 beers, those fermented with the *C. saturnus* yeast strains C. sat 247 and C. sat 4549 stood out with the best ratings at 4.54 each, closely followed by C. sat CSa1. Although the threshold value of isovaleric acid was slightly exceeded in the 4549 beer, no negative sensory influence was described. The high concentration of isoamyl acetate (5.23 mg/L), which is known to cause a masking effect, was most probably responsible for masking the sensory effect of isovaleric acid.[59] The reference yeast S. lud SL17 with a rating of 4.16 was to the lower end of the middle range. While the buttery flavor from diacetyl and the apple-like flavor from acetaldehyde were rated as positive in the beers produced with the yeast strains *C. fabianii* 5650, *K. marxianus* 653, and *S. fibuligera* Lu27, other beers, such as the L. klu 3082, were devalued by it. Nevertheless, it is known from previous studies that diacetyl in particular does not necessarily lead to negative flavor impressions.[25] This illustrates that the threshold values of individual flavor components can be evaluated, however, the overall flavor profile determines how harmoniously the individual flavors interact.

Conclusion

In summary, the 15 yeast strains investigated are suitable for producing non-alcoholic beers. Although not all yeast strains are maltose-negative during fermentation, as *S. fibuligera* Lu27 and the two *C. fabianii* yeast strains 5640 and 5650 possess the ability to fundamentally metabolize maltose, the activity at 20 °C is low. Therefore, the ethanol contents remain below 0.5% (*v/v*) and stay within the German regulation for non-alcoholic beers during a six-day fermentation period at an original wort of 7°P. For the two yeast strains *L. kluyveri* 3082 and *T. delbrueckii* 1I6, the original wort should be adjusted in a reducing manner to remain safely within the alcohol-free range.

The aim of producing non-alcoholic beers with desirable flavor characteristics but without wort and phenolic off-flavors during fermentation was achieved, as a wide range of novel aromas were detected during sensory evaluation. The non-alcoholic beers produced with the three

C. saturnus yeast strains (C. sat 247, C. sat 4549, C. sat CSa1) were particularly impressive with cool mint sweets, pear, and in some cases red berry flavors. There were hardly any sensory flaws in the beers fermented with the yeasts *K. marxianus* 653 and *S. fibuligera* Lu27, which displayed a broad flavor diversity of red berry, honey, stone fruit, and apple. Only one yeast stood out with less desirable wort flavors, namely *T. delbrueckii* 1I6. Many of the secondary metabolites analyzed were below the flavor threshold values known for beer from the literature despite the sensory perception of high flavor diversity. However, since the thresholds relate to regular alcoholic beers, masking effects due to ethanol or certain secondary metabolites can be assumed to be the reason. In addition, GC/MS analysis would need to be extended to include further volatile flavor compounds to obtain a complete picture of which secondary metabolites are responsible for the overall flavor of the analyzed non-alcoholic beers.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Wifö (Wissenschaftsförderung der Deutschen Brauwirtschaft e.V., Berlin, Germany) under Grant number AiF 20658 N.

ORCID

Yvonne Methner http://orcid.org/0000-0002-7489-9333
Mathias Hutzler http://orcid.org/0000-0001-5642-4919

Literature cited

[1] Ahuja, K.; Rawat, A. Market Size of Non-Alcoholic Beer Worldwide from 2016 to 2024 (in Billion U.S. Dollars). **2020**. https://www.gminsights.com/industry-analysis/non-alcoholic-beer-market. (accessed Jun 17, 2021).
[2] Bellut, K.; Arendt, E. K. Chance and Challenge: Non-*Saccharomyces* Yeasts in Nonalcoholic and Low Alcohol Beer Brewing – A Review. *J. Am. Soc. Brew. Chem.* **2019**, *77*, 77–91. DOI: 10.1080/03610470.2019.1569452.
[3] Methner, Y.; Hutzler, M.; Matoulková, D.; Jacob, F.; Michel, M. Screening for the Brewing Ability of Different Non-*Saccharomyces* Yeasts. *Fermentation* **2019**, *5*, 101. DOI: 10.3390/fermentation5040101.
[4] Stewart, G. G. The Horace Brown Medal Lecture: Forty Years of Brewing Research. *J. Inst. Brew.* **2009**, *115*, 3–29. DOI: 10.1002/j.2050-0416.2009.tb00340.x.
[5] Gotsick, J. T.; Benson, R. F. HPLC Analysis of Carbohydrates Important to Beer Brewing Using an Aminopropyl Stationary Phase. *J. Liq. Chromatogr.* **1991**, *14*, 1887–1901.DOI: 10.1080/01483919108049660.
[6] Harris, G.; Thompson, C. C. The Uptake of Nutrients by Yeasts III. The Maltose Permease of a Brewing Yeast. *Biochim. Biophys. Acta* **1961**, *52*, 176–183. DOI: 10.1016/0006-3002(61)90915-5.
[7] Haehn, H.; Glaubitz, M. Beer Manufacture: Patent US1898047 A. **1933**.
[8] Kunz, T.; Methner, F.-J. Verfahren Zur Herstellung Eines Getränkes. (EP2382303A1). **2009**.
[9] Blanco, C. A.; Andrés-Iglesias, C.; Montero, O. Low-Alcohol Beers: Flavor Compounds, Defects, and Improvement Strategies. *Crit. Rev. Food Sci. Nutr.* **2016**, *56*, 1379–1388. DOI: 10.1080/10408398.2012.733979.
[10] Liu, S.-Q.; Quek, A. Y. H. Evaluation of Beer Fermentation with a Novel Yeast *Williopsis saturnus*. *Food Technol. Biotechnol.* **2016**, *54*, 403–412. DOI: 10.17113/ftb.54.04.16.4440.
[11] Bellut, K.; Michel, M.; Zarnkow, M.; Hutzler, M.; Jacob, F.; Atzler, J. J.; Hoehnel, A.; Lynch, K. M.; Arendt, E. K. Screening and Application of *Cyberlindnera* Yeasts to Produce a Fruity, Non-Alcoholic Beer. *Fermentation* **2019**, *5*, 103. DOI: 10.3390/fermentation5040103.
[12] van Rijswijck, I. M. H.; van Mastrigt, O.; Pijffers, G.; Wolkers-Rooijackers, J. C. M.; Abee, T.; Zwietering, M. H.; Smid, E. J. Dynamic Modelling of Brewers' Yeast and *Cyberlindnera fabianii* Co-Culture Behaviour for Steering Fermentation Performance. *Food Microbiol.* **2019**, *83*, 113–121. DOI: 10.1016/j.fm.2019.04.010.
[13] Bellut, K.; Michel, M.; Zarnkow, M.; Hutzler, M.; Jacob, F.; Schutter, D.; de; Daenen, L.; Lynch, K.; Zannini, E.; Arendt, E. Application of Non-*Saccharomyces* Yeasts Isolated from Kombucha in the Production of Alcohol-Free Beer. *Fermentation* **2018**, *4*, 66. DOI: 10.3390/fermentation4030066.
[14] Capece, A.; Fusco, D d.; Pietrafesa, R.; Siesto, G.; Romano, P. Performance of Wild Non-Conventional Yeasts in Fermentation of Wort Based on Different Malt Extracts to Select Novel Starters for Low-Alcohol Beers. *Appl. Sci.* **2021**, *11*, 801. DOI: 10.3390/app11020801.
[15] Holt, S.; Mukherjee, V.; Lievens, B.; Verstrepen, K. J.; Thevelein, J. M. Bioflavoring by Non-Conventional Yeasts in Sequential Beer Fermentations. *Food Microbiol.* **2018**, *72*, 55–66. DOI: 10.1016/j.fm.2017.11.008.
[16] Michel, M.; Kopecká, J.; Meier-Dörnberg, T.; Zarnkow, M.; Jacob, F.; Hutzler, M. Screening for New Brewing Yeasts in the Non-*Saccharomyces* Sector with *Torulaspora delbrueckii* as Model. *Yeast (Chichester, England)* **2016**, *33*, 129–144. DOI: 10.1002/yea.3146.
[17] Gutiérrez, A.; Boekhout, T.; Gojkovic, Z.; Katz, M. Evaluation of Non-*Saccharomyces* Yeasts in the Fermentation of Wine, Beer and Cider for the Development of New Beverages. *J. Inst. Brew.* **2018**, *124*, 389–402. DOI: 10.1002/jib.512.
[18] Saerens, S.; Swiegers, J. H. Production of Low-Alcohol or Alcohol-Free Beer with *pichia kluyveri* Yeast Strains. Google Patents, **2016**.
[19] Vaštík, P.; Šmogrovičová, D.; Kafková, V.; Sulo, P.; Furdíková, K.; Špánik, I. Production and Characterisation of Non-Alcoholic Beer Using Special Yeast. *Kvasný Průmysl/Ferment. Ind.* **2020**, 66, 336–344. DOI: 10.18832/kp2019.66.336.,
[20] Johansson, L.; Nikulin, J.; Juvonen, R.; Krogerus, K.; Magalhães, F.; Mikkelson, A.; Nuppunen-Puputti, M.; Sohlberg, E.; de Francesco, G.; Perretti, G.; Gibson, B. Sourdough Cultures as Reservoirs of Maltose-Negative Yeasts for Low-Alcohol Beer Brewing. *Food Microbiol.* **2021**, *94*, 103629. DOI: 10.1016/j.fm.2020.103629.
[21] Meier-Dörnberg, T.; Schneiderbanger, H.; Jacob, F.; Hutzler, M. Alcohol-Free Wheat Beer with Maltose Negative Yeast Strain *Saccharomycodes ludwigii*. *Young Scientists Symposium on Malting, Brewing and Distilling*. **2014**. DOI: 10.13140/RG.2.2.26169.36968.
[22] De Francesco, G.; Turchetti, B.; Sileoni, V.; Marconi, O.; Perretti, G. Screening of New Strains of *Saccharomycodes ludwigii* and *Zygosaccharomyces rouxii* to Produce Low-Alcohol Beer. *J. Inst. Brew.* **2015**, *121*, 113–121. DOI: 10.1002/jib.185.,
[23] Pahl, R. *Modern Production of Alcohol Free Beers for Varying Breweries and Beer Styles*. 12th Trends in Brewing. Ghent, April 3-7, **2016**.
[24] Parise, A.; Kovaleva, E. G.; Glukhareva, T. V.; Shatunova, S. A. Biotechnological Production of Non-Traditional Beer. In *AIP Conference Proceedings (Online)*. **2017** DOI: 10.1063/1.5002995.
[25] Callejo, M. J.; García Navas, J. J.; Alba, R.; Escott, C.; Loira, I.; González, M. C.; Morata, A. Wort Fermentation and Beer Conditioning with Selected Non-*Saccharomyces* Yeasts in Craft Beers. *Eur. Food Res. Technol.* **2019**, *245*, 1229–1238. DOI: 10.1007/s00217-019-03244-w.

[26] Perpète, P.; Collin, S. Contribution of 3-Methylthiopropionaldehyde to the Worty Flavor of Alcohol-Free Beers. *J. Agric. Food Chem.* **1999**, *47*, 2374–2378. DOI: 10.1021/jf9811323.

[27] Perpète, P.; Collin, S. Influence of Beer Ethanol Content on the Wort Flavour Perception. *Food Chem.* **2000**, *71*, 379–385. DOI: 10.1016/s0308-8146(00)00179-5.

[28] Müller, M.; Bellut, K.; Tippmann, J.; Becker, T. Physical Methods for Dealcoholization of Beverage Matrices and Their Impact on Quality Attributes. *ChemBioEng Rev.* **2017**, *4*, 310–326. DOI: 10.1002/cben.201700010.

[29] Back, W.; Bohak, I. *Ausgewählte Kapitel Der Brauereitechnologie*, 2. aktualisierte Aufl.; Carl: Nürnberg, **2008**.

[30] Hutzler, M.; Methner, Y.; Jacob, F. *Spezialhefen für alkoholfreie und alkoholarme Biere*. **2021**, *25–26*, 634–637.

[31] Esslinger, H. M. *Handbook of Brewing: Processes, Technology, Markets*; John Wiley & Sons: Weinheim, Germany, **2009**.

[32] Bleyer, B.; Bames, E.; Büttner, G.; Diemair, W.; Holthöfer, H.; Reichard, O.; Vogt, E.; Bömer, A.; Juckenack, A.; Tillmans, B. *Alkoholische Genussmittel*; Springer: Berlin Heidelberg, **2013**.

[33] Nikulin, J.; Eerikäinen, R.; Hutzler, M.; Gibson, B. Brewing Characteristics of the Maltotriose-Positive Yeast *Zygotorulaspora* Florentina Isolated from Oak. *Beverages* **2020**, *6*, 58. DOI: 10.3390/beverages6040058.

[34] Honda, S.; Akao, E.; Suzuki, S.; Okuda, M.; Kakehi, K.; Nakamura, J. High-Performance Liquid Chromatography of Reducing Carbohydrates as Strongly Ultraviolet-Absorbing and Electrochemically Sensitive 1-Phenyl-3-Methyl5-Pyrazolone Derivatives. *Anal. Biochem.* **1989**, *180*, 351–357. DOI: 10.1016/0003-2697(89)90444-2.

[35] Hildebrandt, G.; Schneider-Haeder, B. Sensorische Analyse: Methodenüberblick und Einsatzbereiche – DLG-Sensorik, **2009**. www.dlg.org.

[36] Narziß, L.; Back, W.; Miedaner, H.; Lustig, S. Untersuchung Zur Beeinflussung Der Geschmacksstabilität Durch Variation Technologischer Parameter Bei Der Bierherstellung. *Monatsschrift Für Brauwissenschaft* **1999**, *1999*, 192–206.

[37] Boundy-Mills, K.; Stratford, M.; Miller, M. W. Chapter 62 - *Saccharomycodes* E.C. Hansen (1904). In *The Yeasts: A Taxonomic Study*, 5th ed.; Kurtzman, C. P., Ed.; Elsevier: Amsterdam, **2011**; pp 747–750.

[38] Lachance, M.-A. Chapter 35 - *Kluyveromyces Van Der Walt* (1971). In *The Yeasts: A Taxonomic Study*, 5th ed.; Kurtzman, C. P., Ed.; Elsevier: Amsterdam, **2011**; pp 471–481.

[39] Berthels, N. J.; Cordero Otero, R. R.; Bauer, F. F.; Thevelein, J. M.; Pretorius, I. S. Discrepancy in Glucose and Fructose Utilisation during Fermentation by *Saccharomyces cerevisiae* Wine Yeast Strains. *FEMS Yeast Res.* 2004, *4*, 683–689. DOI: 10.1016/j.femsyr.2004.02.005.

[40] Kurtzman, C. P. Chapter 75 - *Torulaspora* Lindner (1904). In *The Yeasts: A Taxonomic Study*, 5th ed.; Kurtzman, C. P., Ed.; Elsevier: Amsterdam, **2011**; pp 867–874

[41] Lachance, M.-A.; Kurtzman, C. P. Chapter 41 - *Lachancea* Kurtzman (2003): *Lachancea* Kluyveri (Phaff, M.W. Miller & Shifrine) Kurtzmann (2003). In *The Yeasts: A Taxonomic Study*, 5th ed.; Kurtzman, C. P., Ed.; Elsevier: Amsterdam, **2011**; pp 514–515.

[42] Kurtzman, C. P. Chapter 42 - *Lindnera* Kurtzman, Robnett & Basehoar-Powers (2008). In: The Yeasts: *A taxonomic study*, 5th ed. Kurtzman, C. P., Ed.; Elsevier: Amsterdam, **2011**; pp 521–543.

[43] Kulshrestha, S.; Tyagi, P.; Sindhi, V.; Yadavilli, K. S. Invertase and Its Applications – A Brief Review. *J. Pharm. Res.* **2013**, *7*, 792–797. DOI: 10.1016/j.jopr.2013.07.014.

[44] Suzuki, H.; Ozawa, Y.; Maeda, H. Studies on the Water-Insoluble Enzyme. *Agric. Biol. Chem.* **1966**, *30*, 807–812. DOI: 10.1080/00021369.1966.10858683.

[45] Kurtzman, C. P. Chapter 57 - *Pichia* E.C. Hansen (1904): *Pichia* Kluyveri Bedford Ex Kudryavtsev (1960). In *The Yeasts: A Taxonomic Study*, 5th ed.; Kurtzman, C. P., Ed.; Elsevier: Amsterdam, **2011**; pp 695–696.

[46] Vaughan-Martini, A.; Martini, A. Chapter 66 - *Schizosaccharomyces* Lindner (1893): *Schizosaccharomyces pombe* Lindner (1893). In *The Yeasts: A Taxonomic Study*, 5th ed. Kurtzman, C. P., Ed.; Elsevier: Amsterdam, **2011**; pp 782–784.

[47] Kurtzman, C. P. Chapter 42 - *Lindnera* Kurtzman, Robnett & Basehoar-Powers (2008): *Lindnera misumaiensis* (Sasaki & Yoshida Ex Kurtzman, Robnett & Basehoar-Powers). In *The Yeasts: A Taxonomic Study*, 5th ed.; Kurtzman, C. P., Ed.; Elsevier: Amsterdam, **2011**; pp 534–535.

[48] Vaughan-Martini, A.; Lachance, M.-A.; Kurtzman, C. P. Chapter 34 - *Kazachstania* Zubkova (1971): *Kazachstania servazzii* (Capriotti) Kurtzman (2003). In *The Yeasts: A Taxonomic Study*, 5th ed. Kurtzman, C. P., Ed.; Elsevier: Amsterdam, **2011**; pp 439–470.

[49] Wickerham, L. J. Evidence of the production of extracellular invertase by certain strains of yeasts. *Archives of Biochemistry and Biophysics* 1958, 76, 439-448. DOI: 10.1016/0003-9861(58)90169-3.

[50] Kurtzman, C. P.; Smith, M. T. Chapter 63 - *Saccharomycopsis* Schiönning (1903): *Saccharomycopsis fibuligera* (Lindner) Klöcker (1924). In *The Yeasts: A Taxonomic Study*, 5th ed.; Kurtzman, C. P., Ed.; Elsevier: Amsterdam, **2011**; pp 755–756.

[51] Chen, L.; Chi, Z.-M.; Chi, Z.; Li, M. Amylase Production by *Saccharomycopsis fibuligera* A11 in Solid-State Fermentation for Hydrolysis of Cassava Starch. *Appl. Biochem. Biotechnol.* **2010**, *162*, 252–263. DOI: 10.1007/s12010-009-8744-3.

[52] Futatsugi, M.; Ogawa, T.; Fukuda, H. Purification and Properties of Two Forms of Glucoamylase From *Saccharomycopsis fibuligera*. *J. Ferment. Bioeng.* **1993**, *76*, 521–523. DOI: 10.1016/0922-338X(93)90252-4.

[53] Hostinová, E. Amylolytic Enzymes Produced by the Yeast *Saccharomycopsis fibuligera*. *BIOLOGIA-BRATISLAVA* **2002**, *57*, 247–252.

[54] Fukuhara, H. The Kluyver Effect Revisited. *FEMS Yeast Res.* **2003**, *3*, 327–331. DOI: 10.1016/S1567-1356(03)00112-0.,

[55] Kluyver, A. J.; Custers, M. T. J. The Suitability of Disaccharides as Respiration and Assimilation Substrates for Yeasts Which Do Not Ferment These Sugars. *Antonie Van Leeuwenhoek* **1939**, *6*, 121–162. DOI: 10.1007/BF02146183.

[56] Meilgaard, M. C. Flavor Chemistry of Beer. II. Flavor and Threshold of 239 Aroma Volatiles. *Tech. Quart. Master. Brew. Assoc. Am.* **1975**, *12*, 151–168.

[57] Sannino, C.; Mezzasoma, A.; Buzzini, P.; Turchetti, B. Non-Conventional Yeasts for Producing Alternative Beers. In *Non-Conventional Yeasts: From Basic Research to Application*; Sibirny, A., Ed.; Springer International Publishing: Cham, **2019**; pp 361–388.

[58] Schieberle, P. Primary Odorants of Pale Lager Beer. *Z Lebensm. Unters. Forch.* **1991**, *193*, 558–565. DOI: 10.1007/BF01190873.

[59] Saison, D.; Schutter, D. P.; de; Uyttenhove, B.; Delvaux, F.; Delvaux, F. R. Contribution of Staling Compounds to the Aged Flavour of Lager Beer by Studying Their Flavour Thresholds. *Food Chem.* **2009**, *114*, 1206–1215. DOI: 10.1016/j.foodchem.2008.10.078.

[60] Collin, S.; Derdelinckx, G.; Dufour, J.-P. Relationships between the Chemical Composition and Sensory Evaluation of Lager Beers. *Food Qual. Preference* **1994**, *5*, 145–149. DOI: 10.1016/0950-3293(94)90021-3.

[61] Gernat, D. C.; Brouwer, E.; Ottens, M. Aldehydes as Wort off-Flavours in Alcohol-Free Beers—Origin and Control. *Food Bioprocess Technol.* **2020**, *13*, 195–216. DOI: 10.1007/s11947-019-02374-z.

[62] Kishimoto, T.; Wanikawa, A.; Kono, K.; Shibata, K. Comparison of the Odor-Active Compounds in Unhopped Beer and Beers Hopped with Different Hop Varieties. *J. Agric. Food Chem.* **2006**, *54*, 8855–8861. DOI: 10.1021/jf061342c.

[63] Olšovská, J.; Vrzal, T.; Štěrba, K.; Slabý, M.; Kubizniaková, P.; Čejka, P. The Chemical Profiling of Fatty Acids during the Brewing Process. *J. Sci. Food Agric.* **2019**, *99*, 1772–1779. DOI: 10.1002/jsfa.9369.

[64] Witrick, K. Comparison of the Flavor and Aroma Compounds Present in Aging Lambic Beer; ASBC Meeting, Fort Myers, Florida, **2017**. https://www.asbcnet.org/events/archives/2017ASBCMeeting/proceedings/2017Proceedings/10_Witrick.pdf.
[65] Narziss, L.; Back, W.; Gastl, M.; Zarnkow, M. *Abriss Der Bierbrauerei*, 8th ed.; John Wiley & Sons Incorporated: Newark, **2017**.
[66] Fritsch, H. T.; Schieberle, P. Identification Based on Quantitative Measurements and Aroma Recombination of the Character Impact Odorants in a Bavarian Pilsner-Type Beer. *J. Agric. Food Chem.* **2005**, *53*, 7544–7551. DOI: 10.1021/jf051167k.
[67] Kobayashi, M.; Shimizu, H.; Shioya, S. Beer Volatile Compounds and Their Application to Low-Malt Beer Fermentation. *J. Biosci. Bioeng.* **2008**, *106*, 317–323. DOI: 10.1263/jbb.106.317.
[68] Schwarz, K. J.; Stübner, R.; Methner, F.-J. Formation of Styrene Dependent on Fermentation Management during Wheat Beer Production. *Food Chem.* **2012**, *134*, 2121–2125. DOI: 10.1016/j.foodchem.2012.04.012.
[69] Hutzler, M.; Zarnkow, M.; Hans, S.; Stretz, D.; Meier-Dörnberg, T.; Methner, Y.; Schneiderbanger, H.; Jacob, F. New Yeasts - New Beers. *Brewing and Beverage Industry International*, **2020**, pp 16–23.
[70] Romano, P.; Marchese, R.; Laurita, C.; Saleano, G.; Turbanti, L. Biotechnological Suitability of *Saccharomycodes ludwigii* for Fermented Beverages. *World Journal of Microbiology and Biotechnology* **1999**, *15*, 451–454. DOI: 10.1023/A:1008948623024.
[71] Schieberle, P.; Komarek, D. Changes in Key Aroma Compounds during Natural Beer Aging. In *Freshness and Shelf Life of Foods*; Cadwallader, K. R., Weenen, H., Eds.; American Chemical Society: Washington, DC, **2003**; pp 70–79.

2.3 Publication 3: Beer fermentation performance and sugar uptake of *Saccharomycopsis fibuligera*–A novel option for low-alcohol beer

The yeast *Saccharomycopsis fibuligera* resulted from the publications 1 and 2 as a promising species for the production of beers with exceptionally positive flavor properties reminiscent of plum and berries. With regard to the sugar metabolism, however, it has not yet been clarified why the yeast strain S. fib Lu27 was able to completely metabolize maltose in the selective medium within 168 h at 20 °C, while under similar conditions, it hardly consumed any maltose from the wort. For this reason, the maltose and maltotriose uptake activities of the two strains S. fib Lu27 and SF4 were analyzed as part of publication 3. It turned out that both yeast strains possessed active maltose transporters, whereas no active maltotriose transporters could be found.

A further challenge when applying *S. fibuligera* on an industrial brewing scale is its mycelial growth. This causes insufficient flocculation, which is disadvantageous for clarification. In order to improve the application properties, an approach using micromanipulation was conducted. Two cells were isolated from the mother culture S. fib SF4, which were cultivated at 20 °C and 28 °C in 10 °P unhopped wort. In parallel, the mother culture was cultivated at both temperatures before a microscopic comparison was carried out on the four samples and a further sample of S. fib SF4 from slant agar. Morphological differences were found in the microscopic picture. While the micromanipulated culture hardly showed any growing filaments and budding cells, the mother culture mainly developed mycelia under the same cultivation conditions. The culture from the slant agar showed filaments and single cells in the microscopic picture. Due to the morphological differences, $(GTG)_5$ rep-PCR fingerprints were affiliated from the micromanipulated cultures cultivated at 20 °C and 28 °C as well as from the mother cultures. Despite the morphological differences, the $(GTG)_5$ rep-PCR fingerprints revealed no genetic differences. Brewing trials were conducted to establish whether the morphological differences had an impact on the beer production. The mother culture cultivated at 20 °C and the sample from the micromanipulated single cell cultivated at 20 °C underwent unpressurized fermentation at 20 °C in 1,800 mL unhopped wort with an original gravity of 10 °P. The same procedure was applied for the 28 °C cultures. The pitching rate was set at 10×10^6 cells/mL and the fermentation lasted for 480 h. All four samples proved to be relatively weak in fermentation compared to the reference yeast strain *Saccharomyces pastorianus* TUM 34/70. While an ethanol concentration of around 4.5% (*v*/*v*) was measured in the reference beer at a fermentation temperature of 20 °C, it stayed between 0.74–0.83% (*v*/*v*) for the two *S. fibuligera* beers. At 28 °C, ethanol concentrations of 0.83–1.20% (*v*/*v*) were achieved, while the reference beer reached a concentration of around 4.6% (*v*/*v*). During the sensory evaluation, the trained panellists could not perceive any differences in the flavor profiles of the different SF4 beers. Plum and berry flavors still remained dominant. However, mycelial formation was significantly reduced in the two beers fermented with the micromanipulated cultures, regardless of the temperature, which would provide a technological advantage for large-scale industrial application.

Authors/Authorship contribution:

Methner, Y.: Conception and design of experiments, performance of experiments, analysis of data using OriginPro 2020 as statistical software, data curation, paper writing; **Magalhães, F.**: Conception and design of sugar uptake activity experiments, performance, analysis of data, data curation and method writing of sugar uptake activity experiments, revision of manuscript; **Raihofer, L.**: Preparation of microscopical images, revision of manuscript; **Zarnkow, M.**: Revision of manuscript; **Jacob, F.**: Supervision of project, approval for submission, funding acquisition; **Hutzler, M.**: Supervision of project, conception and design of experiments, revision of manuscript.

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 05 October 2022
DOI 10.3389/fmicb.2022.1011155

OPEN ACCESS

EDITED BY
Haifeng Zhao,
South China University of Technology, China

REVIEWED BY
Diego Bonatto,
UFRGS, Brazil
Olimpio Montero,
Spanish National Research Council (CSIC), Spain
André Alcarde,
University of São Paulo, Brazil

*CORRESPONDENCE
Mathias Hutzler
m.hutzler@tum.de

SPECIALTY SECTION
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

RECEIVED 03 August 2022
ACCEPTED 06 September 2022
PUBLISHED 05 October 2022

CITATION
Methner Y, Magalhães F, Raihofer L, Zarnkow M, Jacob F and Hutzler M (2022) Beer fermentation performance and sugar uptake of *Saccharomycopsis fibuligera*–A novel option for low-alcohol beer. *Front. Microbiol.* 13:1011155. doi: 10.3389/fmicb.2022.1011155

Beer fermentation performance and sugar uptake of *Saccharomycopsis fibuligera*–A novel option for low-alcohol beer

Yvonne Methner[1], Frederico Magalhães[2,3], Luis Raihofer[1], Martin Zarnkow[1], Fritz Jacob[1] and Mathias Hutzler[1]*

[1]Research Center Weihenstephan for Brewing and Food Quality, Technical University of Munich, Freising, Germany, [2]Chair of Brewing and Beverage Technology, Technical University of Berlin, Berlin, Germany, [3]VTT Technical Research Centre of Finland Ltd., Espoo, Finland

There is a growing trend for beers with novel flavor profiles, as consumers demand a more diversified product range. Such beers can be produced by using non-*Saccharomyces* yeasts. The yeast species *Saccharomycopsis fibuligera* is known to produce exceptionally pleasant plum and berry flavors during brewer's wort fermentation while its mycelia growth is most likely a technological challenge in industrial-scale brewing. To better understand and optimize the physiological properties of this yeast species during the brewing process, maltose and maltotriose uptake activity trials were performed. These revealed the existence of active transmembrane transporters for maltose in addition to the known extracellular amylase system. Furthermore, a single cell isolate of *S. fibuligera* was cultured, which showed significantly less mycelial growth during propagation and fermentation compared to the mother culture and would therefore be much more suitable for application on an industrial scale due to its better flocculation and clarification properties. Genetic differences between the two cultures could not be detected in a $(GTG)_5$ rep-PCR fingerprint and there was hardly any difference in the fermentation process, sugar utilization and flavor profiles of the beers. Accordingly, the characteristic plum and berry flavor could also be perceived by using the culture from the single cell isolate, which was complemented by a dried fruit flavor. A fermentation temperature of 20°C at an original gravity of 10 °P proved to be optimal for producing a low-alcohol beer at around 0.8% (*v*/*v*) by applying the *S. fibuligera* yeast culture from the single cell isolate.

KEYWORDS

***Saccharomycopsis fibuligera*, non-*Saccharomyces* yeasts, fermentation, brewing, low-alcohol beer, sugar uptake, micromanipulation, flavor**

10.3389/fmicb.2022.1011155

Introduction

The yeast species *Saccharomycopsis fibuligera* (synonym: *Endomyces fibuliger*) is known from food fermentations, especially from liqueur production (Aidoo et al., 2006; Xie et al., 2021). It was isolated from traditional fermented foods, such as Indonesian tapé made from glutinous rice or cassava tuber (Djien, 1972). In Southeast Asia in particular, the yeast species is used in rice wine fermentation and is known to produce desirable floral, fruity, and honey-like flavors during fermentation as a result of high production of esters and higher alcohols (Xie et al., 2021; Yang et al., 2021). Characteristic fruity, flowery, and honey flavors were also noted when *S. fibuligera* was cultured in selective media containing different carbohydrate sources (Lee et al., 2018). Due to its positive flavor properties, it was also tested for its suitability to ferment brewer's wort, where there is a demand for diversification of the product range. As a result, *S. fibuligera* produced exceptionally promising plum-like, and also berry-like flavors in the beers (Srithamma, 2009; Methner et al., 2019, 2022). It was noticeable that, depending on the yeast strain, different sugar utilization patterns took place in brewer's wort, so it was possible to produce both non-alcoholic and alcoholic beers. Lindner, who isolated the yeast species in the context of bread spoilage in 1907, described it as strongly utilizing sucrose during fermentation, weakly utilizing glucose, and unable to utilize maltose (Lindner, 1907). In contrast, Kreger-van-Rij stated in 1984 that the yeast species is capable of fermenting glucose as well as sucrose and maltose (Kreger-Van Rij, 1984). Nevertheless, existing literature explains that maltose utilization is slow (Kurtzman and Smith, 2011). Generally, *S. fibuligera* is known to possess amylolytic activity and therefore has the ability to degrade starch. In 1944, Wickerham observed the presence of an extracellular amylase system for *S. fibuligera* (Wickerham et al., 1944). Through several studies, the two enzymes α-amylase and glucoamylase from *S. fibuligera* were isolated and characterized (Futatsugi et al., 1993; Chen et al., 2010). The endoamylase α-amylase is capable of randomly cleaving α-1,4 glycosidic bonds and thus reducing amylose to oligosaccharides such as maltotriose and maltose. Glucoamylase (synonym: amyloglucosidase) yields individual glucose units from the hydrolysis of non-reducing ends of amylose and amylopectin and additionally cleaves maltose and maltotriose (Janeček and Baláž, 1992; Pandey, 1995; Tiwari et al., 2015). Hostinová observed that *S. fibuligera* can synthesize either both enzymes or only one type of amylase in a strain-specific manner (Hostinová, 2002). In a study by Methner et al. the glucoamylase activity gave an indication of why maltose from selective media could be fully metabolized by the yeast strain *S. fibuligera* S. fib Lu27 although hardly any maltose and maltotriose could be utilized by this strain from brewer's wort (Methner et al., 2022). In contrast, the *S. fibuligera* yeast strain S. fib SF4 was able to partially metabolize maltose and maltotriose from brewer's wort in another study (Methner et al., 2019). To date, it has not yet been investigated whether this yeast species utilized maltose due to extracellular amylase activity and subsequent passive glucose transport into the cell or due to transmembrane transporters (permeases) which actively transport maltose and/or maltotriose into the cell. Since the fermentation trials with S. fib Lu27 and S. fib SF4 were performed at different temperatures in the two aforementioned studies by Methner et al., a possible temperature dependence on the sugar utilization needs to be taken into consideration.

A distinctive characteristic of the yeast species *S. fibuligera* is its morphology, as it is dimorphic and forms individual round or oval budding cells as well as mycelia (Nga et al., 1995; Xie et al., 2021). The mycelium represents a challenge in terms of its suitability for brewing, since it does not flocculate, triggering a restriction of clarification of the beer. For this reason, single cells were isolated and recultivated in this study to establish in a direct comparison with the mother culture, whether the single cell isolation would influence the morphology during propagation and fermentation. To ensure that the culture of the isolated single cell had no genetic differences from the mother culture, additional $(GTG)_5$ rep-PCR fingerprints were applied. Furthermore, since the yeast should retain the ability to produce exceptionally fruity flavors during the fermentation of brewer's wort as a consequence of the single cell isolation, the sensory properties of the final beers were studied.

The objective of this study was to elucidate whether the yeast species *S. fibuligera* possesses an active transport system for maltose and maltotriose, which was examined by maltose and maltotriose transport assays. Furthermore, it was investigated – exemplified by the yeast strain S. fib SF4 – whether it was possible to reduce the characteristic mycelial formation by means of micromanipulation and recultivation in order to increase the suitability for brewing on an industrial scale. Possible influences of micromanipulation on brewing potential including sugar utilization and beer flavor profiles were studied and compared with the mother culture and the domesticated reference lager yeast strain *Saccharomyces pastorianus* TUM 34/70 at fermentation temperatures of 20 and 28°C.

Materials and methods

Yeast strains

Table 1 lists the yeast strains with the corresponding abbreviations that were investigated or used as reference yeast strains in this study. While the two *S. fibuligera* yeast strains represented the yeasts under investigation, *S. pastorianus* S. pas 34/70 served as the reference yeast strain for all conducted experiments. *S. eubayanus* S. eub 12357 and *S. ludwigii* S. lud SL17 were used as additional reference yeasts for the maltose and maltotriose transport assays.

10.3389/fmicb.2022.1011155

TABLE 1 Yeast species and strain numbers with corresponding abbreviations used in this study.

Yeast strain number	Yeast strain abbreviation	Yeast species
TUM SL17	S. lud SL17	*Saccharomycodes ludwigii*
VTT C-12902/CBS12357	S. eub 12357	*Saccharomyces eubayanus*
VTT A-13220/TUM 34/70	S. pas 34/70	*Saccharomyces pastorianus*
PI S 6; Lu27	S. fib Lu27	*Saccharomycopsis fibuligera*
PI S 7; Lu 26/SF4	S. fib SF4	*Saccharomycopsis fibuligera*

Maltose and maltotriose uptake activity

S. lud SL17 was used as a negative control as the yeast strain is unable to take up maltose or maltotriose during fermentation (Boundy-Mills et al., 2011) whereas S. eub 12357 is known to be maltose-positive but maltotriose-negative during fermentation and thus represented the maltotriose-negative control (Gibson et al., 2013). S. pas 34/70, which represents a traditional group II lager yeast can utilize both maltose and maltotriose and was therefore used as the positive control. The maltose and maltotriose uptake activity of the two yeast strains S. fib Lu27 and S. fib SF4 were to be determined. For maltose and maltotriose uptake measurement, S. lud SL17 was grown in YP medium prepared from 1.0% yeast extract (Sigma-Aldrich, St. Louis, MO, U.S.A.), 2.0% peptone from casein, pancreatic digest (Sigma-Aldrich, St. Louis, MO, U.S.A.) and 1.0% glucose. S. eub 12357 was grown in YP containing 1.0% maltose and the remaining three strains (S. pas 34/70 and the two *S. fibuligera* S. fib Lu27 and S. fib SF4) were grown in YP medium containing maltose (1% *w/v*) or maltotriose (1% *w/v*). All strains were propagated at 20°C in liquid medium to an $OD_{600\,nm}$ between 4 and 8. The cells were harvested by centrifugation (10 min, 2,968 g, 0°C), washed twice with ice-cold water and once with 0.1 M tartrate-Tris (pH 4.2) before being re-suspended in the same buffer to a concentration of 200 mg/ml fresh yeast. Zero-trans rates of [U-^{14}C]-maltotriose uptake were measured at 20°C essentially as described by Lucero et al. (1997). Briefly, aliquots of 40 µl of yeast suspension were added to 20 µl of 15 mM labeled maltotriose (for a final concentration of 5 mM [U-^{14}C]-maltotriose) and incubated for 60 s at 20°C. The reaction was stopped by adding 5 ml ice-cold water. The suspension was immediately filtered and washed with an additional 5 ml ice-cold water. The filter was submerged in 3.5 ml of Optiphase HiSafe 3 scintillation cocktail (Perkin Elmer, MA, United States) and the radioactivity measured in a Perkin Elmer Tri-carb 2,810 TR scintillation counter. [U-^{14}C]-maltotriose (ARC 627) was obtained from American Radiolabeled Chemicals (St. Louis, MO, United States) and re-purified before use as described by Dietvorst et al. (2005). Maltose (minimum purity, 99%) and maltotriose (minimum purity, 95%) were from Sigma-Aldrich (St. Louis, MO). The cell washing was expected to remove any potential extracellular carbohydrate-hydrolase that could interfere with the results. In addition, keeping cells on ice until the uptake assay, the short incubation time in maltotriose, and subpar temperature and pH conditions for known extracellular glucoamylases of the *Saccharomyces* genus (pH_{Opt} = 4.5–6, T_{Opt} = 40–60°C; (Hostinová and Gašperík, 2010)) were expected to limit the activity of any residual carbohydrate hydrolase that might be present.

Micromanipulation and yeast propagation

For micromanipulation, a TransferMan® 4r micromanipulator with DualSpeed™ Joystic and CellTram® 4r Air, a pneumatic manual microinjector, were used (Eppendorf SE, Hamburg, Germany). The capillary with an inner diameter of 10 µm (BioMedical Instruments, Zöllnitz, Germany) was connected to a Nikon eclipse T*i-e* inverse microscope (Nikon, Tokyo, Japan) using an adapter. For micromanipulation, 3 ml of sterilized wort (100°C, 45 min) with an original gravity of 10 °P was pipetted into three sterile cell culture dishes (35 × 10 mm; Greiner Bio-One GmbH, Frickenhausen, Germany). Wort was prepared from unhopped malt extract (Weyermann®, Bamberg, Germany) by re-dilution with distilled water. The yeast strain S. fib SF4 was transferred from a wort slant agar (mother culture) into the wort of one cell culture dish and was distributed using a sterile inoculation loop. Using the micromanipulator, two single cells of the yeast strain S. fib SF4 were isolated and one cell per cell culture dish was transferred into the wort. Due to the filamentous growth of *S. fibuligera*, care was taken not to isolate filaments from the mother culture along with the single cells. One cell culture dish was incubated at 20°C for 72 h, the other sample was incubated at 28°C for 72 h. Temperatures at 20 and 28°C were selected, since there are already existing studies with *S. fibuligera* yeast strains in brewer's wort on these two approximate temperatures (Methner et al., 2019, 2022). The yeast cultures grown in the cell culture dishes were each transferred to 50 ml sterile wort in 100 ml flasks sealed with cotton plugs and propagated for another 72 h at the respective temperatures on a WiseShake orbital shaker (Witeg Labortechnik GmbH, Wertheim, Germany) at 80 rpm. Simultaneously, S. fib SF4 mother culture from wort slant agar was incubated under sterile conditions into 2 × 50 ml of the identical wort in 100 ml flasks. Here as well, one sample was propagated at 20°C, while the second sample was propagated at 28°C. At the end of the 72 h propagation, 1 ml material of the four different samples was removed in each case into sterile 1.5 ml SafeSeal micro tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany) for $(GTG)_5$ rep-PCR fingerprint analysis. The remaining propagation yeasts were transferred to 250 ml fresh, sterile 10 °P wort in 500 ml flasks and propagated for an additional 72 h at the respective parameters selected at the outset. In a final propagation step, the four samples were transferred to 1800 ml sterile wort in 2500 ml flasks and propagated for another 72 h before the yeasts were further processed for fermentation. Since a reference beer was to be produced for the fermentations in addition to the four experimental beers, the yeast strain S. pas 34/70 was also

10.3389/fmicb.2022.1011155

TABLE 2 Four different propagation approaches with the corresponding varying parameters of the yeast strain *Saccharomycopsis fibuligera* SF4.

Abbreviation	Yeast culture	Propagation and fermentation temperature
SF4-MI-20	Micromanipulated	20°C
SF4-MK-20	Mother culture	20°C
SF4-MI-28	Micromanipulated	28°C
SF4-MK-28	Mother culture	28°C

propagated as described as a commercial domesticated lager yeast strain for brewing. Additionally, comparative microscopic images were taken of the mother culture S. fib SF4 from the wort slant agar and the two yeasts propagated at 28°C (mother culture and micromanipulated culture) to compare the morphologies using the aforementioned brightfield Nikon inverse microscope at a magnification of × 40 (objective) plus digital zoom (scale is visible in microscopic pictures).

$(GTG)_5$ rep-PCR fingerprint

$(GTG)_5$ rep-PCR fingerprint system was applied to determine if the four differently propagated *S. fibuligera* S. fib SF4 yeast cultures had identical or different genetic fingerprints. According to Versalovic et al. (1994), the primer $(GTG)_5$ (5′-GTG GTG GTG GTG GTG-3′) was originally developed for bacteria and was successfully transferred to differentiate various non-*Saccharomyces* yeasts (Meyer et al., 1993; Erdem et al., 2016). After sample preparation, DNA fingerprint amplification was performed, followed by capillary gel electrophoresis and the data processing of the generated fingerprints. Table 2 lists the four different propagation approaches of the *S. fibuligera* yeast strain S. fib SF4 with their corresponding varying parameters and abbreviations, which were subsequently investigated.

For sample preparation, DNA was first isolated from the liquid samples. For this purpose, the yeast-wort suspensions were centrifuged (Mikro 200, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) in sterile 1.5 ml SafeSeal microtubes (Sarstedt AG & Co. KG, Nümbrecht, Germany) at 16,000 g for 2 min and the supernatant was discarded. 200 µl Insta-Gene Matrix was added to the samples before they were incubated in the thermomixer preheated to 56°C for 30 min. Samples were then vortexed in the tubes and placed in the 95°C preheated thermomixer for an additional 8 min. After a further centrifugation step at 16,000 g for 2 min, 100 µl of the supernatant was transferred to fresh sterile tubes. In the next step, DNA concentrations were measured using NanoDrop1000 spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany) and adjusted to 25 ng/µL, containing 12.5 µl RedTaq Mastermix (2×) 2-fold (Genaxxon bioscience, Ulm, Germany), 10 µl Primer Solution (50 pmol/l), 5 µl PCR-clean double distilled water (ddH_2O) and 2.5 µl sample DNA. The PCR temperature protocol of the DNA fingerprint amplification and subsequent capillary gel electrophoresis including data processing were described according to Riedl et al. (2019).

Fermentation and beer analysis

Before starting the small-scale fermentation trials in triplicate, the yeast cell counts for the reference strain S. pas 34/70 and the four individually propagated S. fib SF4 cultures were determined. The Cellometer® Vision (Nexcelom Bioscience LLC, Lawrence, MA, United States) was used to determine cell counts. The pitching rate for the fermentation experiments was set at 10×10^6 cells/mL ($\pm = 1 \times 10^6$ cells/mL). After cell counting, the corresponding calculated propagation yeast volumes were centrifuged (Roto Super 40, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) at 750 g for 5 min in sterilized 500 ml PPCO centrifuge bottles (Nalgene, Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the wort supernatant was discarded and the yeast samples were pitched into 1800 ml unhopped sterilized wort (10.2 °P, pH 5.3) prepared from malt extract (Weyermann®, Bamberg, Germany) in 2000 ml sterile Duran glass bottles (Schott AG, Mainz, Germany). The fermentation bottles were closed with glass fermentation airlocks on top. While yeast cultures already propagated at 28°C were also fermented at 28°C, yeast cultures propagated at 20°C were fermented at 20°C accordingly. By weighing the samples every 24 h, the fermentation progress was monitored by weight loss, which was mainly due to escaping carbon dioxide and based on Balling's assumption that during fermentation, an average of 2.0665 g of extract is converted into 1 g alcohol, 0.9565 g carbon dioxide and 0.11 g yeast (Esslinger, 2009). Following the main fermentation, which lasted for 480 h (20 days), the samples were sealed with sterile screw caps and cooled to 2°C for an additional 168 h (7 days) before the analyses shown in Table 3 were performed. For the sugar utilization results, one-sample *t*-tests were performed using OriginPro 2020 as statistical software to evaluate if the mean values of carbohydrate utilizations of the different fermentations varied significantly.

Sensory evaluation

The beer samples were tempered to 12°C and were profiled at 20°C room temperature by a sensory panel of eight DLG (Deutsche Landwirtschafts-Gesellschaft e.V., Frankfurt, Germany)-certified assessors. The accredited sensory evaluations were conducted according to DIN EN 17025. To exclude external interferences during the tastings, they were held in an appropriately neutral, white-colored room with individual tasting chambers. A sensory test was carried out according to the DLG evaluation scheme, which comprises a rating scale from zero to five. While zero is considered the lowest score and represents insufficient product quality, a score of five points fully meets the quality expectations of the product and matches the quality

10.3389/fmicb.2022.1011155

TABLE 3 Analytical methods of the wort and the beers according to MEBAK[a] and Donhauser et al.[b].

Analysis	Method	Device
Original gravity, apparent attenuation, ethanol content	MEBAK[a] WBBM 2.9.6.3	Bending vibration and NIR spectroscopy, Alcolyzer Plus with DMA 5000 X sample 122 (Anton-Paar GmbH, Ostfildern, Germany)
pH value	MEBAK[a] WBBM 2.13	pH meter with pH electrode, ProfiLine pH3210 pH meter (Xylem Inc., New York, NY, United States)
Sugar composition (glucose, fructose, sucrose, maltose, maltotriose)	Donhauser et al.[b] LS-HPLC 002_2	HPLC UltiMate 3000 (Thermo Fisher Scientific, Waltham, MA, United States)

[a]MEBAK® (2012), Editor: Dr. F. Jacob: The MEBAK collection of brewing analysis methods: Wort, beer and beer-based beverages. Collection of methods of the Mitteleuropäischen Brautechnischen Analysenkommission. Self-published by MEBAK.
[b]Donhauser, S.; Wagner, D. (1990): Zucker- und Endvergärungsgradbestimmung mittels der HPLC, 9:306–309. Monatsschrift für Brauwissenschaft.

description very well (Hildebrandt and Schneider-Haeder, 2009). The odor, purity of taste and body of the beers were evaluated, while the quality of bitterness as well as carbonation were neglected as the beers were produced from unhopped malt extract and the fermentations were conducted without pressure. For the tasting, the samples were assigned randomized three-digit numbers and 50 ml of each sample was served in brown 200 ml tasting glasses. There was also an additional sensory test method – a descriptive tasting. The method was based on the descriptive sensory evaluation of Meier-Dörnberg et al. (2017) with the seven main categories of tropical fruity, fruity (other fruits), citrus, spicy, floral, malty, and other flavors. The sensory assessors were asked separately about beer odor and taste, which they scored from 0 (not noticeable) to 5 (extremely noticeable). The results were statistically analyzed by first determining the interquartile ranges and removing extreme outliers (3 × IQR) to obtain only statistically significant values. For significant results, the mean values of odor and taste were calculated before the beer samples fermented with the *S. fibuligera* yeast strain S. fib SF4 were compared with the reference beers fermented with *S. pastorianus* S. pas 34/70.

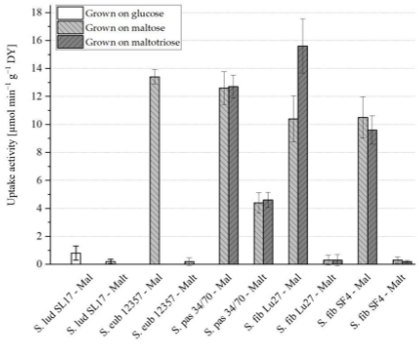


FIGURE 1
Zero-trans rates of maltose (Mal) and maltotriose (Malt) uptake activity (µmol min^{-1} g^{-1} dry yeast (DY)) measured at 20°C for cells propagated on glucose (white), maltose (light grey) or maltotriose (dark grey) with n=4. An uptake activity equal or below 0.5µmol min^{-1} g^{-1} DY is considered negligible. Yeast strain abbreviations according to Table 1.

Results

Maltose and maltotriose uptake activity

Prior to the uptake activity assessment, the strains were propagated when possible in both maltose and maltotriose as sole carbon sources. Due to the inability of S. eub 12357 to grow on maltotriose, it was propagated only on maltose. S. lud SL17 cannot grow on maltose nor maltotriose and was therefore only propagated in glucose. The results of the maltose and maltotriose uptake activity are presented in Figure 1, respectively. Raw data can be found in the Supplementary material Table 1.

S. ludwigii S. lud SL17 maltose uptake activity was just above the limit at 0.8 µmol min^{-1} g^{-1} DY but not significantly (standard deviation of ±0.51; *cf.* Figure 1). This strain is known to be maltose negative and the higher measured value is likely a result of experimental variability. Maltotriose activity was, however, clearly below the minimum activity required (cf. Figure 1). *S. eubayanus* S. eub 12357, as expected, showed high maltose uptake activity and no maltotriose uptake activity. The strain S. pas 34/70 had maltose uptake activity comparable to that of S. eub 12357 and it was the only strain with maltotriose uptake activity significantly above 0.5 µmol min^{-1} g^{-1} DY. Growth on maltose or maltotriose generally did not seem to affect the uptake activity of either sugar. The only exception was S. fib Lu27, which had 50% higher maltose uptake activity when grown on maltotriose. Otherwise, the *S. fibuligera* strains S. fib Lu27 and S. fib SF4 showed very similar behavior. Maltose uptake activity was lower but still comparable to that of S. eub 12357 and S. pas 34/70. Maltotriose activity was below 0.5 µmol min^{-1} g^{-1} DY for both strains irrespective of the sugar used. However, the error is quite high, particularly for S. fib Lu27. This can partly be explained by the difficulty of homogenizing cells with filamentous growth. This created challenges for the uptake experiments and the measurement of cell mass. Regardless, the results strongly suggest that *S. fibuligera* does not have any significantly active transporters able to take up maltotriose into the cells. Maltotriose utilization is therefore

10.3389/fmicb.2022.1011155

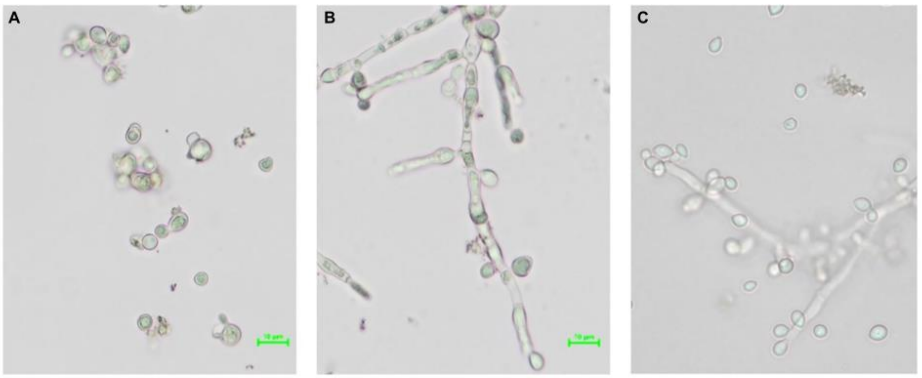


FIGURE 2
Brightfield microscopy pictures at a magnification of × 40 (objective) plus digital zoom [scale is visible in microscopic photos **(A)** and **(B)**] showing different morphologies of the yeast strain *S. fibuligera* S. fib SF4: **(A)** SF4-MI-28, **(B)** SF4-MK-28, **(C)** SF4 from slant agar.

dependent on its extracellular hydrolysis followed by uptake of the resulting glucose or maltose molecules. Maltose, however, can be both hydrolyzed extracellularly or intracellularly due to active maltose transmembrane transporters. Due to the identical sugar transport systems of the two investigated *S. fibuligera* yeast strains, S. fib SF4 and S. fib Lu 27, and the higher fermentation activity of the yeast strain S. fib SF4 which was reported in two previous studies by Methner et al. (2019), the yeast strain S. fib SF4 was selected for all further trials in this study.

Morphology of *Saccharomycopsis fibuligera*

A single cell without filaments of the yeast strain *S. fibuligera* S. fib SF4 was isolated and propagated in brewer's wort as well as the mother culture S. fib SF4 as described in Section 2.3. Figure 2 depicts the comparison of the morphologies in a brightfield microscope at a magnification of × 40 (objective) plus digital zoom [scale is visible in microscopic pictures Figure 2A, B] during growth at 28°C of the micromanipulated yeast culture Figure 2A in comparison with the mother culture during growth at 28°C Figure 2B and the original culture from the wort slant agar before propagation Figure 2C.

Comparing the three microscopic images with each other, there are clear differences in morphology. While the micromanipulated yeast culture hardly formed filaments during growth at 28°C and showed budding cells (A), the mother culture predominantly developed mycelia at the same propagation temperature (B). The original culture from slant agar, which unlike the yeasts in (A) and (B) was not in log but in stationary phase, showed both mycelia and single cells in the microscopic image (C). At a propagation temperature of 20°C, the cell morphology of the three cultures A-C did not differ from the respective clone cultures propagated at 28°C. Therefore, these images are not shown.

$(GTG)_5$ rep-PCR fingerprint

Based on the different morphologies in Section 3.2, a $(GTG)_5$ rep-PCR fingerprint was performed to investigate whether the two micromanipulated isolates were genetically different from the mother culture. Accordingly, the fingerprint analysis was carried out with both micromanipulated cultures propagated at 20°C and 28°C and with the mother culture propagated at 20°C and 28°C. Previous studies revealed that the $(GTG)_5$ fingerprinting is not only a suitable method to differentiate all species of beer-spoiling bacteria, but also to differentiate non-*Saccharomyces* yeasts on strain level (Michel et al., 2016; Riedl et al., 2019). The results are depicted in Figure 3.

Although there were differences in morphology in the brightfield microscopy (*cf.* Section 3.2), no obvious differences could be detected in the $(GTG)_5$ patterns of the capillary gel electrophoresis.

Analytical results

To investigate the fermentation process, the weight losses of the six samples were checked every 24 h during fermentation and can be viewed in Figure 4. Raw data can be viewed in the Supplementary material Table 2. Due to the unpressurized fermentation, the carbon dioxide formed during fermentation was able to escape, resulting in a weight loss. The reference yeast strain S. pas 34/70 generally showed a significantly higher fermentation

10.3389/fmicb.2022.1011155

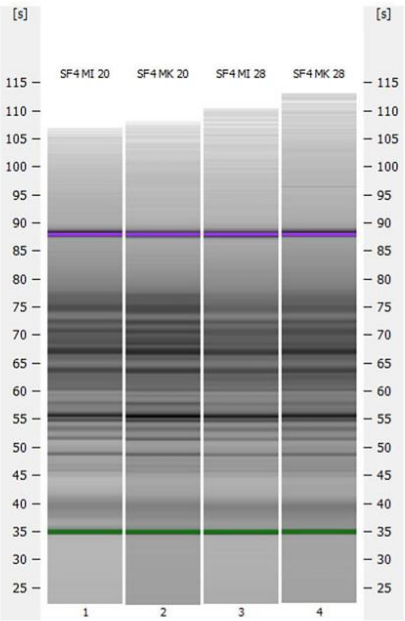


FIGURE 3
Capillary gel electrophoresis $(GTG)_5$ patterns for the investigated yeast strain *S. fibuligera* S. fib SF4 micromanipulated and propagated at 20°C (SF4 MI 20) and 28°C (SF4 MI 28) and the mother culture propagated at 20°C (SF4 MK 20) and 28°C (SF4 MK 28).

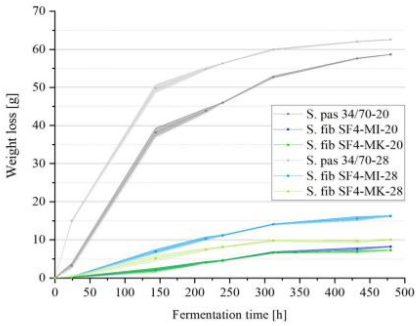


FIGURE 4
Mean values ($n=3$) with standard deviations of the fermentation samples' total weight losses in grams of the investigated yeast strain S. fib SF4 as micromanipulated (MI) and mother culture (MK) and the reference yeast strain S. pas 34/70 during the fermentation process over a fermentation period of 480 h (20 days) at 20 and 28°C.

activity than the yeast S. fib SF4. While S. pas 34/70 already lost around 40 g in weight within the first six fermentation days (144 h) at a temperature of 20°C, the weight loss at 28°C was even higher at around 50 g. For the sample fermented at 20°C, this accounted for around two thirds of the final weight loss after 20 days, while the sample fermented at 28°C had already reached almost 80% of the final weight loss after only six days. In contrast, the S. fib SF4 fermentations lost significantly less weight than those from the reference strains. After 20 days, the sample S. fib SF4-MI-28 lost the most weight at about 16 g in direct comparison with the other S. fib SF4 samples. S. fib SF4-MI-20 only achieved about half of this amount, at approximately 8 g. Despite the higher fermentation temperature, S. fib SF4-MK-28 lost an insignificant amount of weight at around 10 g, and S. fib SF4 -MK-20 brought up the rear with just about 7 g weight loss after 20 days of fermentation.

The high weight losses of the two reference beers fermented with S. pas 34/70 at 20°C and 28°C correspond to the high apparent attenuations over 80% and ethanol concentrations of 4.5–4.6% (*v*/*v*), as can be seen in Table 4. Both apparent attenuation and ethanol concentration of the reference sample fermented at 28°C were slightly higher than the concentrations of the sample fermented at 20°C, which is consistent with the weight losses shown in Figure 4. Consequently, it was expected that the values for the S. fib SF4 samples would be significantly lower, which was in fact the case. The results are also listed in Table 4. Among the four S. fib SF4 beers, the beer fermented with the micromanipulated yeast culture at 28°C had comparatively the highest apparent attenuation at 23% and an ethanol content of 1.2% (*v*/*v*). The other three beers fermented with S. fib SF4, which showed similar weight losses during the fermentation process (*cf.* Figure 4), revealed comparable apparent attenuations (around 14–16%) and ethanol contents (0.74–0.83% (*v*/*v*)). Although the apparent attenuations of the beers fermented with the S. fib SF4 yeast cultures were low compared with the two reference beers, the pH drop was more pronounced. While the two reference beers had a pH of around 4.6, pH values of between 4.16–4.43 were measured in the S. fib SF4 beers. Strikingly, the two beers from the micromanipulated cultures were lower than the beers from the mother culture with pH values of 4.16 (SF4-MI-28) and 4.33 (SF4-MI-20). It cannot be explicitly explained and would need further research to establish why *S. fibuligera* caused a stronger pH drop in the beers than the reference yeast *S. pastorianus*. However, it is known that yeast cells acidify their environment during nutrient transport by a combination of direct secretion of organic acids, excretion of protons and CO_2 dissolution (Budroni et al., 2017). These metabolic processes could be comparatively more pronounced in *S. fibuligera* yeast cells. A reduced buffer capacity could also be a potential reason for the low pH. In order to gain a deeper understanding of why the pH value was comparatively lower than in the two reference beers produced with the *Saccharomyces* yeast strain, organic acids could be measured in future investigations. This was not carried out as part of this study, as the question was not focused.

TABLE 4 Original gravity [%], apparent attenuation [%], ethanol content [% (*v*/*v*)], and pH values in the final beers fermented with the yeast strain *S. fibuligera* S. fib SF4 (mother culture and micromanipulated culture) and the reference yeast strain *S. pastorianus* S. pas 34/70 at fermentation temperatures of 20 and 28°C.

Yeast strain	Original gravity [°P]	Apparent attenuation [%]	Ethanol [% (*v*/*v*)]	pH value
S. pas 34/70-20	10.12 ± σ = 0.01	81.40 ± σ = 0.08	4.47 ± σ = 0.00	4.61 ± σ = 0.01
S. fib SF4-MI-20	10.17 ± σ = 0.01	15.83 ± σ = 0.17	0.83 ± σ = 0.01	4.33 ± σ = 0.01
S. fib SF4-MK-20	10.18 ± σ = 0.01	13.97 ± σ = 1.01	0.74 ± σ = 0.05	4.43 ± σ = 0.02
S. pas 34/70-28	10.13 ± σ = 0.01	82.20 ± σ = 0.08	4.57 ± σ = 0.01	4.58 ± σ = 0.00
S. fib SF4-MI-28	10.18 ± σ = 0.02	22.73 ± σ = 1.39	1.20 ± σ = 0.07	4.16 ± σ = 0.03
S. fib SF4-MK-28	10.18 ± σ = 0.00	15.77 ± σ = 0.92	0.83 ± σ = 0.05	4.38 ± σ = 0.02

TABLE 5 Sugar composition of the wort used for fermentation trials.

Wort sugar	Concentration in g/L
Glucose	9.2
Fructose	1.0
Sucrose	4.1
Maltose	51.0
Maltotriose	13.9

Table 5 illustrates the sugar composition of the main wort carbohydrates glucose, fructose, sucrose, maltose and maltotriose (Narziß et al., 1999) used for the fermentation experiments. Both the wort sugar composition and the sugar composition of the beers were analyzed in order to draw conclusions about the sugar utilization of the individual yeast cultures during fermentation. Figure 5 depicts the sugar utilization by the individual yeast cultures in percent. Raw data can be found in Supplementary material Table 3.

The sugar utilization of the yeasts also reflects that the reference yeast strain S. pas 34/70 was significantly more active than the experimental yeast S. fib SF4 at both 20°C and 28°C fermentation temperatures. One-sample *t*-tests were performed using OriginPro 2020 as statistical software to demonstrate that the mean values of the maltose and maltotriose utilization results were significantly different between the reference yeast strain S. pas 34/70 and the experimental yeast S. fib SF4 at both temperatures (*cf.* Supplementary material Table 4). The reference yeast completely utilized glucose, fructose and sucrose present in the wort, maltose at 98% (20°C) to 99% (28°C), and maltotriose at 87% (20°C) to 88% (28°C). In contrast, none of the wort sugars was fully utilized by the S. fib SF4 cultures during the fermentation time of 20 days. In the beer fermented with S. fib SF4-MI-28, which showed the highest fermentation activity in direct comparison with the other *S. fibuligera* cultures, the highest sugar utilization was measured. Glucose utilization was above 90% and on average not significantly different from the reference S. pas 34/70 at 28°C according to the one-sample *t*-test result (*cf.* Supplementary material Table 4). In general, the range of maltose utilization of the S. fib SF4 cultures was between 43 and 65%, for maltotriose it was between 41 and 50%. For glucose, fructose and sucrose, significantly larger ranges of variation were analyzed. Only sucrose utilization of S. fib SF4-MI-28 and S. fib SF4-MK-28 were not significantly different from each other, while all other results for glucose, fructose, and sucrose were significantly different regardless of the fermentation temperature (*cf.* Supplementary material Table 4). The two beers fermented with the S. fib SF4 mother culture stood out, as hardly any fructose was utilized and only about half of the glucose. The micromanipulated cultures showed higher utilization rates, but here, too, the wort sugars were only partially utilized by the yeasts. The incomplete sugar utilization explains the low apparent attenuations and the low ethanol concentrations from Table 4.

Sensory evaluation

By comparing the flavor profiles of the beers in Figure 6 (raw data are depicted in Supplementary material Table 5), the different fermentation temperatures at 20°C and 28°C had an obvious impact on the flavors of the beers. The difference is most pronounced in the reference beer, which is visible in the radar plot (Figure 6A).

The reference beer fermented at 20°C exhibited a dominant banana flavor, which was complemented by an apple-like and slightly malty and melon-like flavor. At a fermentation temperature of 28°C, the tasters could no longer detect any significant fruity flavor. Instead, the beer was described as neutral with slightly malty, clove and wort-like flavors. Clove-like flavor is unusual for beers fermented with S. pas 34/70. The clove-like flavor is one of the phenolic off-flavors known in beers fermented with certain *S. cerevisiae* yeast strains. These yeast strains must possess the POF1 gene, which enables the yeast to decarboxylate the phenolic acid ferulic acid to 4-vinyl guaiacol (Meaden and Taylor, 1991; Lodolo et al., 2008). This property is not known for S. pas 34/70. Although utmost care was taken, contamination with *S. cerevisiae* cannot be completely excluded, which could have potentially caused a clove-like flavor in the beer. The wort flavor was also reflected as an off-flavor in the total DLG score and resulted in deductions in the overall score, which is visible in Table 6. While the reference beer fermented at 28°C received the lowest DLG rating of the total of six beers with a score of 4.05 points, the reference beer fermented at 20°C achieved the best score of 4.45 points. In contrast, the flavor profiles of the two beers fermented with the micromanipulated S. fib SF4 yeast culture were more alike as shown in the radar plot (Figure 6B). Both beers

10.3389/fmicb.2022.1011155

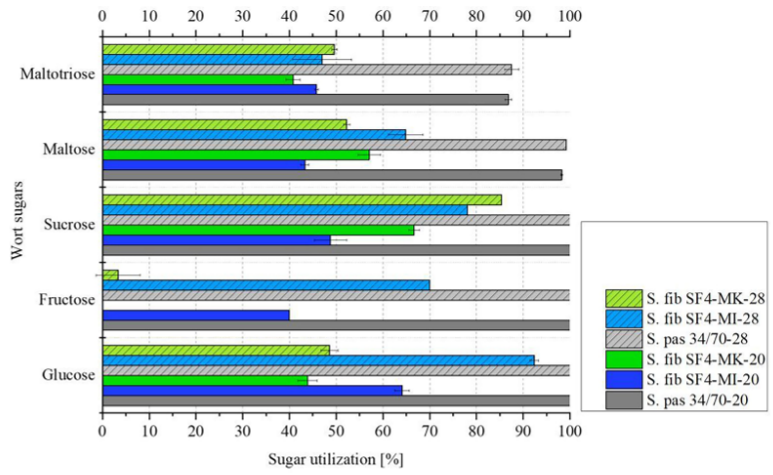


FIGURE 5
Mean values (n=3) with standard deviations of wort sugar utilization in % of the investigated yeast strain S. fib SF4 as micromanipulated (MI) and mother culture (MK) and the reference yeast strain S. pas 34/70 during fermentation from wort to beers at 20 and 28°C.

exhibited a pronounced plum flavor supported by berry and dried fruit flavors. Moreover, slight malty notes were detected in the beers. In general, however, the flavor expressions were significantly stronger in the beer fermented at 20°C than in the beer fermented at 28°C. Slight wort flavors were noticed in the beer fermented at 28°C, which were not perceived in the beer fermented at 20°C. This slight wort flavor led to a devaluation of the total DLG score here, too, and amounted to 4.22 points. The beer fermented at 20°C scored better at 4.43 and was rated very good. In addition to the aforementioned flavors, the sensory assessors mentioned traces of passion fruit in the beer, which were likely to support the positive overall fruity flavor and were not perceptible in the beer produced at 28°C. The flavor characteristics of the beers fermented with the SF4 mother culture were, similarly to the SF4-MI beers, almost independent of temperature. All four beers produced by using *S. fibuligera* SF4 revealed strong similarities. Accordingly, pronounced plum and berry flavors were detected in the beers fermented with the SF4 mother culture as can be seen in the radar plot (Figure 6C). Additionally, the sensory assessors identified malty and dried fruit flavors, although the dried fruit flavors were less pronounced than in the SF4-MI beers. Wort flavor could again only be perceived in the beer fermented at 28°C, which was thus common to all beers produced at 28°C. Comparing the SF4-MI-20 beer with the SF4-MK-20 beer, it is noticeable that the plum and the dried fruit flavors were significantly more distinct in the beer SF4-MI-20, while the two SF4 beers fermented at 28°C differed significantly less regarding the flavor expressions. Just as with the other two beers, which had slight wort off-flavors, the beer fermented with S. fib SF4-MK-28 was downgraded in the total DLG score and received 4.20 points. The beer produced with S. fib SF4-MK-20 was rated better with 4.35 points. However, due to the lower fruitiness, it may have scored lower than the beer produced with S. fib SF4-MI-20.

Discussion

Although extensive studies on the extracellular amylase system of the yeast species *S. fibuligera* already exist (Futatsugi et al., 1993; Hostinová, 2002; Chen et al., 2010; Kurtzman and Smith, 2011), it has not yet been investigated whether the yeast possesses transmembrane transporters for maltose and maltotriose with intracellular enzyme systems for their cleavage in addition to extracellular amylases. Therefore, the use of the maltose and maltotriose transport assay with radiolabeled maltose and maltotriose uncovered new insights into the physiology of this yeast species and revealed that both investigated *S. fibuligera* yeast strains possess an active maltose transport system into the cell, besides the extracellular amylase activity. In contrast, an active transport system for maltotriose was not found. Two fundamentally different mechanisms exist for maltose and maltotriose utilization by yeasts (Krogerus et al., 2019). Either the two sugars can be cleaved extracellularly with the help of amylases or they are taken up directly through the membrane into the cell and are hydrolyzed using an intracellular maltase (La Fuente and Sols, 1962; Novak et al., 2004). Transporters such as AGT1 and MTT1 are known to be responsible for the active transport of maltose and maltotriose (Dietvorst et al., 2005; Vidgren and Londesborough, 2012). However, these transporters are able to carry both sugars and their expression is nonspecific (Magalhães et al., 2016). For *S. fibuligera* it can be suspected that another maltose-specific transport system exists. This could involve MALx1-encoded transporters, which are specific

10.3389/fmicb.2022.1011155

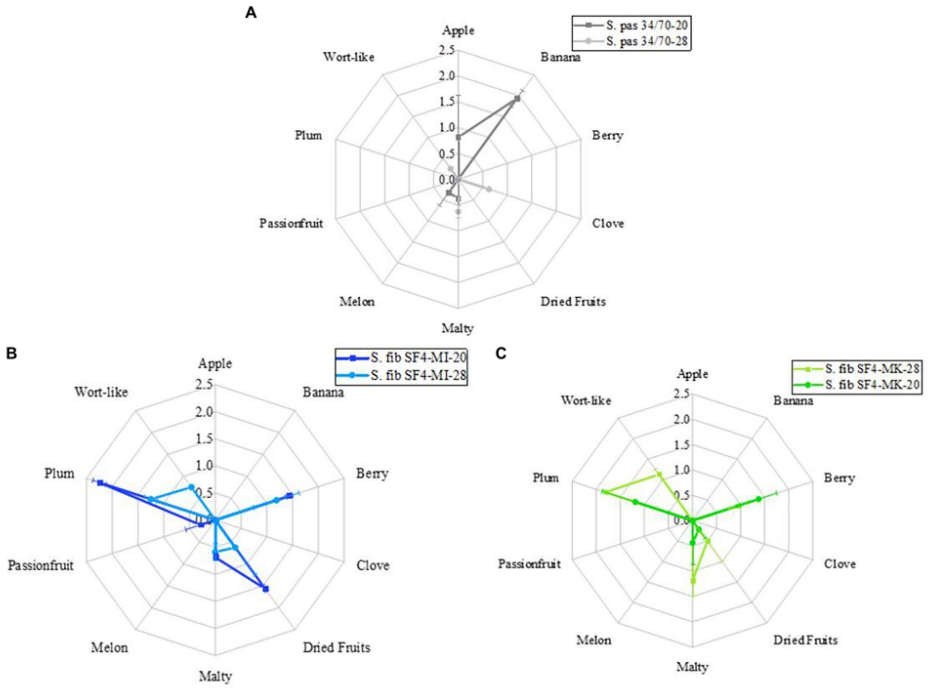


FIGURE 6
Flavor profiles of the beers fermented with the yeast strain *S. fibuligera* S. fib SF4 as micromanipulated (MI) and mother culture (MK) and the reference yeast strain *S. pastorianus* S. pas 34/70 at 20 and 28°C. Values are shown as means of odor and taste with standard deviations. The number of sensory assessors was *n*=8.

TABLE 6 Total DLG score according to the DLG evaluation scheme excluding the quality of bitterness as well as carbonation of the investigated beers fermented with *S. fibuligera* S. fib SF4 yeast cultures and reference yeast strain *S. pastorianus* S. pas 34/70 at 20°C and 28°C.

Yeast culture	Total DLG score
S. pas 34/70-20	4.45
S. fib SF4-MI-20	4.43
S. fib SF4-MK-20	4.35
S. pas 34/70-28	4.05
S. fib SF4-MI-28	4.22
S. fib SF4-MK-28	4.20

to maltose (Rautio and Londesborough, 2003). Nevertheless, for both *S. fibuligera* yeast strains it was irrelevant whether they were propagated on maltose or maltotriose due to the presence of both mechanisms for maltose and maltotriose utilization.

The brewing potential of the two *S. fibuligera* yeast strains was recently demonstrated in two studies by Methner et al. (2019, 2022). Due to the identical sugar transport systems of the two investigated *S. fibuligera* yeast strains, S. fib SF4 and S. fib Lu 27, and the higher fermentation activity of the yeast strain S. fib SF4, the yeast strain S. fib SF4 was selected for all further trials in this study. The filamentous growth of *S. fibuligera* leads to limited usability on an industrial scale. The mycelium does not settle at the bottom of the tank during cold storage, which can lead to difficulties in the clarification of the beer. Since the yeast forms mycelia as well as single cells during the growth phase, single cells were micromanipulated to examine whether they increasingly formed single cells during propagation. Indeed, the micromanipulated single cells developed significantly weaker mycelia than the mother culture during propagation. This occurred independently of the two temperatures selected at 20°C and 28°C. It cannot be conclusively explained yet and would therefore provide potential for further investigation into why significantly less mycelial formation was visible in the micromanipulated cultures. Additionally, future repitching experiments would need to investigate how stable the specific phenotypic trait of the lower mycelial formation of the yeast isolate is in order to reliably apply the benefits of better flocculation

10.3389/fmicb.2022.1011155

and clarification properties on an industrial scale. Investigations with *S. cerevisiae* have shown that so-called FLO genes are responsible for the flocculation behavior, which could possibly also be the case for *S. fibuligera* (Teunissen and Steensma, 1995; Lodolo et al., 2008). It is also known that the flocculation behavior of *S. cerevisiae* can be highly variable, and flocculation behavior could be influenced by selecting specific layers of yeast sediment for reinoculation (Steensels and Verstrepen, 2014). This assumption could be applied to *S. fibuligera* in the context of this study. Another possible suggestion may be that the MIG1 gene was suppressed in the micromanipulated cultures. This gene plays a crucial role in mycelial formation of *S. fibuligera*. Provided it is suppressed, mycelial formation is reduced and cell budding enhanced. Repression of the MIG1 gene in the micromanipulated cultures would also help explain the slightly higher sugar utilization in the corresponding beers in direct comparison to the beers fermented with the mother culture. Repression of the gene enhances the expression of genes such as α-amylase and glucoamylase (Liu et al., 2011).

$(GTG)_5$ rep-PCR fingerprints confirmed that identical patterns emerged during capillary gel electrophoresis. $(GTG)_5$ rep-PCR typing only confirms a stable state within the investigated target DNA-regions. No genetic changes could be observed in terms of the genetic target spectrum. Nevertheless, a more universal approach like whole genome sequencing could be applied in further studies to reveal a genetic origin of the phenotypic difference or not. $(GTG)_5$ rep-PCR typing confirmed that both isolates (clones) still belonged to the same strain origin.

The described morphologies also persisted during the fermentation trials, so micromanipulation might actually be helpful in propagating a culture that exhibits reduced mycelia formation. In this study, the fermentation period was chosen for 20 days in order to bridge a potential prolonged lag phase. Even though such a lag phase was observed in previous studies during the fermentation of wild *Saccharomyces* yeasts (Nikulin et al., 2020; Hutzler et al., 2021) it should not be generally excluded for non-*Saccharomyces* yeasts. While it cannot be completely excluded that the lag phase would have reverted to the active phase at a later stage, based on the extensive stagnation of the fermentation, it could be assumed that there was no delay in fermentation. As shown by the sugar utilization, only about 40–65% of the maltose and 40–50% of the maltotriose were degraded by S. fib SF4 during fermentation across temperatures and regardless of the culture (micromanipulated or mother culture). In contrast, the reference yeast S. pas 34/70 utilized maltose almost completely and maltotriose to nearly 90%. A possible reason for the incomplete utilization of maltose and maltotriose by S. fib SF4 could be an inhibition of the maltose transporter by other substances. Rautio and Londesborough found that, depending on the yeast strain, glucose and maltose transport can be inhibited by each other even though both sugars are carried by different transporters (Rautio and Londesborough, 2003). Since neither glucose nor maltose were fully utilized, this could be a possible explanation. Moreover, it is known that yeast maltose metabolism can be suppressed by glucose metabolism as glucose can repress the transcription of necessary gene loci (such as MIG1) for the corresponding maltose permeases as well as for specific enzymes for the hydrolysis of maltose, which was found in several studies with *Saccharomyces* yeasts (Görts, 1969; Federoff et al., 1983; Klein et al., 1996). Based on the available results, only assumptions can be made at present why the fermentation performance in the brewer's wort was relatively low. It could be an indication of a possible inhibition mechanism of sugar uptake in brewer's wort. Furthermore, a transformation of the sugars to organic acids or other fermentation-by-products or a concentration-dependent feedback of fermentation products could have led to the low fermentation performance, too. Based on existing studies, it is known that yeasts can form different organic acids from carbohydrate sources during fermentation instead of producing ethanol. The formation pathways of organic acids are versatile. Acids are basically present in the raw materials of the wort, however, the acid concentrations can be changed by the yeasts during fermentation. Additionally, acids are produced by the yeasts as by-products of their metabolic pathways (Whiting, 1976). Selected yeast species are known for the production of citric acid or pyruvic acid during fermentation (Yalcin et al., 2010; Chidi et al., 2015; Afolabi et al., 2018), while *Lachancea thermotolerans*, besides other yeast species, possess the ability to form high amounts of lactic acid at the expense of ethanol during fermentation (Sgouros et al., 2020; Rodríguez Madrera et al., 2021). Due to the low pH values in the beers, there is a possibility that S. fib SF4 partly converted carbohydrates to organic acids instead of ethanol. Additionally, the glucose and maltose utilization in Figure 5 may appear lower than the actual results as the extracellular amylase system might have cleaved maltotriose and further oligosaccharides. Consequently, relatively higher proportions of maltose and glucose could be present than in the initial wort. Nevertheless, contrary to expectations, the yeast strain S. fib SF4 degraded only small amounts of the original extract as the two beers fermented at 20°C exhibited apparent attenuations of between 14 and 16%, while the two beers fermented at 28°C had apparent attenuations that ranged between approximately 16 and 23%. A higher apparent attenuation was expected since the same yeast strain was close to 47% at a fermentation temperature of 27°C in a previous study by Methner et al. and was thus about twice as high (Methner et al., 2019). Although the results of this study show that different cultures differed by 7% for apparent attenuation despite identical conditions, further experiments need to be conducted in the future to explain why the values can vary significantly. The apparent attenuations of the beers produced with the reference yeast strain S. pas 34/70 were approximately 82%, reaching the final apparent attenuation for lager beers (Narziss et al., 2017). As expected, the apparent attenuation affected the ethanol content in the final beers, so values between 0.83 and 1.20% (*v*/*v*) were obtained for S. fib SF4 at 28°C, while 3.10% (*v*/*v*) ethanol was obtained in the beer from the earlier study by Methner et al. (2019). However, it must be considered that the original gravity of 12.65% was higher than in the present study and so the direct comparison of the apparent attenuations is more accurate. The pH values were similar in a direct comparison of the two studies.

While the yeast strain S. fib SF4 reached a drop in pH to 4.30 in the earlier study, the pH values in this study ranged from 4.16–4.43. The low pH values could explain why the α-amylase of S. fib SF4 did not work optimally. According to Xie (Xie et al., 2021), the pH optimum of α-amylase is between 5.0–6.0 and the optimal temperature for this enzyme is between 40–50°C. At the beginning of the fermentation process, the pH of the wort was still within the optimal range at 5.3, which could explain the slightly higher fermentation activity at the beginning of the fermentation process (*cf.* Figure 4). The slightly higher fermentation temperature of 28°C likely accelerated the enzyme activity compared to the fermentation temperature of 20°C and thus led to a comparatively faster fermentation process due to a faster extracellular sugar cleavage. Nevertheless, the optimum temperature of α-amylase was far from being reached and the pH values of the beers dropped during fermentation. Therefore, the extracellular enzyme system most probably worked very slowly. An inhibitory effect of the sugar transport systems could nevertheless be likely and could be specifically tested in further experiments.

The sensory properties of the beers play a crucial role for consumers. As shown in Figure 6, the four experimental beers were depicted next to the two reference beers. In this study, the focus was on the direct comparison between the beers fermented with the micromanipulated and the mother culture. In general, despite the different morphologies of the two cultures, the sensory assessors noted almost identical flavor attributes. These coincided with the flavor characteristics of previous studies regardless of temperature, as the beers exhibited pronounced plum- and berry-like flavors. Dried fruit flavors were also perceived, especially in the beers from the micromanipulated culture. While the reference beer fermented with S. pas 34/70 at 20°C exhibited fruity flavors as known from previous studies (Meier-Dörnberg et al., 2017; Hutzler et al., 2021), the reference beer fermented at 28°C was rather neutral and did not exhibit any conspicuous flavor characteristics. Possibly, at 28°C the volatile flavor compounds were more strongly expelled during unpressurized fermentation than at 20°C. In contrast, previous research revealed that *S. fibuligera* produces desirable floral, fruity, and honey-like flavors in rice wine fermentation and in fermentation in selective media containing different carbohydrate sources (Lee et al., 2018; Xie et al., 2021; Yang et al., 2021), while the flavors in beer were previously described to be plum-, and also berry-like (Methner et al., 2019, 2022). It is interesting to note that the floral and honey flavors, produced from rice wine and selective media fermentation, did not appear in the brewer's wort fermentation. The beer had a distinctive focus on fruity flavors. This could be directly related to the carbon source, as it significantly influences the volatile and non-volatile metabolites according to a study by Lee et al. (2018). The same study also describes a significant influence of the cultivation time on flavor expressions. Time appeared to be less influential with respect to the yeast species *S. fibuligera* as the same flavor components were retained as for previous studies of between seven, 14 and 20 days (Methner et al., 2019, 2022). The key aroma compounds of a sweet rice alcoholic beverage (sweet rice wine) fermented with *S. fibuligera* have already been studied in detail in a study by Yang et al. (2021), where 43 volatile compounds were identified, with ethyl butanoate, ethyl hexanoate, β-phenylethyl alcohol and 1-octen-3-one with high OAVs being responsible for the key aroma compounds. Also in beer, ethyl butanoate was measured well above the flavor threshold, while ethyl hexanoate remained below the flavor threshold (Methner et al., 2019). Furthermore, there are findings that relate to the genes responsible in the yeast species *S. fibuligera* for encoding alcohol acetyltransferase for volatile acetate ester formation (Moon et al., 2021). Six of these ATF genes were found in *S. fibuligera*, while in direct comparison *S. cerevisiae* possessed only two ATF genes. This could be an explanation for the more complex flavor attributes in foods fermented with *S. fibuligera*. Nevertheless, it remains unclear which key flavor compounds were responsible for the dominant plum flavor in the beer. A possible approach for future research would be to target substances found in plums. Fricker listed 14 main flavor compounds from four different plum varieties out of a total of about 160 identified compounds (Fricker, 1984). These 14 main flavor substances γ-hexalactone, γ-octalactone, γ-decalactone, γ-dodecalactone, linalool, α-terpineol, benzyl alcohol, cis-3-hexenol, trans-2-hexenol, ethyl cinnamate, benzaldehyde, n-hexanoic acid, n-octanoic acid and trans-2-hexenoic acid could serve as indicators. Comparing these to the primary odorants in pale lager beer, none of the 14 flavor compounds are found. Schieberle published 33 primary odorants of pale lager beer in 1991, of which he listed 3-methylbutanol, 2-phenylethanol, 3- and 2-methylbutanoic acid, 4-vinyl-2-methoxyphenol, furaneol, ethyl-butanoate, (*E*)-*β*-damascenone, sotolone, butanoic acid, ethyl hexanoate, and hexanoic acid as the most important ones (Schieberle, 1991). Although the two flavor compounds linalool and terpineol can also be found in beer, they originate from the added hops (Lam et al., 1986), which were not used in this study. The malty impression derived from the wort, which may be more pronounced due to the sterilization process and the associated increased thermal input. As a result of the increased thermal input, various aldehydes, Maillard reaction products and ketones are formed (Siefker and Pollock, 1956; De Schutter et al., 2008; Piornos et al., 2020). Aldehydes, in turn, can cause characteristic wort flavors that are often perceived as off-flavors (Gernat et al., 2020). A slight wort flavor was perceived in the two beers fermented with *S. fibuligera* at 28°C. It cannot be conclusively explained as to why the wort flavor was noticeable as wort-derived aldehyde off-flavors are often degraded by the yeasts during fermentation. This has already been studied for selected non-*Saccharomyces* yeast strains in the context of cold contact fermentations for the production of non-alcoholic beers (Nikulin et al., 2022). Consequently, it could be assumed that the yeast strain S. fib SF4 was also capable of degrading wort-derived aldehyde off-flavors, as the two beers fermented at 20°C did not exhibit this flavor attribute.

Conclusion

The study clarified whether the yeast species *S. fibuligera* possessed an active transport system for maltose and maltotriose. While an active transport system for maltose was found in the two

investigated *S. fibuligera* strains, there was no such system for maltotriose. Thus, it was concluded that in addition to the extracellular amylase system known for *S. fibuligera*, maltose transport also occurred across the cell membrane. Despite the ability of the yeast to hydrolyze and metabolize both maltose and maltotriose, a very slow fermentation was observed compared to the domesticated reference yeast strain *S. pastorianus* TUM 34/70, resulting in low ethanol contents between 0.8–1.2% (*v*/*v*) in the final beers brewed at 10 °P original gravity. Therefore, the yeast species *S. fibuligera* is ideal for producing beer with a low alcohol content, which at the same time has unique flavor properties. To produce regular beers, it would also be conceivable to use *S. fibuligera* in co-culture with domesticated *Saccharomyces* yeasts in future studies, as this could potentially increase flavor complexity. In this study, the brewing potential was also investigated and optimized by using micromanipulation and re-culturing of the yeast strain S. fib SF4 to reduce the characteristic mycelial formation during propagation and fermentation. This was successfully achieved with the help of micromanipulation. The significantly reduced filamentous growth of the yeast can lead to a positive effect on the flocculation and filtration of the beer on an industrial scale. By further comparing the micromanipulated culture to the mother culture, no genetic differences could be detected in the $(GTG)_5$ rep-PCR fingerprint. The beers fermented with the two different S. fib SF4 cultures revealed only minimal differences as the beers fermented with the micromanipulated culture displayed slightly higher fermentation activity and exhibited stronger dried fruit flavors in addition to the well-known fruity plum and berry flavors. The flavor of the beers fermented at 20°C was preferred by the tasters over the beer flavor at 28°C fermentation temperature while the beer produced with the micromanipulated S. fib SF4 culture at 20°C scored best.

Data availability statement

The original contributions presented in the study are included in the article/ Supplementary materials, further inquiries can be directed to the corresponding author.

Author contributions

YM, FM, and MH conceived the study. YM, FM, and LR performed the experiments. YM and FM analyzed the data. YM and FM wrote the manuscript. YM, FM, LR, MZ, and MH reviewed and edited the manuscript. MZ, FJ, and MH acquired the funding. FJ and MH supervised the study. All authors contributed to the article and approved the submitted version.

Funding

This research was funded by the BMWK (German Ministry of Economic Affairs and Climate Action), Forschungsvereinigung Wifoe (Association for the Promotion of Science of the German Brewing Industry, Berlin). The IGF Project (grant number AiF 20658 N) of the Wifoe is supported *via* AiF within the program for promoting the Industrial Collective Research (IGF) of the BMWK, based on a decision by the German Bundestag.

Acknowledgments

The authors would like to thank Franziska Elisath and Susan Illing (BLQ) for their assistance in analyzing the samples in the laboratories.

Conflict of interest

Author FM was employed by VTT Technical Research Centre of Finland Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1011155/full#supplementary-material

References

Afolabi, F. T., Adeyemo, S. M., and Balogun, H. O. (2018). Fermentation conditions and process optimization of citric acid production by yeasts. *Int. J. Biotechnol.* 7, 51–63. doi: 10.18488/journal.57.2018.71.51.63

Aidoo, K. E., Nout, M. J. R., and Sarkar, P. K. (2006). Occurrence and function of yeasts in Asian indigenous fermented foods. *FEMS Yeast Res.* 6, 30–39. doi: 10.1111/J.1567-1364.2005.00015.X

Boundy-Mills, K., Stratford, M., and Miller, M. W. (2011). "Chapter 62-Saccharomycodes E.C. Hansen (1904)," in *The Yeasts: A taxonomic Study*. ed. C. P. Kurtzman (Amsterdam: Elsevier), 747–750. doi: 10.1016/B978-0-444-52149-1.00062-8

Budroni, M., Zara, G., Ciani, M., and Comitini, F. (2017). "*Saccharomyces* and non-*saccharomyces* starter yeasts," in *Brewing Technology*. ed. M. Kanauchi (London: Intech Open) doi: 10.5772/intechopen.68792

Chen, L., Chi, Z.-M., Chi, Z., and Li, M. (2010). Amylase production by *Saccharomycopsis Fibuligera* A11 in solid-state fermentation for hydrolysis of cassava starch. *Appl. Biochem. Biotechnol.* 162, 252–263. doi: 10.1007/S12010-009-8744-3

Chidi, B. S., Rossouw, D., Buica, A. S., and Bauer, F. F. (2015). Determining the impact of industrial wine yeast strains on organic acid production under white and red wine-like fermentation conditions. *South Afr. J. Enol. Viticult.* 36, 316–327. doi: 10.21548/36-3-965

De Schutter, D. P., Saison, D., Delvaux, F., Derdelinckx, G., Rock, J.-M., Neven, H., et al. (2008). Release and evaporation of volatiles during boiling of Unhopped wort. *J. Agric. Food Chem.* 56, 5172–5180. doi: 10.1021/Jf800610x

Dietvorst, J., Londesborough, J., and Steensma, H. Y. (2005). Maltotriose utilization in lager yeast strains: Mtt1 encodes a Maltotriose transporter. *Yeast* 22, 775–788. doi: 10.1002/Yea.1279

Djien, K. S. (1972). Tapé Fermentation. *Appl. Microbiol.* 23, 976–978. doi: 10.1128/Am.23.5.976-978.1972

Erdem, M., Kesmen, Z., Özbekar, E., Çetin, B., and Yetim, H. (2016). Application of high-resolution melting analysis for differentiation of spoilage yeasts. *J. Microbiol.* 54, 618–625. doi: 10.1007/S12275-016-6017-8

Esslinger, H. M. (2009). *Handbook of Brewing: Processes, Technology, Markets*. Weinheim, Germany: John Wiley & Sons

Federoff, H. J., Eccleshall, T. R., and Marmur, J. (1983). Carbon catabolite repression of maltase synthesis in *saccharomyces Carlsbergensis*. *J. Bacteriol.* 156, 301–307. doi: 10.1128/jb.156.1.301-307.1983

Fricker, A. (1984). "Die Sensorisch Wirksamen Stoffe In Lebensmitteln," in *Lebensmittel — Mit Allen Sinnen Prüfen!* (Springer, Berlin: Heidelberg), 46–117. doi: 10.1007/978-3-642-82325-1_3

Futatsugi, M., Ogawa, T., and Fukuda, H. (1993). Purification and properties of two forms of Glucoamylase from *Saccharomycopsis Fibuligera*. *J. Ferment. Bioeng.* 76, 521–523. doi: 10.1016/0922-338x(93)90252-4

Gernat, D. C., Brouwer, E., and Ottens, M. (2020). Aldehydes as wort off-Flavours in alcohol-free beers—origin and control. *Food Bioprocess Technol.* 13, 195–216. doi: 10.1007/s11947-019-02374-z

Gibson, B. R., Storgårds, E., Krogerus, K., and Vidgren, V. (2013). Comparative physiology and fermentation performance of Saaz and Frohberg lager yeast strains and the parental species *saccharomyces Eubayanus*. *Yeast* 30, 255–266. doi: 10.1002/Yea.2960

Görts, C. P. M. (1969). Effect of glucose on the activity and the kinetics of the maltose-uptake system and of A-glucosidase in *saccharomyces cerevisiae*. *Biochim. Biophys. Acta Gen. Subj.* 184, 299–305. doi: 10.1016/0304-4165(69)90032-4

Hildebrandt, G., and Schneider-Haeder, B. (2009). Sensorische Analyse: Methodenüberblick Und Einsatzbereiche–Dlg-Sensorik, 2/2009, Www.Dlg.Org

Hostinová, E. (2002). Amylolytic enzymes produced by the yeast *Saccharomycopsis Fibuligera*. *Biologia* 57, 247–252.

Hostinová, E., and Gašperík, J. (2010). Yeast Glucoamylases. *Mol. Genet. Struct. Charact.* 65, 559–568. doi: 10.2478/S11756-010-0077-8

Hutzler, M., Michel, M., Kunz, O., Kuusisto, T., Magalhães, F., Krogerus, K., et al. (2021). Unique brewing-relevant properties of a strain of *saccharomyces Jurei* isolated from ash (Fraxinus Excelsior). *Front. Microbiol.* 12:645271. doi: 10.3389/Fmicb.2021.645271

Janeček, Š., and Baláž, Š. (1992). A-amylases and approaches leading to their enhanced stability. *FEBS Lett.* 304, 1–3. doi: 10.1016/0014-5793(92)80575-2

Klein, C. J., Olsson, L., Rønnow, B., Mikkelsen, J. D., and Nielsen, J. (1996). Alleviation of glucose repression of maltose metabolism by Mig1 disruption in *saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 62, 4441–4449. doi: 10.1128/Aem.62.12.4441-4449.1996

Kreger-Van Rij, N.J.W. (1984). *The Yeasts: a Taxonomic Study*. Amsterdam: Elsevier Science Publishers B. V.

Krogerus, K., Magalhães, F., Kuivanen, J., and Gibson, B. (2019). A deletion in the Sta1 promoter determines Maltotriose and starch utilization in Sta1+ *saccharomyces cerevisiae* strains. *Appl. Microbiol. Biotechnol.* 103, 7597–7615. doi: 10.1007/S00253-019-10021-Y

Kurtzman, C. P., and Smith, M. T. (2011). "Chapter 63- Saccharomycopsis Schiönning (1903): *Saccharomycopsis Fibuligera* (Lindner) Klöcker (1924)," in *The Yeasts: A Taxonomic Study*. ed. C. P. Kurtzman (Amsterdam: Elsevier), 755–756.

La Fuente, G., and Sols, A. (1962). Transport of sugars in yeasts. *Biochim. Biophys. Acta* 56, 49–62. doi: 10.1016/0006-3002(62)90526-7

Lam, K. C., Foster, R. T., and Deinzer, M. L. (1986). Aging of hops and their contribution to beer flavor. *J. Agric. Food Chem.* 34, 763–770. doi: 10.1021/jf00070a043

Lee, S. M., Jung, J. H., Seo, J.-A., and Kim, Y.-S. (2018). Bioformation of volatile and nonvolatile metabolites by *Saccharomycopsis Fibuligera* Kjj81 cultivated under different conditions-carbon sources and cultivation times. *Molecules* 23:2762. doi: 10.3390/Molecules23112762

Lindner, P. (1907). *Endomyces Fibuliger* N. Sp. Ein Neuer Gärungspilz Und Erzeuger Der Sog. *Kreidekrankheit Des Brotes. Wochenschrift Für Brauerei* 24, 469–474.

Liu, G.-L., Wang, D.-S., Wang, L.-F., Zhao, S.-F., and Chi, Z.-M. (2011). Mig1 is involved in mycelial formation and expression of the genes encoding extracellular enzymes in *Saccharomycopsis Fibuligera* A11. *Fungal Genet. Biol.* 48, 904–913. doi: 10.1016/j.fgb.2011.04.008

Lodolo, E. J., Kock, J. L. F., Axcell, B. C., and Brooks, M. (2008). The yeast *saccharomyces cerevisiae*-the Main character in beer brewing. *FEMS Yeast Res.* 8, 1018–1036. doi: 10.1111/J.1567-1364.2008.00433.X

Lucero, P., Peñalver, E., Moreno, E., and Lagunas, R. (1997). Moderate concentrations of ethanol inhibit endocytosis of the yeast maltose transporter. *Appl. Environ. Microbiol.* 63, 3831–3836. doi: 10.1128/Aem.63.10.3831-3836.1997

Magalhães, F., Vidgren, V., Ruohonen, L., and Gibson, B. (2016). Maltose and Maltotriose utilisation by group I strains of the hybrid lager yeast *saccharomyces Pastorianus*. *FEMS Yeast Res.* 16:fow053. doi: 10.1093/Femsyr/Fow053

Meaden, P. G., and Taylor, N. R. (1991). Cloning of a yeast gene which causes phenolic off-Flavours in beer. *J. Inst. Brew.* 97, 353–357. doi: 10.1002/J.2050-0416.1991.Tb01075.X

Meier-Dörnberg, T., Hutzler, M., Michel, M., Methner, F.-J., and Jacob, F. (2017). The importance of a comparative characterization of *saccharomyces cerevisiae* and *saccharomyces Pastorianus* strains for brewing. *Fermentation* 3:41. doi: 10.3390/Fermentation3030041

Methner, Y., Hutzler, M., Matoulková, D., Jacob, F., and Michel, M. (2019). Screening for the brewing ability of different non-*saccharomyces* yeasts. *Fermentation* 5:101. doi: 10.3390/Fermentation5040101

Methner, Y., Hutzler, M., Zarnkow, M., Prowald, A., Endres, F., and Jacob, F. (2022). Investigation of non-*saccharomyces* yeast strains for their suitability for the production of non-alcoholic beers with novel flavor profiles. *J. Am. Soc. Brew Chem.* 1–15, 1–15. doi: 10.1080/03610470.2021.2012747

Meyer, W., Mitchell, T. G., Freedman, E. Z., and Vilgalys, R. (1993). Hybridization probes for conventional Dna fingerprinting used as single primers in the polymerase chain Reaction to distinguish strains of *Cryptococcus Neoformans*. *J. Clin. Microbiol.* 31, 2274–2280. doi: 10.1128/Jcm.31.9.2274-2280.1993

Michel, M., Kopecká, J., Meier-Dörnberg, T., Zarnkow, M., Jacob, F., and Hutzler, M. (2016). Screening for new brewing yeasts in the non-*saccharomyces* sector with *Torulaspora Delbrueckii* as model. *Yeast* 33, 129–144. doi: 10.1002/Yea.3146

Moon, H. Y., Kim, H. J., Kim, K. S., Yoo, S. J., Lee, D. W., Shin, H. J., et al. (2021). Molecular characterization of the *Saccharomycopsis Fibuligera* Atf genes, encoding alcohol acetyltransferase for volatile acetate Ester formation. *J. Microbiol.* 59, 598–608. doi: 10.1007/S12275-021-1159-8

Narziss, L., Back, W., Gastl, M., and Zarnkow, M. (2017). *Abriss Der Bierbrauerei*. Newark: John Wiley & Sons Incorporated

Narziß, L., Back, W., Miedaner, H., and Lustig, S. (1999). Untersuchung Zur Beeinflussung Der Geschmacksstabilität Durch Variation Technologischer Parameter Bei Der Bierherstellung. *Monatsschrift Für Brauwissenschaft* 1999, 192–206.

Nga, B. H., Yip, C. W., Koh, S. I., and Chiu, L. L. (1995). Variation of electrophoretic karyotypes in genetically different strains of *Saccharomycopsis Fibuligera* and *Yarrowia Lipolytica*. *Microbiology* 141, 705–711. doi: 10.1099/13500872-141-3-705

Nikulin, J., Aisala, H., and Gibson, B. (2022). Production of non-alcoholic beer via cold contact fermentation with *Torulaspora Delbrueckii*. *J. Inst. Brew.* 128, 28–35. doi: 10.1002/Jib.681

Nikulin, J., Eerikäinen, R., Hutzler, M., and Gibson, B. (2020). Brewing characteristics of the Maltotriose-positive yeast *Zygotorulaspora Florentina* isolated from oak. *Beverages* 6:58. doi: 10.3390/Beverages6040058

Novak, S., Zechner-Krpan, V., and Marić, V. (2004). Regulation of maltose transport and metabolism in *saccharomyces cerevisiae*. *Food Technol. Biotechnol.* 42, 213–218.

Pandey, A. (1995). Glucoamylase research: an overview. *Starch/Stärke* 47, 439–445. doi: 10.1002/Star.19950471108

Piornos, J. A., Balagiannis, D. P., Methven, L., Koussissi, E., Brouwer, E., and Parker, J. K. (2020). Elucidating the odor-active aroma compounds in alcohol-free beer and their contribution to the Worty flavor. *J. Agric. Food Chem.* 68, 10088–10096. doi: 10.1021/acs.jafc.0c03902

Rautio, J., and Londesborough, J. (2003). Maltose transport by Brewer's yeasts in Brewer's wort. *J. Inst. Brew.* 109, 251–261. doi: 10.1002/J.2050-0416.2003.Tb00166.X

Riedl, R., Dünzer, N., Michel, M., Jacob, F., and Hutzler, M. (2019). Beer enemy number one: genetic diversity, physiology and biofilm formation of *lactobacillus brevis*. *J. Inst. Brew.* 125, 250–260. doi: 10.1002/Jib.553

Rodríguez Madrera, R., Pando Bedriñana, R., and Suárez Valles, B. (2021). Evaluation of indigenous non-*saccharomyces* cider yeasts for use in brewing. *Eur. Food Res. Technol.* 247, 819–828. doi: 10.1007/S00217-020-03665-Y

Schieberle, P. (1991). Primary odorants of pale lager beer. *Eur. Food Res. Technol.* 193, 558–565. doi: 10.1007/BF01190873

Sgouros, G., Mallouchos, A., Filippousi, M.-E., Banilas, G., and Nisiotou, A. (2020). Molecular characterization and enological potential of a high lactic acid-producing *Lachancea Thermotolerans* vineyard strain. *Foods* 9:595. doi: 10.3390/Foods9050595

Siefker, J. A., and Pollock, G. E. (1956). Melanoidins in the brewing processes. I. Formation of aldehydes during wort boiling. Proceedings. *Annu. Meet. Am. Soc. Brew. Chem.* 14, 5–12. doi: 10.1080/00960845.1956.12006472

Srithamma, L. (2009). Defining and optimization of Satho production technology. Dissertation. Technische Universität München, Fakultät Wissens chaftszentrum Weihenstephan.

Steensels, J., and Verstrepen, K. J. (2014). Taming wild yeast: potential of conventional and nonconventional yeasts in industrial fermentations. *Annu. Rev. Microbiol.* 68, 61–80. doi: 10.1146/Annurev-Micro-091213-113025

Teunissen, A. W., and Steensma, H. Y. (1995). Review: the dominant flocculation genes of *saccharomyces cerevisiae* constitute a new Subtelomeric gene family. *Yeast* 11, 1001–1013. doi: 10.1002/Yea.320111102

Tiwari, S. P., Srivastava, R., Singh, C. S., Shukla, K., Singh, R. K., Singh, P., et al. (2015). Amylases: an overview with special reference to alpha amylase. *J. Glob. Biosci.* 4, 1886–1901.

Versalovic, J., Schneider, M., De Brujin, F. J., and Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Meth. Mol. Biol.* 5, 25–40.

Vidgren, V., and Londesborough, J. (2012). Characterization of the *saccharomyces Bayanus*-type Agt1 transporter of lager yeast. *J. Inst. Brew.* 118, 148–151. doi: 10.1002/Jib.22

Whiting, G. C. (1976). Organic acid metabolism of yeasts during fermentation of alcoholic beverages - a review. *J. Inst. Brew.* 82, 84–92. doi: 10.1002/J.2050-0416.1976.Tb03731.X

Wickerham, L. J., Lockwood, L. B., Pettijohn, O. G., and Ward, G. E. (1944). Starch hydrolysis and fermentation by the yeast *Endomycopsis Fibuliger*. *J. Bacteriol.* 48, 413–427. doi: 10.1128/Jb.48.4.413-427.1944

Xie, Z.-B., Zhang, K.-Z., Kang, Z.-H., and Yang, J.-G. (2021). *Saccharomycopsis Fibuligera* in liquor production: a review. *Eur. Food Res. Technol.* 247, 1569–1577. doi: 10.1007/S00217-021-03743-9

Yalcin, S. K., Bozdemir, M. T., and Ozbas, Z. Y. (2010). Citric acid production by yeasts: fermentation conditions, process optimization and strain improvement. *Curr. Res. Technol. Educ. Topics Appl. Microbiol. Microbi. Biotechnol.* 9, 1374–1382.

Yang, Y., Zhong, H., Yang, T., Lan, C., and Zhu, H. (2021). Characterization of the key aroma compounds of a sweet Rice alcoholic beverage fermented with *Saccharomycopsis Fibuligera*. *J. Food Sci. Technol.* 58, 3752–3764. doi: 10.1007/S13197-020-04833-4

2.4 Publication 4: Influence of Varying Fermentation Parameters of the Yeast Strain *Cyberlindnera saturnus* on the Concentrations of Selected Flavor Components in Non-Alcoholic Beer Focusing on (*E*)-*β*-Damascenone

In publication 2, the flavor profiles of the three beers fermented with different *Cyberlindnera saturnus* yeast strains stood out positively. In particular, the beer fermented with the yeast strain C. sat 247 stood out for its flavor profile, which was reminiscent of red berries as well as pear and cool mint sweets. Additionally, a less dominant banana and apple flavor was also perceptible. It was unclear which flavor-active substances the red berry and apple flavors could be attributed to and how different fermentation parameters influenced the flavor of the NAB. In publication 4, these two questions were clarified. Hence, a fermentation model with a total of 20 samples, six of which were center points, was set up with the aid of a response surface methodology (RSM). The target was to optimize for maximum fruitiness, minimum off-flavors and minimum ethanol concentration. Temperature (12–28 °C), original gravity (7–11 °P), and pitching rate (5×10^6–25×10^6 cells/mL) were selected as variable factors. Fermentations were unpressurized in 1,800 mL unhopped wort, in each case for 168 h.

Using headspace gas chromatography (HS-GC) with olfactometry (-O), flame ionization (-FID) and mass spectrometry (-MS), the odor qualities of the odorants in the 20 beer samples were analyzed for specific berry and apple flavors. During this analysis, (*E*)-*β*-damascenone was detected and quantified by GC × GC-MS system. The OAVs correlated with the berry and apple flavors perceived by the trained sensory assessors in the beers as part of a multivariate data analysis. Moreover, statistical analysis revealed that pear, banana and cool mint sweets could be traced back to the volatile compound isoamyl acetate. Accordingly, isoamyl acetate and (*E*)-*β*-damascenone were significantly responsible for the fruity character of the beers fermented with C. sat 247. As responses of the RSM, concentrations of ethanol, (*E*)-*β*-damascenone, the sum of esters – isoamyl acetate in particular –, isovaleric acid, diacetyl, the fruitiness of the beer, and the sum of DLG points to rate the sensory purity were considered and measured accordingly by instrumental analysis and sensory tests, respectively. To maximize fruitiness and thus (*E*)-*β*-damascenone, the sum of esters, isoamyl acetate, and the sum of DLG points, while minimizing isovaleric acid, diacetyl, and ethanol concentration, a fermentation temperature of 16.1 °C, an original gravity of 10.5 °P, and a pitching rate of 5×10^6 cells/mL were selected. The unpressurized fermentation for the optimization trial was carried out in 1,800 mL unhopped wort for 168 h. The set parameters indeed led to the desired optimization, resulting in a successful application of the RSM. Moreover, the methodology provided comprehensive information on the changes in the flavor profiles and ethanol concentrations of the beers at varying fermentation parameters.

Authors/Authorship contribution:

Methner, Y.: Conception and design of experiments using RSM, performance of experiments, analysis of data and visualization, data curation, paper writing, revision of manuscript; **Dancker, P**.: Analysis of data, data curation and method writing of HS-GC with -O, -FID and -MS, revision of manuscript; **Maier, R.**: Performance, analysis of data and data curation of GC × GC-MS system; **Latorre, M.**: Organization and provision of yeast strain TUM 247, revision of manuscript; **Hutzler, M.**: Revision and validation of conception and methodology, organization and provision of yeast strain TUM 247, revision of manuscript; **Zarnkow, M.**: Revision and validation of conception of RSM, organization and provision of yeast strain TUM 247, revision of manuscript; **Steinhaus, M.**: Revision of manuscript, project administration, funding acquisition; **Libkind, D.**: Organization and provision of yeast strain TUM 247, revision of manuscript; **Frank, S.**: Data curation and method writing of GC × GC-MS system, revision of manuscript, supervision of project; **Jacob, F.**: Supervision of project, project administration, funding acquisition.

foods

MDPI

Article

Influence of Varying Fermentation Parameters of the Yeast Strain *Cyberlindnera saturnus* on the Concentrations of Selected Flavor Components in Non-Alcoholic Beer Focusing on (*E*)-*β*-Damascenone

Yvonne Methner [1], Philipp Dancker [1], Robin Maier [2], Mailen Latorre [3], Mathias Hutzler [1], Martin Zarnkow [1,*], Martin Steinhaus [2], Diego Libkind [3], Stephanie Frank [2] and Fritz Jacob [1]

[1] Research Center Weihenstephan for Brewing and Food Quality, Technical University of Munich, Alte Akademie 3, 85354 Freising, Germany; yvonne.methner@tum.de (Y.M.); philipp.dancker@tum.de (P.D.); m.hutzler@tum.de (M.H.); f.jacob@tum.de (F.J.)
[2] Leibniz Institute for Food Systems Biology, Technical University of Munich (Leibniz-LSB@TUM), 85354 Freising, Germany; r.maier.leibniz-lsb@tum.de (R.M.); martin.steinhaus@tum.de (M.S.); s.frank.leibniz-lsb@tum.de (S.F.)
[3] Centro de Referencia en Levaduras y Tecnología Cervecera (CRELTEC), Instituto Andino Patagónico de Tecnologías Biológicas y Geoambientales (IPATEC), CONICET—Universidad Nacional del Comahue, Quintral 1250, San Carlos de Bariloche CP8400, Argentina; mailenlatorre@gmail.com (M.L.); libkindfd@comahue-conicet.gob.ar (D.L.)
* Correspondence: martin.zarnkow@tum.de; Tel.: +49-8161-71-3530

check for updates

Citation: Methner, Y.; Dancker, P.; Maier, R.; Latorre, M.; Hutzler, M.; Zarnkow, M.; Steinhaus, M.; Libkind, D.; Frank, S.; Jacob, F. Influence of Varying Fermentation Parameters of the Yeast Strain *Cyberlindnera saturnus* on the Concentrations of Selected Flavor Components in Non-Alcoholic Beer Focusing on (*E*)-*β*-Damascenone. *Foods* **2022**, *11*, 1038. https://doi.org/10.3390/foods11071038

Academic Editor: Francesco Grieco

Received: 25 February 2022
Accepted: 1 April 2022
Published: 2 April 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Abstract: The diversification of beer flavor is becoming increasingly popular, especially in the field of non-alcoholic beers, where sales are growing steadily. While flavor substances of traditional beers can largely be traced back to defined secondary metabolites, the production of non-alcoholic beers with non-*Saccharomyces* yeasts generates novel fruity flavors, some of which cannot yet be assigned to specific flavor substances. In a recently published study, besides pear, cool mint sweets, and banana-like flavor, distinctive red berry and apple flavors were perceived in a non-alcoholic beer fermented with the yeast strain *Cyberlindnera saturnus* TUM 247, whose secondary metabolites were to be elucidated in this study. The trials were carried out using response surface methodology to examine the fermentation properties of the yeast strain and to optimize the beer with maximum fruitiness but minimal off-flavors and ethanol content. It turned out that a low pitching rate, a moderate fermentation temperature, and an original gravity of 10.5 °P gave the optimal parameters. Qualitative analysis of the secondary metabolites, in addition to standard analysis for traditional beers, was first performed using headspace-gas chromatography with olfactometry. (*E*)-*β*-damascenone emerged as the decisive substance for the red berry and apple flavor and so this substance was then quantitated. Although (*E*)-*β*-damascenone is a well-known secondary metabolite in beer and this substance is associated with apple or cooked apple- and berry-like flavors, it has not yet been reported as a main flavor component in non-alcoholic beers.

Keywords: non-*Saccharomyces* yeast; *Cyberlindnera saturnus*; fermentation; brewing; non-alcoholic beer; response surface methodology; gas chromatography-mass spectrometry; olfactometry; secondary metabolites; (*E*)-*β*-damascenone

1. Introduction

The importance of non-alcoholic beers has grown in recent years and the trend continues to grow into this direction [1,2]. The use of maltose-negative non-*Saccharomyces* yeasts is a significant production method for non-alcoholic beers to reduce or even eliminate undesirable wort off-flavors. The potential to generate pleasant flavors using the microbiological method has been shown to be effective in numerous studies. There is a particular

focus on generating different fruity flavor characteristics due to a more diverse and higher ester concentration in beer [2–8].

Although the secondary metabolites of regular traditional beers produced with conventional *Saccharomyces* brewing yeasts have been deciphered, the use of non-*Saccharomyces* yeasts occasionally result in flavor compounds that cannot be clearly assigned in standard analysis [2,9]. A recent study by Methner et al. [9] revealed that in some non-alcoholic beers produced with various non-*Saccharomyces* yeasts, the sensory assessors perceived red berry and apple flavors that could not be directly attributed to any of the volatiles analyzed. In the specified study, one yeast strain stood out positively in terms of its flavor-generating properties in brewer's wort: *Cyberlindnera saturnus* TUM 247. Besides a dominant cool mint sweets and red berry flavor, the beer fermented with this yeast strain was further described to exhibit less intense but perceptible pear, banana, solvent, and apple flavors. The yeast species *C. saturnus* is generally known from existing literature sources to be suitable for the production of non-alcoholic beers due to its maltose-negative properties [9–11]. It also synthesizes significant amounts of 3-methylbutyl acetate (isoamyl acetate) during the fermentation process in brewer's wort resulting in a fruity, mainly banana flavor [5,10]. However, the fact that certain yeast strains of this species can also produce unusual red berry and apple flavors is a recent finding.

Apple flavor in beer is well known and can, for example, be attributed to 2-phenylethyl acetate or to ethyl decanoate. As a sour apple flavor, it can be traced back to ethyl hexanoate and ethyl octanoate. Some sources also attribute an apple flavor to isoamyl acetate. The flavor threshold concentration of 2-phenylethyl acetate in beer is 3.8 mg/L, for isoamyl acetate this is 1.2 mg/L, ethyl hexanoate 0.21 mg/L, ethyl octanoate 0.9 mg/L, and ethyl decanoate 1.5 mg/L. In addition to these five esters, acetaldehyde is also known for its apple flavor, being recognized rather as green apple and grassy when exceeding a flavor threshold concentration of 10 mg/L [6–9]. Furthermore, the ketone (2*E*)-1-(2,6,6-trimethylcyclohexa-1,3-dien-1-yl)but-2-en-1-one ((*E*)-*β*-damascenone) can also induce an apple or cooked apple-like impression in beer [12–16]. With regard to red berry flavor, there are currently no studies that present findings that attribute this flavor in beer to volatile substances, although berry-like flavors are principally known when using non-*Saccharomyces* yeasts for beer production [17,18]. Interestingly, however, (*E*)-*β*-damascenone has already been described in the literature as causing red fruits and strawberry flavor [19–21].

Therefore, this study investigated which volatile substance or substances were responsible for the red berry-like flavor expression in beer. Moreover, it was examined which volatile substances were responsible for the apple-like flavor. In addition, the question was answered as to what extent different fermentation parameters influence the flavor characteristics of the non-alcoholic beer produced with TUM 247. Studies by Pires et al. and Verstrepen et al. showed that fermentation parameters such as original gravity, temperature, and pitching rate have a demonstrable influence on the formation of esters during beer production [22,23]. Further studies by Bellut et al., Michel et al., and Puerari et al. also provided insight that the fermentation parameters have a decisive impact on the beer flavor. These three studies used response surface methodology (RSM) to optimize the fermentation parameters with regard to e.g., a maximum ester yield [10,17,24]. Based on the successful application of the method, RSM with a central composite design was used in this study to combine the variation in fermentation temperature, original gravity and pitching rate. This method was applied specifically to optimize the beer flavor and also to investigate the general influence of the different fermentation parameters on the flavor of the beers.

Since the fermentation trials were conducted in 2-L small-scale fermentation trials, sterilized wort produced from malt extract was used for this study in order to ensure a comparable, standardized wort base and avoid microbiological contamination. However, it must be taken into consideration that the thermal influence on the wort during the sterilization process generates Strecker aldehydes, resulting in the formation of various aldehydes, Maillard reaction products and ketones, such as (*E*)-*β*-damascenone [15,25,26]. Aldehydes, can cause the characteristic wort flavors and are often referred to as off-flavors [27]. However,

in addition to undesirable wort off-flavors, further adverse flavor compounds, such as 3-methylbutanoic acid (isovaleric acid) or butane-2,3-dione (diacetyl), can emerge during wort fermentation, which also applies to the yeast species *C. saturnus* [9]. For this reason, these two flavor compounds were explicitly investigated. In addition to sensory evaluations by trained panelists, extensive gas chromatography-mass spectrometry (GC-MS) analyses were performed as part of this study. As a result of headspace-gas chromatography (HS-GC) with olfactometry (-O), flame ionization (-FID) and mass spectrometry (-MS) for a qualitative assessment, a GC × GC-MS system was applied for quantitation.

The main objective of this study was to find out to which flavor components the red berry and the apple flavor can be attributed in order to reconstruct which secondary metabolites were responsible for the overall fruity flavor. Additionally, the influence of different fermentation parameters was investigated by RSM to determine optimum parameters resulting in maximum fruitiness of the beer with as few off-flavor compounds as possible. At the same time, the ethanol content was to be kept as low as possible, since the target application was a non-alcoholic beer.

2. Materials and Methods

2.1. Yeast Strain and Wort

The maltose-negative yeast strain *Cyberlindnera saturnus* TUM 247 proved suitable for producing non-alcoholic beers in preliminary trials based on its fermentation properties. The favorable fermentation properties were due to the fact that only glucose, fructose, and sucrose could be metabolized from brewer's wort, while exhibiting sufficient hop tolerance and producing an exceptionally pleasant fruity flavor [9,18]. The yeast strain was isolated from soil underneath an ash tree (*Fraxinus excelsior*) in the district Kelheim, state Bavaria, Germany. Identification was performed by D1/D2 26S rDNA sequencing using primers according to Kurtzman [28] fp NL1 rp NL4, while NL1 was used as sequencing primer. The 26S sequence data from sequencing with NL1 primer can be found in the supplementary material Sequence S1.

For the RSM experiments, the wort was prepared from unhopped malt extract (Weyermann®, Bamberg, Germany), which was adjusted to the different, desired original gravities. The wort was then sterilized (100 °C, 45 min) to exclude microbiological contamination.

Furthermore, to determine the extent to which (*E*)-*β*-damascenone can be formed via thermal processing, three different thermally treated worts were prepared from unhopped malt extract (Weyermann®, Bamberg, Germany). The worts were all adjusted to an original gravity of 7 °P and 1800 mL of each was filled into 2000 mL Duran glass bottles (Schott AG, Mainz, Germany). While one wort was kept at 100 °C for 5 min (pH 5.6), another wort was sterilized at 100 °C for 45 min (pH 5.5). The third wort was autoclaved at 121 °C for 10 min (pH 5.4). The wort with the lowest thermal input was used for small-scale fermentation trials as described in Methner et al. (2022) [9].

2.2. Yeast Propagation and Fermentation

For propagation, the yeast strain TUM 247 was inoculated from wort slope agar under sterile conditions into five 500 mL flasks each containing 250 mL of unhopped wort (9.0 °P, pH 5.4). After propagating for 72 h at 20 °C on a WiseShake orbital shaker (Witeg Labortechnik GmbH, Wertheim, Germany) at 80 rpm, the yeast suspensions were transferred to five sterile 2500 mL flasks, each containing 1800 mL of comparable wort. After a further propagation period of 60 h and 12 h of settling time, the wort supernatant was poured off, accounting for approximately one third of the total volume, before yeast cell numbers were counted using the Cellometer® Vision (Nexcelom Bioscience LLC, Lawrence, MA, USA). Subsequently, the required amounts of propagation yeast could be calculated for the fermentation trials.

The wort for the fermentation trials was prepared analogously to the propagation wort. Before pitching, the propagation yeasts were centrifuged (Roto Super 40, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) in sterilized 500 mL PPCO centrifuge

bottles (Nalgene, Thermo Fisher Scientific, Waltham, MA, USA) at 750× *g* for 5 min and the supernatant was discarded. The yeasts were then washed in sterile physiological sodium chloride solutions before being used for fermentation. In total, 20 small-scale fermentation trials and an optimization trial in triplicate were conducted, each unpressurized in 1800 mL sterilized unhopped wort in 2000 mL Duran glass bottles (Schott AG, Mainz, Germany) equipped with fermentation locks on top. After fermentation for seven days (168 h), the fermentation locks were replaced with sterile screw caps and the 20 samples were cooled down to 3 °C. Following storage for 72 h, the samples were analyzed and tasted.

2.3. Response Surface Methodology (RSM)

To investigate the influence of different fermentation parameters on selected flavor components and ethanol content of the beers fermented with the yeast strain *C. saturnus* TUM 247, RSM was applied using Design-Expert 13 software (StatEase, Minneapolis, MN, USA). A face-centered central composite design with three independent factors and six replications of the center point was chosen. The three variable factors were original gravity (7 to 11 °P), fermentation temperature (12 to 28 °C), and pitching rate (5×10^6 to 25×10^6 cells/mL). Therefore, in total, 20 small-scale fermentation trials were performed. This results in a center point with the factors of temperature at 20 °C, original gravity of 9 °P, and pitching rate of 15×10^6 cells/mL. Table 1 presents the experimental design.

Table 1. Experimental design of response surface methodology (RSM) with face-centered, three-factorial (temperature, original gravity, and pitching rate) central composite design, and six replications of the center point (20 °C, 9 °P, 15×10^6 cells/mL).

Run	Factor 1 A: Temperature (°C)	Factor 2 B: Original Gravity (°P)	Factor 3 C: Pitching Rate ($\times 10^6$ cells/mL)
1	20	7	15
2	28	9	15
3 *	20	9	15
4	12	7	25
5	12	9	15
6	20	11	15
7 *	20	9	15
8 *	20	9	15
9	28	7	5
10 *	20	9	15
11	20	9	25
12	12	7	5
13	28	11	25
14	12	11	5
15	12	11	25
16	20	9	5
17	28	11	5
18	28	7	25
19 *	20	9	15
20 *	20	9	15

* Center points.

All data were statistically evaluated with analysis of variance (ANOVA). A one-sample *t*-test was performed using OriginPro 2020 as statistical software to evaluate whether all values of the six center points were insignificantly different from each other. If there was no significant difference in the one-sample *t*-test, it may be assumed that all remaining 14 measurement points reveal statistically significant results.

As responses, concentrations of ethanol, (*E*)-*β*-damascenone, the sum of esters—isoamyl acetate in particular—isovaleric acid, diacetyl, the fruitiness of the beer, and the sum of DLG points were taken into account. For optimization using RSM, the aim was

to maximize the concentrations of (*E*)-*β*-damascenone, the sum of esters, isoamyl acetate, the fruitiness of the beer, and the sum of DLG points, while minimizing the concentrations of ethanol, isovaleric acid and diacetyl. Only the responses to be optimized were analyzed in the beer produced in the course of the RSM optimization.

2.4. Analytical Methods

Regarding the analytical methods, MEBAK [1] standard methods for wort and beer were initially applied for the 20 beer samples (cf. Table 1) and additionally in the final stages for the optimized beer samples. Since the standard methods were not sufficient to elucidate the red berry- and apple-like flavors of the beers, qualitative HS-GC-O, -FID, and -MS were performed first, followed by a stable isotope dilution assay (SIDA) using a GC × GC-MS system for quantitation.

2.4.1. Analytical Standard Methods

The analytical standard methods applied for the initial wort and the beers are listed in Table 2.

Table 2. Analytical standard methods of the wort and the beers according to MEBAK [1].

Analysis	Method	Device
Original gravity, ethanol content, apparent attenuation	MEBAK WBBM 2.9.6.3	Bending vibration and NIR spectroscopy, Alcolyzer Plus with DMA 5000 X sample 122 (Anton-Paar GmbH, Ostfildern, Germany)
pH value	MEBAK WBBM 2.13	pH meter with pH electrode, ProfiLine pH3210 pH meter (Xylem Inc., New York, NY, USA)
(Methylsulfanyl) methane (dimethyl sulfide)	MEBAK WBBM 2.23.1.1	GC-FID Clarus 580 (Perkin Elmer, Waltham, MA, USA), Column: 50 m × 0.32 mm Phenomenex FFAP, 0.25 µm
Fatty acid esters, fatty acids, 2-phenylethan-1-ol	MEBAK WBBM 2.23.6	GC-FID Clarus 580 (Perkin Elmer, Waltham, MA, USA), Column: 50 m × 0.32 mm Phenomenex FFAP, 0.25 µm
Acetaldehyde, ethyl acetate, isoamyl acetate, higher alcohols (propan-1-ol (n-propanol), 2-methylpropan-1-ol (isobutanol), amyl alcohols), ethyl methanoate (ethyl formate), ethyl propanoate (ethyl propionate)	MEBAK WBBM 2.21.1	GC-FID Clarus 580, Turbo Matrix 40, Head Space (Perkin Elmer, Waltham, MA, USA), Column: INNOWAX cross-linked polyethylene glycol, 60 m × 0.32 mm × 0.5 µm
Diacetyl, pentane-2,3-dione	MEBAK WBBM 2.21.5.1	GC-FID Clarus 580, Turbo Matrix 40, Head Space (Perkin Elmer, Waltham, MA, USA), Column: INNOWAX cross-linked polyethylene glycol, 60 m × 0.32 mm × 0.5 µm

[1] MEBAK® (2012), Editor: Dr. F. Jacob: The MEBAK collection of brewing analysis methods: Wort, beer and beer-based beverages. Collection of methods of the Mitteleuropäischen Brauchtechnischen Analysenkommission. Self-published by MEBAK.

2.4.2. Headspace-Gas Chromatography (HS-GC) with Olfactometry (-O), Flame Ionization (-FID) und Mass Spectrometry (-MS)

For this method, a multidimensional GC system consisting of two GC-2010Plus (Shimadzu, Neufahrn bei Freising, Germany) connected by a Deans' switching device was used. GC-1 was equipped with a polar fused silica capillary ZB-WAX (30 m × 0.25 mm × 0.25 µm, Phenomenex Ltd., Aschaffenburg, Germany) and GC-2 with a non-polar fused silica capillary column ZB-5MS (30 m × 0.32 mm × 0.5 µm, Phenomenex Ltd., Aschaffenburg, Germany). As a static headspace sampler and injection port, the HS-20 series (Shimadzu, Neufahrn bei Freising, Germany) was used. After equilibration of the 10 mL beer samples, each in 20 mL HS vials, a portion of the HS was transferred, splitless, for 1 min onto the GC column using a 1 mL sample loop. The conditions for the HS-20 are specified in the

supplementary material Table S1. The GC oven temperature was started at 40 °C to improve the separation of early eluting components. After that, the temperature was increased in 10 °C/min increments up to 125 °C and in 20 °C/min increments up to 240 °C and held there for 5 min. This equals a total runtime of 19.75 min. The temperature program was the same for both GCs and the inlet pressure was set for an optimal carrier gas velocity for helium between 20–40 cm/s. After elution on GC-1 the components were split between either the FI-/O-Detector on GC-1 or transferred onto the GC-2 for further separation and to reduce overlays. The partition between the FI-/O-Detector were achieved via a SGE SilFlow GC 4 Port Splitter (Trajan Scientific Pty Ltd., Ringwood, VIC, Australia) in a 1:1 ratio. On the GC-2, the eluting components were analyzed by a mass spectrometer QP2010-Ultra SE (Shimadzu, Neufahrn bei Freising, Germany) with electron ionization (EI) at 70 eV. The interface I/F was set at 250 °C and the ion source at 200 °C while the scan was between 50–250 amu. The emission current was at 60 µA.

Using GC-O, the odor qualities of the odorants in the beer samples in Runs 1–20 with the fermentation parameters shown in Table 1 were analyzed for the specific berry and apple flavor. For the final identification, the odor quality, retention index (RI), and mass spectrum was compared with that of the corresponding reference compound. The linear RI was calculated from its retention time and the retention times of adjacent *n*-alkanes by linear interpolation [29].

2.4.3. GC × GC-MS System

A 6890 Plus gas chromatograph (Agilent Technologies, Waldbronn, Germany) was equipped with a Combi PAL autosampler (CTC Analytics, Zwingen, Switzerland), a KAS4 injector (Gerstel, Mülheim/Ruhr, Germany), and a fused silica column, DB-FFAP, 30 m × 0.25 mm i.d., 0.25 µm film (Agilent Technologies, Waldbronn, Germany). The carrier gas was helium at 2.0 mL/min constant flow. The end of the column was connected to a second fused silica column, DB-5, 2 m × 0.15 mm i.d., 0.30 µm film (Agilent Technologies, Waldbronn, Germany). The front part of this column passed through a liquid nitrogen-cooled dual stage quad-jet thermal modulator (Leco, Mönchengladbach, Germany). The modulator was used to collect the volatiles eluting from the first column in discrete portions (4 s), which were then rechromatographed on the major part of the second column. This part was installed in a secondary oven mounted inside the primary oven of the gas chromatograph. The end of this column was connected via a heated (250 °C) transfer line to the inlet of a Pegasus III time-of-flight (TOF) mass spectrometer (Leco, Mönchengladbach, Germany). The temperature of the first oven was 40 °C for 2 min, followed by a gradient of 6 °C/min to a final temperature of 230 °C, which was held for 5 min. The temperature of the secondary oven was 80 °C for 2 min, followed by a gradient of 6 °C/min to a final temperature of 250 °C, which was held for 5 min. Mass spectra were generated in the EI mode at 70 eV, a scan range of m/z 35−300, and a scan rate of 100 spectra/s. The ChromSpace software (Markes International Ltd., Llantrisant, UK) was employed for data analysis.

(*E*)-*β*-damascenone was a gift from Symrise to the Leibniz-LSB@TUM (Holzminden, Germany) and ($^2H_{4-7}$)-(*E*)-*β*-damascenone was synthesized as detailed in the literature [30]. Diethyl ether (VWR, Darmstadt, Germany) was freshly distilled before use. For the work-up, beer was filtered through a paper filter to remove carbon dioxide. For the quantitation of (*E*)-*β*-damascenone, diethyl ether (100 mL) and ($^2H_{4-7}$)-(*E*)-*β*-damascenone (0.013 µg) were added to the samples (50 mL). The mixture was stirred for 30 min at ambient temperature. The organic phase was separated, and the aqueous phase was shaken with a second portion of diethyl ether (100 mL). The combined organic phases were washed with saturated aqueous sodium chloride solution (2 × 50 mL) and dried over anhydrous sodium sulfate. Nonvolatiles were removed by solvent-assisted flavor evaporation (SAFE) [31] at 40 °C. The distillate was concentrated to 500 µL by using a Vigreux column (50 × 1 cm) and a Bemelmans microdistillation device [32]. The concentrate was analyzed using the GC × GC-MS system. Peak volumes of the (*E*)-*β*-damascenone peak and the ($^2H_{4-7}$)-(*E*)-

β-damascenone peak were extracted using ions m/z 121 for (*E*)-β-damascenone and m/z 125−128 for ($^2H_{4-7}$)-(*E*)-β-damascenone. The concentration of (*E*)-β-damascenone in the samples was then calculated from the volume of the (*E*)-β-damascenone peak, the volume of the ($^2H_{4-7}$)-(*E*)-β-damascenone peak, the amount of sample used, and the amount of ($^2H_{4-7}$)-(*E*)-β-damascenone added, by employing a calibration line equation. To obtain the calibration line equation, diethyl ether solutions of (*E*)-β-damascenone and ($^2H_{4-7}$)-(*E*)-β-damascenone mixtures in different concentration ratios were analyzed under the same conditions followed by linear regression. The resulting calibration line equation was y = 0.9608x + 0.1701, with y = [concentration of ($^2H_{4-7}$)-(*E*)-β-damascenone]/[concentration of (*E*)-β-damascenone] and x = [peak volume of ($^2H_{4-7}$)-(*E*)-β-damascenone]/[peak volume of (*E*)-β-damascenone].

2.5. Sensory Evaluation

The tastings were performed by twelve DLG-certified (Deutsche Landwirtschafts-Gesellschaft e.V., Frankfurt, Germany) assessors in a specially designated room (neutral white-colored with individual tasting chambers) at 20 °C. The samples were tempered to 12 °C before tasting and were assigned three-digit randomized numbers. First, the tasters performed an evaluation according to the DLG scheme, which comprises a rating scale from zero (minimum score) to five (maximum score) [33], to evaluate only the odor, the purity of taste and the body of the beers. Since the beers were unhopped and fermented without pressure, the quality of the bitterness and carbonation were not taken into account in the sensory evaluation. By conducting the DLG test, beers were, therefore, mainly evaluated for their purity in terms of odor and taste, so that a devaluation was based on perceptible off-flavors such as a buttery flavor from diacetyl or vegetable-like flavor from dimethyl sulfide.

The tasters then evaluated the fruitiness of the beers. In this context, a scale of zero to five was used to indicate how fruity the beer was rated, with zero standing for not at all fruity and five for maximum fruitiness. Due to the large number of samples, a composite sample was prepared from the six center points, each in equal proportions so that the assessors evaluated a total of 15 samples. Prior to this, as described in Section 2.3. the six center point samples were analyzed and statistically evaluated using a one-sample *t*-test to ensure that the six samples did not differ significantly. The fruitiness was converted to a percentage based on a maximum score of 60 points for the evaluation. Since the flavor profile of the yeast strain *C. saturnus* TUM 247 is already known from a recent study [9] and is described as being like cool mint sweets and solvent-like besides fruity expressions of pear, banana, red berries, and apple, the tasters were specifically asked if they could perceive these flavors in the beers. Additionally, based on the analyzed secondary metabolites, the flavor characteristics honey, cheesy, and watery/pale were included. The methods for sensory evaluation were applied both to the 15 beer samples from the RSM central composite design and to the beer sample optimized by RSM in triplicate.

To establish a possible correlation between the specific volatile compounds measured analytically and the specified flavor characteristics identified by the tasters, a principal component analysis (PCA) was performed after normalizing the values using OriginPro 2020 statistical software.

3. Results

In this chapter, the analyses of the 20 beer samples (cf. Table 1) are presented, which were carried out according to the experimental design of the RSM (cf. Section 2.3). This includes standard analytical methods for beer (cf. Table 2) and two additional GC methods (Section 2.4). Furthermore, the sensory evaluation of the fermented samples is depicted before the RSM optimization results are presented. The yeast strain TUM 247 (cf. Section 2.1) was used for all experiments without exception using the propagation and fermentation methods described in Section 2.2.

3.1. Analytical Results

Initially, the one-sample *t*-test was performed for selected values. Since the mean population was insignificantly different from the test mean at the 0.05 level without exception (cf. supplementary material Table S2), it may be assumed that all remaining 14 measurement points show statistically significant results in a single experiment.

In Table 3, an analysis of variance (ANOVA) was performed for the purposes of RSM for predetermined responses (cf. Section 2.3) that were later used for the optimization experiment. Occasionally, values were identified as outliers in the statistical analyses. Thus, for the sum of esters and isoamyl acetate, Run 16 was excluded; for diacetyl, Run 2; for (*E*)-*β*-damascenone, Run 13; and for fruitiness, Run 12. Additionally, for (*E*)-*β*-damascenone Runs 7, 8, 10, 19, and 20 were excluded, which is explained in the further course.

Table 3. Analysis of variance (ANOVA) of selected responses using the three factors A: Temperature (°C), B: Original Gravity (°P), C: Pitching Rate ($\times 10^6$ cells/mL) for response surface methodology (RSM).

Response	Unit	Minimum	Maximum	Model	*p*-Value	LOF *p*-Value
∑ Esters	mg/L	2.1	3.6	Quadratic	0.0012	0.1928
Isoamyl acetate	mg/L	0.7	1.9	Linear	0.0066	0.0065 *
Isovaleric acid	mg/L	0.53	1.30	Quadratic	<0.0001	0.8221
Diacetyl	mg/L	0.06	0.19	Linear	0.0005	0.4825
(*E*)-*β*-damascenone	µg/L	0.873	1.57	Linear	0.0058	<0.0001 *
Ethanol	% (*v*/*v*)	0.11	0.63	2FI [1]	<0.0001	0.0210 *
Fruitiness	%	50	77	Linear	<0.0001	<0.0001 *
Sum DLG	points	3.93	4.70	Quadratic	0.0002	<0.0001 *

* Lack of fit (LOF) significant; target: LOF *p*-value > 0.10 insignificant; *p*-value < 0.05: significant. [1] 2FI = two-factor interaction.

All eight responses revealed significant models. Although some responses marked with asterisks* showed a significant lack of fit (LOF), these were included in the analysis due to the significant *p*-values. However, only three-dimensional (3D) plots were created for significant models with insignificant LOF.

In addition to the responses to be optimized in the RSM, the results of the standard analytical methods from Section 2.4.1 are depicted below. The analytical results of ethanol, apparent attenuation, and pH values of the 20 samples depicted in Table 4 showed correlations depending on the selected fermentation parameters.

Regarding the ethanol content, the general trend was that the higher the original gravity and the temperature, the higher the ethanol content. In case a lower pitching rate was used, correspondingly less ethanol was produced by the yeasts. Conversely, the higher the pitching rate, the higher the ethanol content. With the exception of Run 13, all beers were below 0.5% (*v*/*v*) ethanol content. The low ethanol contents can be explained by the fact that the yeast species *C. saturnus* is known to be maltose- and maltotriose-negative [11].

Table 4. Analyzed values of original gravity (°P), ethanol (% *v*/*v*), apparent attenuation (%), and pH value in the 20 beers fermented with parameters of the response surface methodology (RSM) design.

Run	Original Gravity (°P)	Ethanol (% *v*/*v*)	Apparent Attenuation (%)	pH Value Beer
1	6.90	0.26	7.6	4.83
2	8.90	0.48	7.6	4.76
3 *	8.94	0.29	6.4	4.86
4	6.88	0.18	5.2	4.93
5	8.88	0.15	3.3	5.00
6	10.84	0.29	5.1	4.91
7 *	8.95	0.27	5.8	4.87
8 *	8.89	0.26	5.7	4.88
9	6.89	0.19	5.5	4.89
10 *	8.86	0.28	6.2	4.91
11	8.92	0.32	6.9	4.86
12	6.89	0.11	3.3	5.01
13	10.81	0.63	8.8	4.88
14	10.87	0.14	2.5	5.10
15	10.79	0.20	3.6	4.99
16	8.90	0.16	3.4	5.00
17	10.79	0.48	8.5	4.75
18	6.88	0.35	10.1	4.74
19 *	8.90	0.25	5.5	4.89
20 *	8.87	0.29	6.4	4.85

* Center points.

The situation was similar for the apparent attenuation. Since the apparent attenuation and the ethanol content are directly related, this behavior was to be expected. The lower the original gravity, the fewer sugars are available for the yeast, so that correspondingly fewer sugars can be converted into ethanol and carbon dioxide. It is interesting to note that at a fermentation temperature of 12 °C, the yeast activity was consistently weak, irrespective of the original gravity. Only with increasing fermentation temperatures was there an increase in the yeast metabolizing the available wort sugars. The strongest activity within the selected temperature range was at the maximum of 28 °C.

Evaluating the pH values of the beers, a linear correlation can be assumed between the original gravity and the temperature. A lower yeast pitching rate resulted in a higher pH value, while the pH value decreased as the pitching rate increased. The stronger pH drop at higher fermentation temperatures can be attributed to the fact that the yeasts form more fixed and volatile organic acids due to their higher fermentation activity [34]. Nevertheless, it could be assumed that the pH drop would be correspondingly stronger at an original gravity of 11 °P than at 7 °P due to the higher substrate concentration, which was not the case. The yeast species *C. saturnus*, as already mentioned, can only metabolize glucose, fructose, and sucrose. Since these three sugars are only present in small amounts in the wort (7–9% hexoses, 3% sucrose [34]), the pH drop was relatively weak compared to a conventional brewing yeast such as *Saccharomyces cerevisiae*, which causes a pH drop in the beer to 4.2–4.6 [35].

As Table 5 depicts, out of the ten quantitated fruit esters, ethyl acetate, isoamyl acetate, 2-phenylethyl acetate, ethyl hexanoate, and, to some extent, isobutyl acetate, ethyl butanoate, and ethyl octanoate were formed in concentrations above the limit of quantitation. Considering the relevant seven fruit esters analyzed in Figure 1, the 3D diagram illustrates the relationship between original gravity, temperature and the sum of esters concentration at a pitching rate of 15×10^6 cells/mL.

Table 5. Secondary metabolites analyzed by standard methods according to MEBAK [1] in the 20 beer samples fermented according to RSM design. Ethyl decanoate, ethyl formate, ethyl propionate, hexanoic acid, octanoic acid, decanoic acid, pentane-2,3-dione, 2-phenylethan-1-ol were measured additionally, however, are not shown as the concentrations stayed below the limit of quantitation (LOQ). An additional graphical representation can be found in the supplementary material Figure S1.

	Run	1	2	3 *	4	5	6	7 *	8 *	9	10 *	11	12	13	14	15	16	17	18	19 *	20 *
Esters (mg/L)	Ethyl acetate	1.3	1.2	1.2	1.0	0.93	1.3	1.1	1.2	0.96	1.3	1.2	0.83	1.5	0.79	1.1	1.3	1.0	1.1	1.1	1.2
	Isoamyl acetate	1.4	0.8	1.1	0.8	0.7	1.0	1.0	1.2	1.9	1.2	1.3	1.3	0.8	1.2	0.7	2.7	1.3	0.8	1.1	1.1
	2-Phenylethyl acetate	0.73	0.36	0.62	0.50	0.38	0.62	0.65	0.59	0.67	0.57	0.70	0.58	0.39	0.49	0.42	1.1	0.43	0.45	0.62	0.81
	Isobutyl acetate	0.01	0.01	0.01	<LOQ	<LOQ	0.01	0.01	0.01	0.02	0.01	0.01	<LOQ	0.01	0.01	<LOQ	0.04	0.01	0.01	0.01	0.01
	Ethyl butanoate	<LOQ	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
	Ethyl hexanoate	0.02	0.02	0.02	0.03	0.03	0.02	0.02	0.01	0.03	0.02	0.02	0.03	0.01	0.03	0.03	0.02	0.02	0.02	0.02	0.02
	Ethyl octanoate	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.01	<LOQ	<LOQ	<LOQ	0.01	0.01	<LOQ	<LOQ	<LOQ
Organic acids (mg/L)	Isovaleric acid	1.2	1.2	1.1	0.76	0.61	1.1	1.1	1.1	0.74	1.0	1.1	0.54	1.2	0.53	0.62	0.83	0.86	1.2	1.1	1.3
Higher alcohols (mg/L)	n-Propanol	1.8	3.1	2.0	1.0	1.1	1.9	1.5	1.8	2.0	2.1	2.0	0.9	3.7	1.0	1.1	1.1	2.4	2.4	1.6	2.1
	Isobutyl alcohol	7.3	11.1	6.8	2.8	2.4	6.3	5.7	5.9	9.6	7.4	7.2	1.8	13.2	1.9	2.8	5.4	9.7	10.5	6.1	6.9
	Isoamyl alcohols	15.9	18.6	15.6	10.3	8.8	16.2	13.4	14.7	13.0	16.1	19.6	5.4	23.4	5.9	9.7	9.1	12.8	18.4	14.5	16.0
Aldehydes (mg/L)	Acetaldehyde	1.4	1.7	1.7	1.6	1.2	1.2	1.3	1.6	1.8	1.8	1.7	1.3	2.1	1.2	1.4	1.5	1.7	1.8	1.3	1.6
Ketones (mg/L)	Diacetyl	0.11	0.29	0.14	0.12	0.10	0.10	0.11	0.10	0.15	0.13	0.10	0.06	0.19	0.08	0.12	0.08	0.15	0.16	0.10	0.14
Sulfide (mg/L)	Dimethyl sulfide	0.016	0.022	0.024	0.015	0.023	0.028	0.022	0.021	0.017	0.022	0.024	0.020	0.029	0.029	0.030	0.021	0.031	0.019	0.023	0.022

* Center points; LOQ = Limit of quantitation; [1] MEBAK® (2012), Editor: Dr. F. Jacob: The MEBAK collection of brewing analysis methods: Wort, beer, and beer-based beverages. Collection of methods of the Mitteleuropäischen Brauchtechnischen Analysenkommission. Self-published by MEBAK.

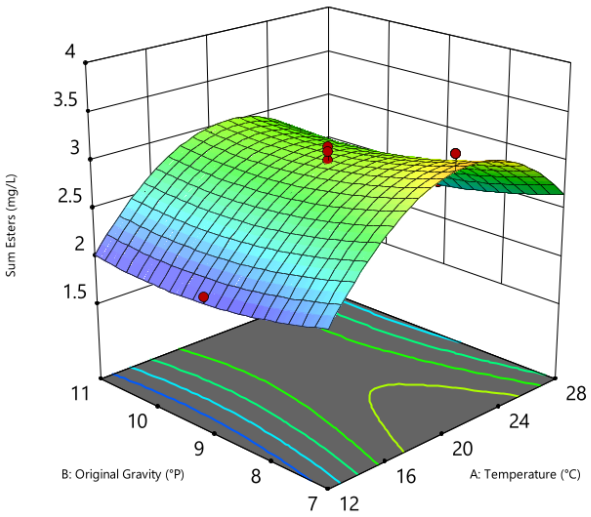


Figure 1. Three-dimensional (3D) diagram of the quadratic correlation between original gravity (°P), temperature (°C), and the sum of esters concentration (mg/L) of the beers at a pitching rate of 15×10^6 cells/mL.

It is particularly interesting that the concentration of the sum of esters was only slightly dependent on the original gravity, as it showed an increasing tendency with decreasing original gravity, which would not have been expected based on existing literature. The higher the original gravity, the higher the concentration of nitrogen, fermentable sugars, and unsaturated fatty acids. These are required for ester synthesis, since fusel alcohols are formed via nitrogen metabolism and acetyl-CoA via sugar and lipid metabolism, which, in turn, react via ester synthase to form fatty acid esters. Accordingly, with increasing original gravity, more substrate is available for ester synthesis [23,34]. *C. saturnus* TUM 247 exhibited a different metabolism to conventional brewing yeasts, since the investigations showed that the original gravity had almost no influence on ester formation. There was even a tendency for more esters to be metabolized at lower original gravity. Thus, the highest concentration of the sum of esters was measured at 7 °P original gravity and at a pitching rate of 5×10^6 cells/mL (cf. Table 5), which was not to be expected as the literature explains that higher pitching rates often lead to higher ester concentrations [34,36,37]. Up to a pitching rate of approximately 18×10^6 cells/mL, the concentration of the sum of esters decreased continuously and independently of temperature. Regardless of the pitching rate and the original gravity, the ester peak was always between 20 and 22 °C. Up to 22×10^6 cells/mL the total ester concentration increased slightly in the higher investigated original gravity concentrations, so that from these cell numbers the ester concentrations were almost independent of the selected original gravity range between 7 and 11 °P. Above 22×10^6 cells/mL, the total esters in the beers increased again, although at the maximum applied cell count of 25×10^6 cells/mL and 7 °P, this was still approximately 1.5 mg/L lower than at 5×10^6 cells/mL at unchanged temperature as well as original gravity. Nevertheless, a similar observation was made in a study by Bellut et al. [10] in which a *Cyberlindnera subsufficiens* also exhibited enhanced fruitiness in beer at a comparatively low pitching rate. However, this was at a pitching rate of 1×10^7 cells/mL. Measured on the basis of regular beers containing alcohol, only the isoamyl acetate concentrations were in the range of the flavor threshold concentration, which is at approximately 1.2 mg/L, causing fruity, banana, pear, and also solvent-like flavors [14,38–40]. Nevertheless, due to the low alcohol concentrations and the associated low concentrations of higher alcohols,

it could be assumed that the flavor threshold concentrations are significantly lower for these kinds of beers. Accordingly, a solvent-like, fruity and sweet flavor could occur due to the presence of ethyl acetate, whereas 2-phenylethyl acetate leads to floral, honey, apple-like flavor impressions [41,42]. While ethyl acetate is increasingly formed by the yeasts at higher fermentation temperatures, the pitching rate had a significant influence as well. For the selected range, it could be determined that the higher the pitching rate, the higher the ethyl acetate concentration in the beer. Interestingly, the opposite conclusion could be drawn for isoamyl acetate with regard to the pitching rate. In addition, a lower original gravity resulted in higher isoamyl acetate concentrations, and, just as with ethyl acetate, higher temperatures also led to increased formation of the ester. 2-phenylethyl acetate was formed most strongly by the yeast at a fermentation temperature of 20 °C and decreased with both increasing and decreasing fermentation temperatures. The original gravity only played a subordinate role, since only a slightly higher 2-phenylethyl acetate formation was recorded at lower original gravity than at higher concentrations. Similar to the isoamyl acetate formation, a lower pitching rate led to higher 2-phenylethyl acetate concentrations in the beers. It is noticeable that the trend continued between pitching rates of approximately 5 to 20 $\times$ 10^6 cells/mL. Above 20 $\times$ 10^6 cells/mL, the flavor compound concentration increased again up to the observation limit of 25 $\times$ 10^6 cells/mL.

Looking at the sum of the organic acids analyzed, which are composed of isovaleric, hexanoic, octanoic, and decanoic acid, Table 5 shows that, apart from isovaleric acid, all other quantitated acids were below the limit of quantitation and, thus, seemed not to effect the beers. However, the isovaleric acid concentration varied among the beers. Figure 2 shows the quadratic correlation of the original gravity and fermentation temperature in the isovaleric acid concentration of the beers at a pitching rate set at 15 $\times$ 10^6 cells/mL.

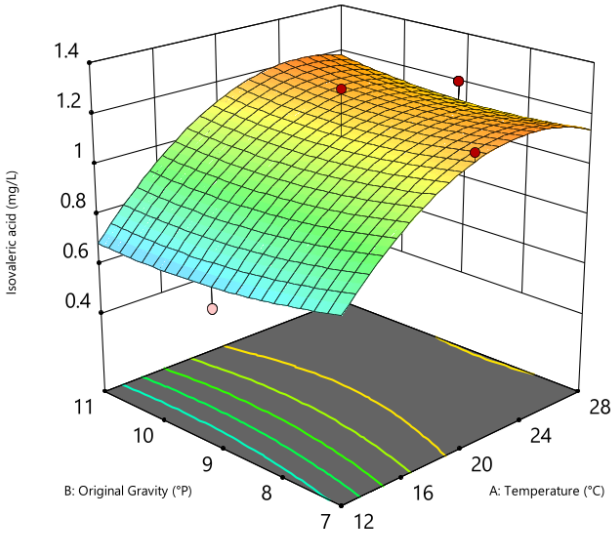


Figure 2. Three-dimensional (3D) diagram of the quadratic correlation of original gravity (°P), temperature (°C), and isovaleric acid concentration (mg/L) of the beers at a pitching rate of 15 $\times$ 10^6 cells/mL.

It was not possible to ascertain a dependence of the isovaleric acid concentration on the original gravity. However, the fermentation temperature and the pitching rate played a significant role. Rising temperatures between 12 °C and 24 °C led to an increase in isovaleric acid production by the yeast. A plateau can be seen at fermentation temperatures

between 24 °C and 28 °C. The lower the selected pitching rate, the less isovaleric acid was formed (cf. Table 5). However, at a pitching rate of 15 × 10^6 cells/mL, the concentration did not increase further in the beers. One possible reason for this could be that certain amino acids were depleted from the wort and were, therefore, no longer available for yeast metabolism, as isovaleric acid is a by-product of the amino acid metabolism formed from leucine during catabolic pathway [43]. In regular beers containing ethanol, the flavor threshold concentration of isovaleric acid is at 1.5–2.5 mg/L, which was not measured in any of the beer samples. The maximum concentration was 1.3 mg/L. In addition, it is known from literature that isoamyl acetate has a masking effect on isovaleric acid and, therefore, based on the measured values, it could be assumed that isovaleric acid had no influence on the beers by exposing a cheesy or rancid flavor impression [38,44].

Higher alcohols were present in lower concentrations at lower fermentation temperatures, regardless of the original gravity, and increased linearly with increasing temperature as well as increasing pitching rate (cf. Table 5). Acetaldehyde and diacetyl in the beers behaved similarly. While acetaldehyde in regular beers leads to fruity, green apple, grassy flavors only at concentrations above 10 mg/L, the flavor threshold concentration for diacetyl is 0.10 to 0.15 mg/L, causing a butter-like flavor [20,38]. The maximum value of acetaldehyde was 2.1 mg/L (cf. Table 5), which is far below the flavor threshold concentration. The situation was different for diacetyl. With values of up to 0.19 mg/L, diacetyl reached concentrations above the flavor threshold concentration. The diacetyl concentration of Run 2 at 0.29 mg/L was excluded as it was an outlier based on statistical analysis (cf. Table 5). Nevertheless, a sensory input could not be excluded and was investigated in the further course of the study. Looking at the 3D diagram in Figure 3, there is a visible linear correlation between the original gravity and the fermentation temperature on the diacetyl concentration.

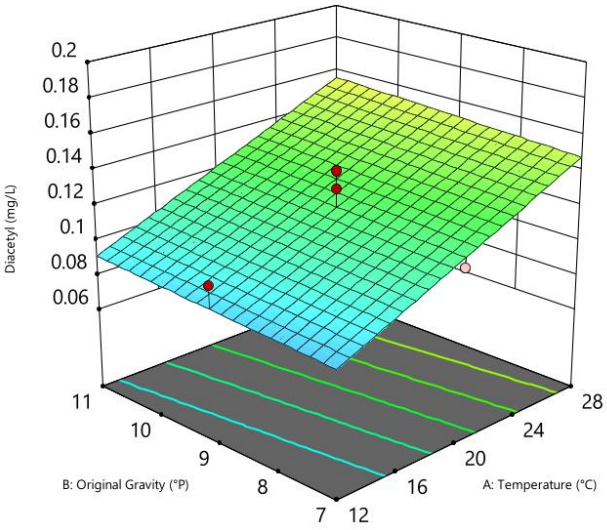


Figure 3. Three-dimensional (3D) diagram of the linear correlation of original gravity (°P), temperature (°C), and diacetyl concentration (mg/L) of the beers at a pitching rate of 15 × 10^6 cells/mL.

The lower the selected pitching rate, the lower the measurable diacetyl concentration in the beers (cf. Table 5). While the influence of the original gravity was negligible, the fermentation temperature had a decisive influence. The 3D diagram clearly depicts that significantly less diacetyl was formed at a fermentation temperature of 12 °C than at 28 °C. The reason for this is that higher fermentation temperatures lead to stronger yeast growth,

which, in turn, results in initially higher diacetyl production rates. Although diacetyl would be degraded more rapidly again at higher temperatures in the course of maturation, the short fermentation and maturation time of only seven days was not sufficient for this [45]. Moreover, the pH value was not optimal for the fastest possible diacetyl degradation, as the beers had relatively high pH values (cf. Table 4). According to the literature, the optimum pH value for acetoin dehydrogenase is 3.5 and this decreases steadily with increasing pH values [46]. Thus, the diacetyl concentration increases linearly with increasing temperature and pitching rate.

HS-GC-O, -FID, and -MS were performed on the beers listed in Table 1 to determine which flavor compound beyond standard analysis (cf. Table 2) was causing the red berry- and apple-like flavors in the beers. (*E*)-*β*-damascenone was clearly perceptible as red fruits and apple in olfactometry and was perceived as very pleasant. Using this method, it was possible to identify the odor-active compound qualitatively, although not quantitatively. Therefore, in a further step, (*E*)-*β*-damascenone was quantitated by SIDA using a GC × GC-MS system. The results are depicted in Table 6. As there was no significant difference between the six center point samples based on the one-sample *t*-test, only Run 3 was analyzed as the center point. This was the reason why Runs 7, 8, 10, 19, and 20 were excluded for ANOVA in the context of the RSM.

Table 6. Concentrations of (*E*)-*β*-damascenone (µg/L) in the beer samples fermented according to response surface methodology (RSM) design.

Run	Conc. (*E*)-*β*-Damascenone [1] (µg/L)	Range (*E*)-*β*-Damascenone (µg/L)
1	0.873	0.787–0.941
2	1.01	0.967–1.06
3 *	1.43	1.37–1.48
4	1.13	1.09–1.18
5	1.27	1.21–1.34
6	1.54	1.50–1.59
9	0.978	0.943–1.04
11	1.46	1.35–1.52
12	0.999	0.934–1.03
13	1.07	0.971–1.13
14	1.25	1.17–1.34
15	1.57	1.53–1.61
16	1.29	1.25–1.33
17	1.15	1.07–1.22
18	1.05	1.01–1.08

* Center point; [1] Mean of triplicates; coefficients of variation ≤9%.

A general trend is visible that the higher the pitching rate, the higher the (*E*)-*β*-damascenone concentrations. The fermentation temperature played more of a subordinate role, with lower temperatures tending to result in somewhat higher concentrations than higher temperatures. The original gravity, on the other hand, had a significant influence on the (*E*)-*β*-damascenone concentration, as the higher it was, the higher the measured concentrations. These conclusions can only be drawn when Run 13 is considered an outlier. Although the general variation is small, as can be seen Table 6, the *p*-value (<0.0058) for (*E*)-*β*-damascenone (cf. Table 3) showed that the values were significant. As the linear model showed a significant LOF, no 3D plot was created. Instead, a Pearson's correlation was created to determine the linear correlation between two variables, which is depicted in Figure 4.

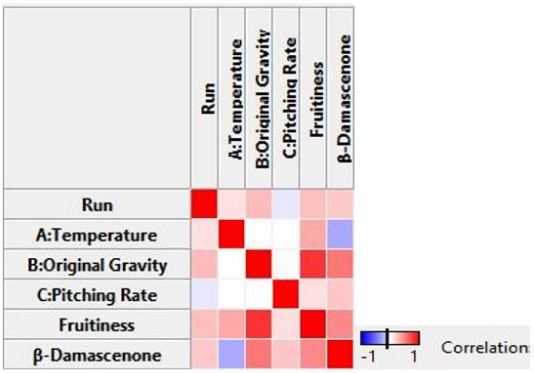


Figure 4. Visualization of Pearson's correlation of selected responses (fruitiness and (*E*)-*β*-damascenone) and factors A, Temperature; B, Original Gravity; and C, Pitching Rate of the response surface methodology (RSM) for the determination of linear correlations between two variables where 1 stands for a strong positive correlation, 0 for no correlation, and −1 for a strong negative correlation.

Pearson's correlation confirmed that the pitching rate was positively correlating with the (*E*)-*β*-damascenone concentration and that there was an even stronger positive correlation between the original gravity and the (*E*)-*β*-damascenone concentration. The fruitiness of the beers also showed a decisive positive correlation with the (*E*)-*β*-damascenone concentration. The raw data can be found in the supplementary material Figure S2.

Based on existing literature sources, it would have been assumed that (*E*)-*β*-damascenone originated from precursors in the malt and was formed during and after the wort boiling process [47]. Therefore, as described in Section 2.1, three different thermally treated worts were analyzed for (*E*)-*β*-damascenone. In the cooked wort (100 °C for 5 min), the (*E*)-*β*-damascenone concentration stayed below the limit of quantitation. The sterilized wort (100 °C for 45 min) exhibited a (*E*)-*β*-damascenone concentration of 0.634 µg/L on average so that the thermal influence is visible. In a direct comparison to the sterilized wort, a mean value of 1.01 µg/L was measured in the five beers from the RSM with an original gravity of 7 °P. Accordingly, besides the thermal influence on the (*E*)-*β*-damascenone formation in the wort, it could be assumed that the yeast strain TUM 247 formed about 0.4 µg/L (*E*)-*β*-damascenone during fermentation. The thermal influence of the (*E*)-*β*-damascenone formation becomes even clearer by considering the autoclaved wort (121 °C for 10 min). A comparatively high concentration was determined at an average of 2.15 µg/L. To determine if (*E*)-*β*-damascenone could actually be formed by the yeast strain during fermentation, an additional triplicate fermentation experiment was performed based on the cooked wort with the lowest thermal impact. Both the initial wort and the experimental beers were analyzed by SIDA using a GC × GC-MS system. While ≤0.1 µg/L (*E*)-*β*-damascenone was detected in the wort, a concentration between 0.451 and 0.571 µg/L was measured in the three beers. It is, therefore, reasonable to assume that the yeast strain TUM 247 was able to form (*E*)-*β*-damascenone during fermentation.

3.2. Sensory Evaluation Results

As described in Section 2.5, due to the large number of samples, a composite sample was prepared from the six center points, each in equal proportions. This was statistically possible as, using a one-sample *t*-test at $\alpha = 0.05$, there were non-significant differences in the center point samples. Table 7 shows the sum of the DLG ratings as well as the fruitiness in percent (n = 12) of the 15 beers fermented with different parameters. As the LOF of the DLG ratings and fruitiness was insignificant within the scope of the RSM (cf. Table 3), no 3D diagrams were created.

Table 7. Sensory evaluation results as sum DLG and fruitiness (%) of 15 beer samples with six center points summarized as composite sample (CS).

Run	Sum DLG	Fruitiness (%)
1	4.25	53
2	4.60	68
CS *	4.70	68
4	4.23	50
5	4.60	65
6	4.50	68
9	4.32	57
11	4.63	62
12	3.93	38
13	4.65	77
14	4.67	75
15	4.70	68
16	4.48	62
17	4.60	73
18	4.40	63

* CS (Composite sample).

Regarding the sum of DLG points, at a pitching rate of 15×10^6 cells/mL, the fermentation temperature had more of a subordinate influence, while the sum of DLG points increased with increasing original gravity up to 11 °P and was decidedly good with around 4.7 out of a total of 5.0 possible points. By reducing the pitching rate, the DLG sums decreased, while with an increasing pitching rate the DLG sums continued to rise, especially in the lower original gravity ranges between 7 and 9 °P. As off-flavors in beer lead to a devaluation in the DLG evaluation, it can be assumed, based on the results, that diacetyl had no negative influence. Diacetyl concentrations above the flavor threshold concentration were measured, especially at high fermentation temperatures and high pitching rates (cf. Table 5 and Figure 3). Nevertheless, the beers with high pitching rates, in particular, received the best DLG ratings, while the fermentation temperature had hardly any influence. Thus, neither diacetyl could be perceived as a buttery off-flavor, nor was found to be pleasant in the beers, without exception. Isovaleric acid also did not seem to affect the beer flavor negatively, as the beers with higher isovaleric acid concentrations were rated very good in the DLG testing. In terms of the fruitiness of the beers, the selected fermentation temperatures between 12 and 28 °C had little influence. Only a slight drop in fruitiness could be observed at lower temperatures. As with the sum of DLG points, however, the original gravity played a decisive role, and the fruitiness increased linearly from 7 °P to 11 °P. A change in the pitching rate did not cause any significant alteration in fruitiness.

Considering the results of the descriptive tasting with predetermined flavor attributes (raw data can be found in the supplementary material Table S3), the correlations with the 15 beers can be seen in Figure 5 as a Principal Component Analysis (PCA) plot. The odor activity values (OAV) of (*E*)-*β*-damascenone, isoamyl acetate, ethyl hexanoate, 2-phenylethyl acetate, and acetaldehyde were included in the statistical analysis to determine whether there was a direct correlation between the flavor attributes identified during the sensory evaluation and the five analyzed secondary metabolites. As mentioned in the introduction, (*E*)-*β*-damascenone, isoamyl acetate, ethyl hexanoate, 2-phenylethyl acetate, and acetaldehyde are possibly known to be responsible for apple-like flavors. Although ethyl octanoate and ethyl decanoate can also refer to apple-like flavors, their OAVs < 1 are unlikely to cause a noticeable effect on the beer flavor in comparison to the other five flavor compounds. The OAVs are depicted in Table 8. They are calculated as the ratio of the concentration to the orthonasal odor threshold concentration in water.

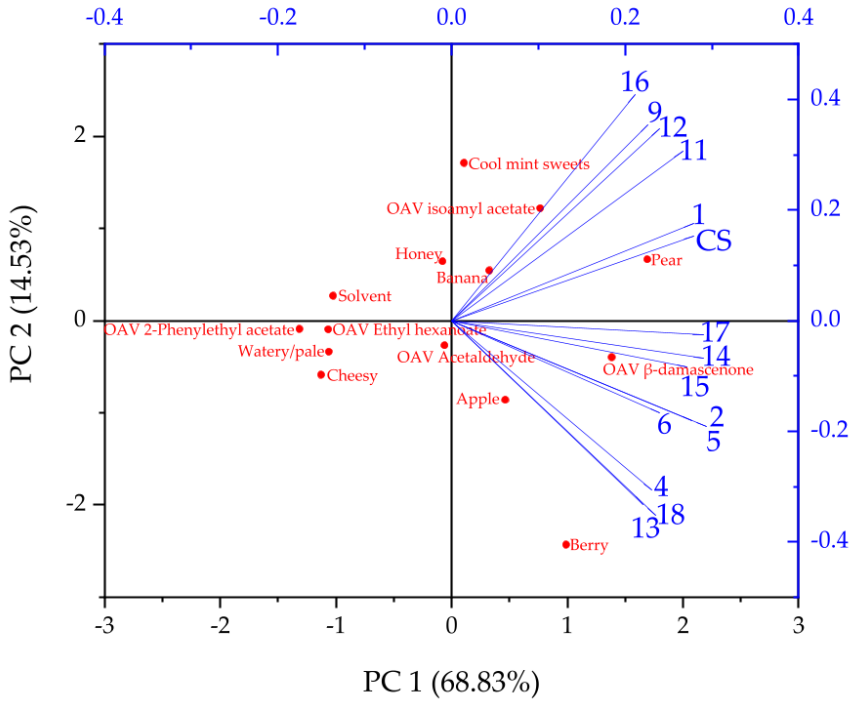


Figure 5. Principal Component Analysis (PCA) biplot with two principal components PC 1 and PC 2 showing the correlation of the 15 beers produced with the yeast strain C. saturnus TUM 247 as loading plot and the flavor characteristics described by the sensory assessors as well as odor activity values (OAV) of (*E*)-*β*-damascenone, isoamyl acetate, ethyl hexanoate, 2-phenylethyl acetate, and acetaldehyde.

Principal Components (PC) 1 and 2 cover 83.36% of the variation in the data, which represents a high share. The flavor attributes cheesy, solvent, and watery/pale have no correlation with the beers, so they are not significant. Since isovaleric acid contributes to a cheesy flavor it can be assumed that this substance did not affect the flavor of the beers. Similarly, the OAVs of 2-phenylethyl acetate as well as ethyl hexanoate showed no direct correlation with the evaluated flavor attributes of the beers. A majority of the beers had a slight banana flavor, while pear was clearly perceived in many beers and was strongest in the center point composite sample (CS) and in sample Run 1. A cool mint sweet flavor was detected primarily in beers from Runs 9, 11, 12, and 16 where a direct correlation with the OAV of isoamyl acetate could be observed. In addition, a direct correlation between the OAV of isoamyl acetate and the flavor attributes banana and pear perceived by the tasters was shown. A honey flavor was also recognized in these samples, however, only to a weak degree. It could not be assigned to any secondary metabolite investigated in the PCA. (*E*)-*β*-damascenone, 2-phenylethyl acetate, and isobutyl acetate could be responsible for the slightly honey-like character based on the results of the investigations [41,42,48–51]. Even though the three substances could not be clearly identified as honey-like by olfactometry and showed no correlation in the PCA, an additive or synergistic effect might be possible [52,53]. Furthermore, phenylacetaldehyde is known for its honey-like flavor, however, it was not analyzed in this study [54].

Table 8. Odor activity values (OAV) of the flavor compounds (*E*)-*β*-damascenone, isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, 2-phenylethyl acetate, and acetaldehyde.

Run	OAV [1]						
	(*E*)-*β*-Damascenone	**Isoamyl Acetate**	**Ethyl Hexanoate**	**Ethyl Ocanoate**	**Ethyl Decanoate**	**2-Phenylethyl Acetate**	**Acetaldehyde**
1	150	190	17	<1	<1	2.0	88
2	170	110	17	<1	<1	1.0	110
CS *	240	150	17	<1	<1	1.8	97
4	190	110	25	<1	<1	1.4	100
5	210	97	25	<1	<1	1.1	75
6	260	140	17	<1	<1	1.7	75
9	160	260	25	<1	<1	1.9	110
11	240	180	17	<1	<1	1.9	110
12	170	180	25	1.1	<1	1.6	81
13	180	110	8.3	<1	<1	1.1	130
14	210	170	25	<1	<1	1.4	75
15	260	97	25	<1	<1	1.2	88
16	220	380	17	1.1	<1	3.1	94
17	190	180	17	1.1	<1	1.2	110
18	180	110	17	<1	<1	1.3	110

* CS (Composite sample). [1] The OAVs are calculated as a ratio of the concentration to the orthonasal odor threshold concentration in water. The orthonasal odor threshold concentrations in water are for (*E*)-*β*-damascenone 0.006 µg/kg, isoamyl acetate 7.2 µg/kg, ethyl hexanoate 1.2 µg/kg, ethyl octanoate 8.7 µg/kg, 2-phenylethyl acetate 360 µg/kg, and acetaldehyde 16 µg/kg [16].

According to the PCA, there was a direct correlation between the apple and berry flavors, which were perceived primarily in the beers from Runs 4, 6, 13, and 18, occurring in conjunction with the berry flavor being much stronger than the apple flavor. Beers from Runs 2, 5, 14, 15, and 17 were also dominated by a light apple and stronger berry flavor. A direct correlation is visible between apple and berry flavor with the OAV of (*E*)-*β*-damascenone. The results from GC-O associated with PCA suggest that the berry and apple flavors can indeed be attributed to the metabolite (*E*)-*β*-damascenone. The influence of acetaldehyde was negligible although the OAVs from Table 8 might have suggested an effect. In this case, it must be considered that the orthonasal odor threshold concentrations are related to individual flavor compounds in water, while the tasters perceived the complex flavor interplay in the beers. It has already been discussed that the acetaldehyde concentrations were far below the flavor threshold concentration in beer, and there is also the possibility that it was masked by other flavor substances.

3.3. Optimization Using RSM

Based on the analyses of ethanol, secondary metabolites, and sensory evaluation, the aim was to produce a beer with optimized properties. Since the focus was on the production of a non-alcoholic beer, the optimized beer should have the lowest possible ethanol content. At the same time, the DLG sum and the fruitiness of the beer should be maximized. Since isoamyl acetate with the flavor attributes of banana and pear is decisive for a fruity flavor, the aim was to maximize the concentration of this ester as well as the sum of esters. As (*E*)-*β*-damascenone had a direct correlation to the flavor characteristics apple and berry, its concentration should also be maximized as far as possible. In contrast, the aim was to minimize isovaleric acid and diacetyl concentrations to avoid undesirable off-flavors. To meet these specifications with a desirability of 66.5%, the optimization would reach the predicted interval shown in Table 9 at a fermentation temperature of 16.1 °C, an original gravity of 10.5 °P and a pitching rate of 5×10^6 cells/mL.

Table 9. Validation of response surface methodology (RSM) model of selected responses with a two-sided predicted interval (PI) of 95%. Values based on triplicates.

Response	Unit	Predicted Mean	Observed Mean	Standard Deviation	95% PI Low	95% PI High
∑ Esters	mg/L	3.2	6.1	0.18	2.73	3.57
Isoamyl acetate *	mg/L	1.2	3.8	0.22	0.88	1.59
Isovaleric acid	mg/L	0.73	0.53	0.08	0.56	0.90
Diacetyl	mg/L	0.09	0.08	0.02	0.06	0.12
(*E*)-*β*-damascenone *	µg/L	1.30	1.41	0.14	1.07	1.54
Ethanol *	% (*v*/*v*)	0.19	0.19	0.04	0.13	0.26
Fruitiness *	%	71	87	3.67	64	76
Sum DLG *	points	4.62	4.61	0.08	4.45	4.78

* Significant models with significant LOF (lack of fit).

Although asterisk labelled responses showed a significant LOF, in case of (*E*)-*β*-damascenone, ethanol, and sum DLG, the observed mean was within the predicted interval (PI). Only the isoamyl acetate concentration was almost three times higher than the predicted mean. However, since the aim of the optimization was to maximize the isoamyl acetate concentration, this was achieved by means of the selected fermentation parameters. The same applies to the fruitiness. Although the sum of esters showed an insignificant LOF, the analyzed value in the application case was almost twice as high as the predicted mean, so that the objective was also exceeded here. Nevertheless, the desired maximization was reached. The PI was not met for isovaleric acid. Still, since the specified target was to minimize the concentration, this target was exceeded while diacetyl stayed within the PI. Consequently, the optimized fermentation parameters, a moderate fermentation temperature, a low pitching rate, and—related to non-alcoholic beers—relatively high original gravity, delivered the desired results and even exceeded them in a positive way. Based on the desirability of 66.5%, it was expected that the predicted means would not be fully met within a 95% confidence interval. In fact, 50% of the values were within the predicted confidence interval, with the other values positively exceeding the prediction. In the supplementary material Table S4 and Figure S3, the tasting results of the predetermined flavor attributes of the optimized beer (mean values of triplicates) can be found including a spiderweb model showing the flavor profile. Compared to the beers Runs 1–20, in the optimized beer, the banana flavor in particular was much more noticeable and supported the strong fruitiness. This finding from the tasting is consistent with the high concentration of isoamyl acetate.

4. Discussion

As this study focused on (*E*)-*β*-damascenone, the associated findings and their implications are discussed below in the broadest context possible. The investigations of this study revealed that the flavor substance (*E*)-*β*-damascenone correlated with the apple and red berry flavor and, thus, contributed significantly to these flavor characteristics. Although (*E*)-*β*-damascenone is a well-known secondary metabolite in beer, and this substance is associated with apple or cooked apple- and berry-like flavors, it has not yet been reported as a main flavor component in non-alcoholic beers that contributes to berry flavors. A useful process proved to be first qualitatively analyzing which flavor component was causing the apple- and berry-like character in the beers using GC-O and subsequently quantitating the identified flavor substance (*E*)-*β*-damascenone by SIDA using a GC × GC-MS system. Although (*E*)-*β*-damascenone is predominantly associated with the apple or cooked apple flavor impression in the literature, further sources describe the flavor substance as fruity, peach-, rose-, and honey-like [12,13,15,47,48]. In general, (*E*)-*β*-damascenone is found naturally in various fruits [12,55]. The applied PCA clearly showed that the apple-like flavor detected during the sensory evaluations in this study could be associated with the presence of (*E*)-*β*-damascenone. Although it was already described in the introduction

that numerous volatile substances are known to be responsible for apple flavor, no other correlations except for (*E*)-*β*-damascenone could be detected.

The fact that (*E*)-*β*-damascenone was perceived even more distinctively as a red berry flavor by the sensory assessors was also in agreement with existing literature. Gijs et al. described the flavor compound as red fruits, strawberry, and rhubarb [21]. Moreover, Saison et al. designated the flavor of the substance as red fruits, with attributes such as coconut and tobacco mentioned as well. The fact that less pleasant flavor impressions are partly associated with (*E*)-*β*-damascenone could be due to the fact that this secondary metabolite is often related to the aging of beers and is, therefore, used as an aging indicator [12,19,20]. Nevertheless, this study illustrates that the compound can be perceived as very pleasant. In addition, studies confirm that although (*E*)-*β*-damascenone may be an aging indicator, due to this, it does not necessarily have a direct sensory impact on the deterioration of the beer during the aging process [12,55]. Moreover, the concentration of (*E*)-*β*-damascenone is, thereby, decisive for the perceived character [56].

Due to its low orthonasal odor threshold concentration in water (0.006 µg/kg), only small amounts are required to perceive an impact by (*E*)-*β*-damascenone on the overall flavor, which was already demonstrated in different types of beers [16,41,57]. A study by Chevance et al. [58] explained that in a series of fresh Belgian beers, only low levels of (*E*)-*β*-damascenone of 6–25 µg/kg were found, which increased up to 210 µg/kg during aging. High concentrations of 450 µg/kg also occurred in investigations of the wort; however, these were reduced in the course of fermentation. The same phenomenon occurred not only in brewer's wort, but could also be detected in a papaya juice substrate. Interestingly, different *C. saturnus* yeast strains reduced (*E*)-*β*-damascenone from papaya juice to trace levels during fermentation [13]. During this study, the opposite occurred. While ≤0.1 µg/L (*E*)-*β*-damascenone could be detected in the cooked brewer's wort (100 °C for 5 min), the concentration range in the beers was between 0.451 and 0.571 µg/L. Thus, the concentration of (*E*)-*β*-damascenone did not decrease during the fermentation process, but increased instead. The fact that (*E*)-*β*-damascenone can also be formed during fermentation was also described in a wine study by Lloyd et al. [59], in the context of which six different grape musts were examined for this secondary metabolite. Without exception, the concentration in each sample increased over the course of fermentation, demonstrating that (*E*)-*β*-damascenone occurred via a sequential two-step process that required not only acidic conditions, but also yeast activity. Although the formation of (*E*)-*β*-damascenone by acid hydrolysis cannot be excluded in the context of this study, it is possible that the yeast metabolism of *C. saturnus* could also be responsible for its formation. However, the exact pathway of (*E*)-*β*-damascenone formation during fermentation cannot be explained so further research would need to be conducted in the future. In the literature, different pathways are described regarding the formation of the secondary metabolites [15]. It was reported that (*E*)-*β*-damascenone can be formed by oxidative cleavage from the carotenoid neoxanthin or from acid hydrolysis of plant secondary metabolites/glycosides during beer aging [48,55,58,60]. During wort boiling, it was observed that (*E*)-*β*-damascenone was mainly formed by the acid hydrolysis of glycoside precursors [21], though studies by Chevance et al. showed that the acid-catalyzed hydrolysis of glycosides in beer hardly occurred at a pH above 4.2 [58]. Nevertheless, it is likely that, in our study, the increasing (*E*)-*β*-damascenone formation in the brewer's wort with increasing thermal input is due to acid hydrolysis. In terms of the (*E*)-*β*-damascenone formation during fermentation in our study, it is questionable whether acid-catalyzed hydrolysis of glycosides occurred. Since the pH values of the non-alcoholic beers were above 4.2 without exception (the lowest value was 4.74, cf. Table 4), this pathway is less likely based on the literature. Nevertheless, an oxidative reaction that may occur during beer aging is also questionable due to the freshness of the beers. Therefore, it can be assumed that yeast activity may have played a role in the formation of (*E*)-*β*-damascenone.

5. Conclusions

The main objective of this study was achieved, namely, to find out which volatile components the red berry as well as the apple flavor of the non-alcoholic beers produced with the yeast strains *C. saturnus* TUM 247 can be attributed to and which volatile substances were responsible for the overall fruity flavor. (*E*)-*β*-damascenone turned out to be a main flavor component of the red berry and apple flavor. The general fruitiness, besides (*E*)-*β*-damascenone, was mainly determined by isoamyl acetate, which was clearly assigned to the flavor characteristics pear, banana, and cool mint sweets. The influence of different fermentation parameters could be investigated and successfully optimized within the framework of response surface methodology by setting a maximum fruitiness without perceptible off-flavors during sensory evaluations. In addition, the aim of achieving the lowest possible ethanol content was reached. It should be emphasized with regard to yeast cell metabolism that *C. saturnus* TUM 247 can produce the highest ester quantity at a low pitching rate of 5×10^6 cells/mL, almost regardless of the original gravity and at a moderate fermentation temperature. If the pitching rate increased, the sum of esters in the beers decreased, which is in contrast to the behavior often observed for traditional brewer's yeasts.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods11071038/s1, Sequence S1: *Cyberlindnera saturnus* TUM 247 26S sequence data from sequencing with NL1 primer, Table S1: Static headspace (HS) conditions, Table S2: One-sample *t*-test results of selected analyzed values of the six center point beers (Runs 3, 7, 8, 10, 19 and 20) determined for response surface methodology, Figure S1: Additional graphical representation of secondary metabolites analyzed by standard methods according to MEBAK in the 20 beer samples fermented according to RSM design, Figure S2: Pearson's correlation of selected responses (fruitiness and (*E*)-*β*-damascenone) and factors A: Temperature, B: Original Gravity and C: Pitching Rate of the RSM for the determination of linear correlations between two variables where 1 stands for a strong positive correlation, 0 for no correlation and −1 for a strong negative correlation, Table S3: Predetermined flavor attributes for the tasting of the 15 beer samples. Numbers indicate how many sensory assessors recognized the specific flavor in the corresponding beer (n = 12), Table S4 + Figure S3 (Spiderweb): Predetermined flavor attributes for the tasting of the optimized beer sample (mean values of triplicates). Numbers indicate how many sensory assessors recognized the specific flavor in the beer (n = 12).

Author Contributions: Conceptualization, Y.M.; methodology M.Z., M.H., S.F., P.D. and Y.M.; validation, M.Z., M.H. and S.F.; formal analysis, Y.M.; investigation, Y.M., P.D. and R.M.; resources—yeast strain TUM 247, M.L., M.H., M.Z. and D.L.; data curation, Y.M., P.D., R.M. and S.F.; writing—original draft preparation, Y.M., P.D. and S.F.; writing—review and editing, M.Z., M.H., M.S., S.F., P.D., D.L., M.L. and Y.M.; visualization, Y.M.; supervision, F.J. and S.F.; project administration, F.J. and M.S.; funding acquisition, F.J. and M.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the BMWK (German Ministry of Economic Affairs and Climate Action), Forschungsvereinigung Wifoe (Association for the Promotion of Science of the German Brewing Industry, Berlin). The IGF Project (grant number AiF 20658 N) of the Wifoe is supported via AiF within the program for promoting the Industrial Collective Research (IGF) of the BMWK, based on a decision by the German Bundestag.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Ahuja, K.; Rawat, A. Market Size of Non-Alcoholic Beer Worldwide from 2016 to 2024 (in Billion U.S. Dollars). Available online: https://www.gminsights.com/industry-analysis/non-alcoholic-beer-market (accessed on 17 June 2021).
2. Bellut, K.; Arendt, E.K. Chance and Challenge: Non-*Saccharomyces* Yeasts in Nonalcoholic and Low Alcohol Beer Brewing—A Review. *J. Am. Soc. Brew. Chem.* **2019**, *77*, 77–91. [CrossRef]
3. Gibson, B.; Krogerus, K.; Ekberg, J.; Mikkelson, A.; Pentikäinen, S.; Wilpola, A.; Vidgren, V. Non-conventional Yeast as a New Tool for Beer Flavour Modification. In Proceedings of the EBC Congress, Porto, Portugal, 24–28 May 2015.
4. de Francesco, G.; Turchetti, B.; Sileoni, V.; Marconi, O.; Perretti, G. Screening of new strains of *Saccharomycodes ludwigii* and *Zygosaccharomyces rouxii* to produce low-alcohol beer. *J. Inst. Brew.* **2015**, *121*, 113–121. [CrossRef]
5. Liu, S.-Q.; Quek, A.Y.H. Evaluation of Beer Fermentation with a Novel Yeast *Williopsis saturnus*. *Food Technol. Biotechnol.* **2016**, *54*, 403–412. [CrossRef] [PubMed]
6. Michel, M.; Kopecká, J.; Meier-Dörnberg, T.; Zarnkow, M.; Jacob, F.; Hutzler, M. Screening for new brewing yeasts in the non-*Saccharomyces* sector with *Torulaspora delbrueckii* as model. *Yeast* **2016**, *33*, 129–144. [CrossRef] [PubMed]
7. Varela, C. The impact of non-*Saccharomyces* yeasts in the production of alcoholic beverages. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 9861–9874. [CrossRef]
8. Basso, R.F.; Alcarde, A.R.; Portugal, C.B. Could non-*Saccharomyces* yeasts contribute on innovative brewing fermentations? *Food Res. Int.* **2016**, *86*, 112–120. [CrossRef]
9. Methner, Y.; Hutzler, M.; Zarnkow, M.; Prowald, A.; Endres, F.; Jacob, F. Investigation of Non-*Saccharomyces* Yeast Strains for Their Suitability for the Production of Non-Alcoholic Beers with Novel Flavor Profiles. *J. Am. Soc. Brew. Chem.* 2022; *Ahead-of-print*. [CrossRef]
10. Bellut, K.; Michel, M.; Zarnkow, M.; Hutzler, M.; Jacob, F.; Atzler, J.J.; Hoehnel, A.; Lynch, K.M.; Arendt, E.K. Screening and Application of *Cyberlindnera* Yeasts to Produce a Fruity, Non-Alcoholic Beer. *Fermentation* **2019**, *5*, 103. [CrossRef]
11. Kurtzman, C.P. (Ed.) *The Yeasts: A Taxonomic Study*, 5th ed.; Elsevier: Amsterdam, The Netherlands, 2011; ISBN 9780123847072.
12. Guido, L.F.; Carneiro, J.R.; Santos, J.R.; Almeida, P.J.; Rodrigues, J.A.; Barros, A.A. Simultaneous determination of E-2-nonenal and β-damascenone in beer by reversed-phase liquid chromatography with UV detection. *J. Chrom. A* **2004**, *1032*, 17–22. [CrossRef]
13. Lee, P.-R.; Ong, Y.-L.; Yu, B.; Curran, P.; Liu, S.-Q. Evolution of volatile compounds in papaya wine fermented with three *Williopsis saturnus* yeasts. *Int. J. Food Sci. Technol.* **2010**, *45*, 2032–2041. [CrossRef]
14. Schieberle, P. Primary odorants of pale lager beer. *Eur. Food Res. Technol.* **1991**, *193*, 558–565. [CrossRef]
15. Piornos, J.A.; Balagiannis, D.P.; Methven, L.; Koussissi, E.; Brouwer, E.; Parker, J.K. Elucidating the odor-active aroma compounds in alcohol-free beer and their contribution to the worty flavor. *J. Agric. Food Chem.* **2020**, *68*, 10088–10096. [CrossRef] [PubMed]
16. Kreissl, J.; Mall, V.; Steinhaus, P.; Steinhaus, M. *Leibniz-LSB@TUM Odorant Database Version 1*; Leibniz Institute for Food Systems Biology at the Technical University of Munich: Freising, Germany, 2022; Available online: https://www.leibniz-lsb.de/en/databases/leibniz-lsbtum-odorant-database (accessed on 24 January 2022).
17. Michel, M.; Meier-Dörnberg, T.; Jacob, F.; Schneiderbanger, H.; Haselbeck, K.; Zarnkow, M.; Hutzler, M. Optimization of Beer Fermentation with a Novel Brewing Strain *Torulaspora delbrueckii* Using Response Surface Methodology. *TQ* **2017**, *54*, 23–33. [CrossRef]
18. Methner, Y.; Hutzler, M.; Matoulková, D.; Jacob, F.; Michel, M. Screening for the Brewing Ability of Different Non-*Saccharomyces* Yeasts. *Fermentation* **2019**, *5*, 101. [CrossRef]
19. Vanderhaegen, B.; Neven, H.; Verachtert, H.; Derdelinckx, G. The chemistry of beer aging—A critical review. *Food Chem.* **2006**, *95*, 357–381. [CrossRef]
20. Saison, D.; de Schutter, D.P.; Uyttenhove, B.; Delvaux, F.; Delvaux, F.R. Contribution of staling compounds to the aged flavour of lager beer by studying their flavour thresholds. *Food Chem.* **2009**, *114*, 1206–1215. [CrossRef]
21. Gijs, L.; Chevance, F.; Jerkovic, V.; Collin, S. How low pH can intensify beta-damascenone and dimethyl trisulfide production through beer aging. *J. Agric. Food Chem.* **2002**, *50*, 5612–5616. [CrossRef]
22. Pires, E.J.; Teixeira, J.A.; Brányik, T.; Vicente, A.A. Yeast: The soul of beer's aroma—a review of flavour-active esters and higher alcohols produced by the brewing yeast. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 1937–1949. [CrossRef]
23. Verstrepen, K.J.; Derdelinckx, G.; Dufour, J.-P.; Winderickx, J.; Thevelein, J.M.; Pretorius, I.S.; Delvaux, F.R. Flavor-active esters: Adding fruitiness to beer. *J. Biosci. Bioeng.* **2003**, *96*, 110–118. [CrossRef]
24. Puerari, C.; Strejc, J.; Souza, A.C.; Karabín, M.; Schwan, R.F.; Brányik, T. Optimization of alcohol-free beer production by lager and cachaça yeast strains using response surface methodology. *J. Instig. Brew.* **2016**, *122*, 69–75. [CrossRef]
25. de Schutter, D.P.; Saison, D.; Delvaux, F.; Derdelinckx, G.; Rock, J.-M.; Neven, H.; Delvaux, F.R. Release and evaporation of volatiles during boiling of unhopped wort. *J. Agric. Food Chem.* **2008**, *56*, 5172–5180. [CrossRef]
26. Siefker, J.A.; Pollock, G.E. Melanoidins in the Brewing Processes. I. Formation of Aldehydes during Wort Boiling. *Proc. Annu. Meet.—Am. Soc. Brew. Chem.* **1956**, *14*, 5–12. Available online: https://www.tandfonline.com/doi/abs/10.1080/00960845.1956.12006472?journalCode=ujbc19 (accessed on 17 June 2021). [CrossRef]
27. Gernat, D.C.; Brouwer, E.; Ottens, M. Aldehydes as Wort Off-Flavours in Alcohol-Free Beers—Origin and Control. *Food Bioprocess Technol.* **2020**, *13*, 195–216. [CrossRef]
28. Kurtzman, C.P.; Robnett, C.J. Phylogenetic relationships among yeasts of the ? complex? determined from multigene sequence analyses. *FEMS Yeast Res.* **2003**, *3*, 417–432. [CrossRef]

29. van den Dool, H.; Kratz, P.D. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *J. Chromatogr.* **1963**, *11*, 463–471. [CrossRef]
30. Sen, A.; Laskawy, G.; Schieberle, P.; Grosch, W. Quantitative determination of beta-damascenone in foods using a stable isotope dilution assay. *J. Agric. Food Chem.* **1991**, *39*, 757–759. [CrossRef]
31. Engel, W.; Bahr, W.; Schieberle, P. Solvent assisted flavour evaporation—A new and versatile technique for the careful and direct isolation of aroma compounds from complex food matrices. *Eur. Food Res. Technol.* **1999**, *209*, 237–241. [CrossRef]
32. Bemelmans, J.M.H. Review of isolation and concentration techniques. *Prog. Flavour Res.* **1979**, *8*, 79–98.
33. Hildebrandt, G.; Schneider-Haeder, B. *Sensorische Analyse: Methodenüberblick und Einsatzbereiche—DLG-Sensorik*; DLG e.V.: Frankfurt am Main, Germany, 2016; Available online: www.dlg.org (accessed on 29 November 2021).
34. Narziss, L.; Back, W.; Gastl, M.; Zarnkow, M. *Abriss der Bierbrauerei*, 8th ed.; John Wiley & Sons Incorporated: Newark, NJ, USA, 2017; ISBN 9783527340361.
35. Leskošek, I.; Stojanović, M. A possible application of ale brewery strains of *Saccharomyces cerevisiae* in lager beer production. *World J. Microbiol. Biotechnol.* **1993**, *9*, 70–72. [CrossRef]
36. KRUGER, L. Yeast metabolism and its effect on flavour: Part. *Brew. Guard.* **1998**, *127*, 27–30.
37. Back, W.; Bohak, I. *Ausgewählte Kapitel der Brauereitechnologie*; 2. aktualisierte Aufl.; Carl: Nürnberg, Germany, 2008; ISBN 9783418008028.
38. Meilgaard, M.C. Flavor chemistry of beer. II. Flavor and threshold of 239 aroma volatiles. *Tech. Quart. Master. Brew. Assoc. Am.* **1975**, *12*, 151–168.
39. Sannino, C.; Mezzasoma, A.; Buzzini, P.; Turchetti, B. Non-conventional Yeasts for Producing Alternative Beers. In *Non-Conventional Yeasts: From Basic Research to Application*; Sibirny, A., Ed.; Springer International Publishing: Cham, Switzerland, 2019; pp. 361–388. ISBN 978-3-030-21109-7.
40. Collin, S.; Derdelinckx, G.; Dufour, J.-P. Relationships between the chemical composition and sensory evaluation of lager beers. *Food Qual. Prefer.* **1994**, *5*, 145–149. [CrossRef]
41. Fritsch, H.T.; Schieberle, P. Identification based on quantitative measurements and aroma recombination of the character impact odorants in a Bavarian Pilsner-type beer. *J. Agric. Food Chem.* **2005**, *53*, 7544–7551. [CrossRef] [PubMed]
42. Kobayashi, M.; Shimizu, H.; Shioya, S. Beer volatile compounds and their application to low-malt beer fermentation. *J. Biosci. Bioeng.* **2008**, *106*, 317–323. [CrossRef]
43. Olšovská, J.; Vrzal, T.; Štěrba, K.; Slabý, M.; Kubizniaková, P.; Čejka, P. The chemical profiling of fatty acids during the brewing process. *J. Sci. Food Agric.* **2019**, *99*, 1772–1779. [CrossRef] [PubMed]
44. Hawthorne, D.B.; Jones, R.D.; Barrbett, P.A.; Kavanagh, T.E.; Clarke, B.J. Methods for the analysis of C4 to C10 fatty acids in beer, wort and carbohydrate syrups. *J. Inst. Brew.* **1986**, *92*, 181–184. [CrossRef]
45. Krogerus, K.; Gibson, B.R. 125th Anniversary Review: Diacetyl and its control during brewery fermentation. *J. Inst. Brew.* **2013**, *119*, 86–97. [CrossRef]
46. Bamforth, C.W.; Kanauchi, M. Enzymology of Vicinal Diketone Reduction in Brewer's Yeast. *J. Inst. Brew.* **2004**, *110*, 83–93. [CrossRef]
47. Kishimoto, T.; Wanikawa, A.; Kagami, N.; Kawatsura, K. Analysis of hop-derived terpenoids in beer and evaluation of their behavior using the stir bar-sorptive extraction method with GC-MS. *J. Agric. Food Chem.* **2005**, *53*, 4701–4707. [CrossRef]
48. Belitz, H.-D.; Grosch, W.; Schieberle, P. *Food Chemistry*; Springer: Berlin/Heidelberg, Germany, 2009.
49. Humia, B.V.; Santos, K.S.; Barbosa, A.M.; Sawata, M.; Mendonça, M.d.C.; Padilha, F.F. Beer Molecules and Its Sensory and Biological Properties: A Review. *Molecules* **2019**, *24*, 1568. [CrossRef]
50. Xu, Y.; Wang, D.; Li, g.; Hao, J.; Jiang, W.; Liu, Z.; Qin, Q. Flavor Contribution of Esters in Lager Beers and an Analysis of Their Flavor Thresholds. *J. Am. Soc. Brew. Chem.* **2017**, *75*, 201–206. [CrossRef]
51. Rettberg, N.; Biendl, M.; Garbe, L.-A. Hop Aroma and Hoppy Beer Flavor: Chemical Backgrounds and Analytical Tools—A Review. *J. Am. Soc. Brew. Chem.* **2018**, *76*, 1–20. [CrossRef]
52. Engan, S. Organoleptic Threshold Values of Some Alcohols and Esters in Beer. *J. Inst. Brew.* **1972**, *78*, 33–36. [CrossRef]
53. Verstrepen, K.J.; Derdelinckx, G.; Delvaux, F.R. Esters in beer-part 1: The fermentation process: More than ethanol formation. *Cerevisia* **2003**, *28*, 41–49.
54. Schieberle, P.; Komarek, D. Changes in Key Aroma Compounds during Natural Beer Aging. In *Freshness and Shelf Life of Foods*; Cadwallader, K.R., Weenen, H., Eds.; American Chemical Society: Washington, DC, USA, 2003; pp. 70–79. ISBN 9780841238015.
55. Sefton, M.A.; Skouroumounis, G.K.; Elsey, G.M.; Taylor, D.K. Occurrence, sensory impact, formation, and fate of damascenone in grapes, wines, and other foods and beverages. *J. Agric. Food Chem.* **2011**, *59*, 9717–9746. [CrossRef] [PubMed]
56. Kotseridis, Y.; Baumes, R.; Skouroumounis, G.K. Synthesis of labelled [$^2H_4$] β-damascenone, [$^2H_2$] 2-methoxy-3-isobutylpyrazine, [$^2H_3$] α-ionone, and [$^2H_3$] β-ionone, for quantification in grapes, juices and wines. *J. Chrom. A* **1998**, *824*, 71–78. [CrossRef]
57. Langos, D.; Granvogl, M.; Schieberle, P. Characterization of the key aroma compounds in two bavarian wheat beers by means of the sensomics approach. *J. Agric. Food Chem.* **2013**, *61*, 11303–11311. [CrossRef]
58. Chevance, F.; Guyot-Declerck, C.; Dupont, J.; Collin, S. Investigation of the beta-damascenone level in fresh and aged commercial beers. *J. Agric. Food Chem.* **2002**, *50*, 3818–3821. [CrossRef]

59. Lloyd, N.D.R.; Capone, D.L.; Ugliano, M.; Taylor, D.K.; Skouroumounis, G.K.; Sefton, M.A.; Elsey, G.M. Formation of Damascenone under both commercial and model fermentation conditions. *J. Agric. Food Chem.* **2011**, *59*, 1338–1343. [CrossRef]
60. de Schutter, D.P.; Saison, D.; Delvaux, F.; Derdelinckx, G.; Rock, J.-M.; Neven, H.; Delvaux, F.R. Characterization of volatiles in unhopped wort. *J. Agric. Food Chem.* **2008**, *56*, 246–254. [CrossRef]

2.5 Publication 5: Investigations into metabolic properties and selected nutritional metabolic byproducts of different non-*Saccharomyces* yeast strains when producing nonalcoholic beer

NABs produced by using maltose-negative or weakly fermenting non-*Saccharomyces* yeasts are becoming increasingly important due to their novel, positive flavor profiles. While *Saccharomycodes ludwigii* is already known as a commercial yeast species for the production of NABs, the safety considerations for the other yeast strains that were identified as promising in publications 2 to 4 have not yet been conclusively clarified. Although the yeast species investigated in the publications 2 to 4 were known from previous studies and/or existing food fermentations, only yeast strains that hold either GRAS (Generally Recognized As Safe) status by the US Food and Drug Administration (FDA) or QPS (Qualified Presumption of Safety) status by the European Food Safety Authority (EFSA) should be used in food fermentations.

Biogenic amines, for example, are an important aspect of the safety assessment. Moderate concentrations of about 50 mg/kg food are considered safe, but negative health effects can occur if these amounts are exceeded. For this reason, nine selected biogenic amines from beers fermented in 300 mL unhopped 12 °P wort each at 20 °C and a pitching rate of 30×10^6 cells/mL were analyzed with the 15 yeast strains from publication 2. In addition, a positive control and two reference beers fermented with *S. ludwigii* SL17 and *Saccharomyces cerevisiae* TUM 68 were analyzed. With the exception of the positive control, the concentrations of biogenic amines remained below the limit of determination of 5.0 mg/L in all beer samples. Thus, a negative influence on health by the analyzed biogenic amines can be excluded when using the investigated yeast strains for beer production.

In order to gain further insights into the safe use of the 15 yeast strains mentioned, stress tolerance tests were carried out. At 37 °C, the physiological growth of the yeasts was investigated. *Cyberlindnera fabianii*, *Kluyveromyces marxianus*, *Lachancea kluyveri*, *Pichia kluyveri*, *Saccharomycopsis fibuligera* and *Schizosaccharomyces pombe* showed growth at 37 °C and were subsequently tested in vitro for human gastric tolerance. All yeast strains survived the in vitro stress test with viabilities above 90%.

However, NABs fermented with non-*Saccharomyces* yeasts not only pose safety concerns, but also offer potential for positive health effects. Beers are generally known to have relevant B vitamin concentrations. These were analyzed in this publication for the beers and yeasts S. lud SL17, C. sat CSa1, C. Sat 247, and K. mar 653. B vitamin concentrations of both propagated and sedimented yeasts were measured as well as in the beers that were fermented without pressure at 20 °C and a pitching rate of 15×10^6 cells/mL for 144 h in 1,800 mL of 7 °P unhopped wort. All beers contained nutritionally relevant amounts of B vitamins so they could serve as a supplement for a balanced diet. However, the B vitamins most likely derived from the raw materials, as there was no evidence that the investigated yeast strains could synthesize B vitamins via their own cellular metabolism.

Authors/Authorship contribution:

Methner, Y.: Conception and design of experiments, performance of experiments, analysis of data using OriginPro 2020 as statistical software, data curation, paper writing; **Weber, N.**: Performance, analysis of data, data curation and method writing of folate analysis, revision of manuscript; **Kunz, O.**: Performance, analysis of data and data curation of MALDI-TOF, revision of manuscript; **Zarnkow, M.**: Revision of manuscript; **Rychlik, M.**: Supervision of folate analysis, revision of folate analysis; **Hutzler, M.**: Organization and provision of a large amount of yeast strains, conception and design of experiments, revision of manuscript; **Jacob, F.**: Supervision of project, approval for submission, funding acquisition.

FEMS Yeast Research, 2022, **22**, 1–13
DOI: 10.1093/femsyr/foac042
Advance access publication date: 25 August 2022
Research Article

Investigations into metabolic properties and selected nutritional metabolic byproducts of different non-*Saccharomyces* yeast strains when producing nonalcoholic beer

Yvonne Methner[1], Nadine Weber[2], Oliver Kunz[1], Martin Zarnkow[1], Michael Rychlik[2,3], Mathias Hutzler[1,*], Fritz Jacob[1]

[1]Research Center Weihenstephan for Brewing and Food Quality, Technical University of Munich, Alte Akademie 3, 85354 Freising, Germany
[2]Chair of Analytical Food Chemistry, Technical University of Munich, Maximus-von-Imhof-Forum 2, 85354 Freising, Germany
[3]Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, 306 Carmody Road, St Lucia QLD 4072, Australia
*Corresponding author: Research Center Weihenstephan for Brewing and Food Quality, Technical University of Munich, Alte Akademie 3, 85354 Freising, Germany. Tel: +49 8161 71-3100; Fax: +49 8161 71-4181; E-mail: m.hutzler@tum.de
Editor: Isak Pretorius

Abstract

Nonalcoholic beers are becoming increasingly popular, in part due to consumers' awareness of a healthier lifestyle. Additionally, consumers are demanding diversification in the product range, which can be offered by producing nonalcoholic beers using non-*Saccharomyces* yeasts for fermentation to create a wide variety of flavors. So far, little is known about the nutritionally relevant byproducts that these yeasts release during wort fermentation and whether these yeasts can be considered safe for food fermentations. To gain insights into this, the B vitamins of four different nonalcoholic beers fermented with the yeast species *Saccharomycodes ludwigii*, *Cyberlindnera saturnus* (two strains), and *Kluyveromyces marxianus* were analyzed. Furthermore, a total of 16 beers fermented with different non-*Saccharomyces* yeast strains were analyzed for biogenic amines. Additionally, stress tolerance tests were performed at 37°C and in synthetic human gastric juice *in vitro*. B vitamins were found in the four nonalcoholic beers in nutritionally relevant amounts so they could serve as a supplement for a balanced diet. Biogenic amines remained below the limit of determination in all 16 beers, and thus likely had no influence, while the stress tolerance tests gave a first indication that seven yeast strains could possibly tolerate the human gastric juice milieu.

Keywords: non-*Saccharomyces* yeasts, fermentation, nonalcoholic beers, vitamin B, biogenic amines, stress tolerance tests

Introduction

Nonalcoholic beers are on trend. Market forecasts predict growth and studies also indicate that this product sector has grown in recent years (Bellut and Arendt 2019, Ahuja and Rawat 2020, Kokole et al. 2021). There are various reasons for this development, ranging from an increasingly healthier lifestyle to religious restrictions and consumer preferences (Salanță et al. 2020). One option for producing nonalcoholic beers is to use maltose- and maltotriose-negative non-*Saccharomyces* yeasts, which have only a low fermentative activity based on this property. Accordingly, these kind of yeast strains offer a simple way to produce nonalcoholic or low-alcohol beers. In a recently published study by Methner et al. (2022), 16 different non-*Saccharomyces* yeast strains were investigated for their suitability to produce nonalcoholic beers with novel flavor profiles. Promising flavor profiles emerged and their suitability for the production of nonalcoholic beers was confirmed. An increasing number of maltose-negative yeast strains have been investigated in recent years to produce nonalcoholic beers with pronounced flavor diversity. However, not much is known about the nutritional properties of these beers such as B vitamins or biogenic amines (BAs).

The B vitamin group is composed of eight different water-soluble vitamins. These eight B vitamins act as coenzymes in numerous catabolic and anabolic reactions and are indispensable for optimal physiological and neurological functions in human metabolism. Kennedy summarized the key mechanisms, Recommended Daily Allowance (RDA) and efficacy (Kennedy 2016). His research reveals that thiamine (B_1), riboflavin (B_2), niacin (B_3), pantothenic acid (B_5), pyridoxine (B_6), biotin (B_7), and folate (B_9) are mainly synthesized by plants, whereas cobalamin (B_{12}) is synthesized by bacteria. Moreover, yeasts may have the ability to synthesize B vitamins. For example, it is known from existing studies that brewer's spent yeast is rich in vitamins, especially B_3, B_6, and B_9 (Ferreira et al. 2010, Vieira et al. 2016). In this context, *Saccharomyces cerevisiae* was found to produce particularly high amounts of folate during yeast extract production (Jacob et al. 2019). Regular beers produced by using traditional *Saccharomyces* yeasts have already been analyzed for B vitamins, which may be present in sufficient amounts to meet daily requirements (Bamforth 2002). In a study by Mayer et al. (2001), moderate beer consumption was even found to have certain positive health effects due to an increase in folate and vitamin B_{12} intake. Nevertheless, it remains undisputed that ethanol can have a negative impact on health

Received: May 27, 2022. **Revised:** July 29, 2022. **Accepted:** August 22, 2022

(Marten et al. 2020). Accordingly, nonalcoholic beer could provide positive health benefits, given that B vitamins are present in significant concentrations. Since all eight B vitamins are essential for the human metabolism, they must be consumed through diet. Little is known to date about the vitamin B composition of nonalcoholic beers produced with maltose-negative yeast strains. As the health trend steadily increases (Habschied et al. 2020), the purpose of this study was to screen nonalcoholic beers for B vitamins. A total of four nonalcoholic beers were selected, which were produced with maltose-negative yeasts strains found in a previous study to form particularly fruity flavors during wort fermentation (Methner et al. 2022). These were the yeast strains *Cyberlindnera saturnus* C. sat 247 and C. sat CSa1 as well as *Kluyveromyces marxianus* K. mar 653. Additionally, a nonalcoholic beer produced with the well-known and commercially used yeast strain *Saccharomycodes ludwigii* S. lud SL17 was chosen, which was also analyzed.

BAs also play a decisive role from a health perspective. For example, they perform important tasks as messenger substances in the neural system. However, intolerance, and thus adverse health effects can occur if certain intake concentrations are exceeded (European Food Safety Authority 2011, Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit 2020). Moderate amounts of about 50 mg/kg food are considered safe (Loret et al. 2005). Since BAs can be formed during the fermentation process, there are numerous studies on their occurrence in beers, which were summarized in a review by Kalac and Krízek (2003). This states that it is mainly bacterial contamination that leads to BA formation. Nevertheless, Ternes (2007) found that the BAs putrescine, spermine, and spermidine occur naturally in beer, whereas histamine, tyramine, and cadaverine are formed by microbes. Few studies report that yeasts such as *S. cerevisiae* and also several non-*Saccharomyces* yeast strains are capable of synthesizing BAs in a species- or even strain-dependent manner, which is why this formation pathway should also be considered (Caruso et al. 2002, Torrea and Ancín 2002, Granchi et al. 2005, Beneduce et al. 2010, Wang et al. 2021). Similar concentrations of BAs were found in alcoholic as well as alcohol-free beers (Buiatti et al. 1995, Izquierdo-Pulido et al. 1996, Kalac and Krízek 2003). In foods, the highest importance is attributed to the BAs histamine, putrescine, cadaverine, tyramine, tryptamine, phenylethylamine, spermine, and spermidine (Shalaby 1996). In the aforementioned study by Methner et al. (2022), a total of 16 different non-*Saccharomyces* yeast strains turned out to be suitable for producing nonalcoholic beers by means of microbiological manufacturing processes. To date, nothing is known about the yeast species *Cyberlindnera fabianii*, *Cyberlindnera misumaiensis*, *C. saturnus*, *Kazachstania servazzii*, *Kluyveromyces lactis*, *K. marxianus*, *Lachancea kluyveri*, *Pichia kluyveri*, and *Saccharomycopsis fibuligera* with regard to the formation of BAs. For the yeast species *P. kluyveri*, this assumption has already been made in the literature (Landete et al. 2007, Vicente et al. 2021). Regarding the yeast species *Schizosaccharomyces pombe* and *S. ludwigii*, it is reported from wine fermentations that they can even cause a reduction in BAs (Benito et al. 2015, Ivit et al. 2018). Only for *Torulaspora delbrueckii* it was reported that in winemaking this yeast species might be able to slightly increase BA precursors (Benito 2018). Therefore, it was investigated in this study, whether the 16 specified yeast strains could release the most food-relevant BAs during fermentation.

Besides BAs, there are further factors that can lead to safety concerns in the context of non-*Saccharomyces* yeasts for the production of nonalcoholic beers. Thus, for reasons of precautionary consumer protection, only yeasts that have been granted GRAS (Generally Recognized As Safe) status by the US Food and Drug Administration (FDA) or QPS (Qualified Presumption of Safety) status by the European Food Safety Authority (EFSA) should be used (Steensels and Verstrepen 2014, U.S. Food and Drug Administration 2017). While QPS status mainly considers the safety of the microorganism regardless of application, GRAS status refers to specific food applications. In the USA, microorganisms hold GRAS status if they have been commonly used in food applications before 1958, and thus are known to have a history of safe use (U.S. Food and Drug Administration 2018). EFSA, on the other hand, grants QPS status based on risk assessments of biological agents conducted by the Panel on Biological Hazards (BIOHAZ; EFSA-BIOHAZ Panel 2007, 2021). A more detailed overview of the differences between QPS and GRAS is provided in a review by Laulund et al. (2017). Of the 16 yeast species investigated in this study, only three species besides the reference yeast *S. cerevisiae* have QPS status: *K. lactis*, *K. marxianus*, and *S. pombe* (Ricci et al. 2018). Unless yeasts can grow at 37°C, potential pathogenicity is largely excluded. However, if yeasts are viable at 37°C, this does not necessarily mean that the yeasts are pathogenic, as there may also be a potential probiotic benefit, as is known for both *S. cerevisiae* var. *boulardii* and *S. cerevisiae* (Gil-Rodriguez 2015, Lara-Hidalgo et al. 2017). However, before probiotic potential can be considered, it must be established that microorganisms can be used without safety concerns. Since few findings in this regard have been found in literature for the 16 yeast strains studied, they were tested to determine whether they could grow at 37°C. In addition, they were subjected to a stress tolerance test in synthetic gastric juice to gain initial insights into their physiological properties.

The aim of this study was, therefore, to examine nonalcoholic beers fermented with selected non-*Saccharomyces* yeast strains for selected BAs and B vitamins in order to make a statement about these selected nutritionally relevant metabolic yeast byproducts. Furthermore, the yeasts were investigated *in vitro* for certain physiologically relevant properties to give an initial indication that they can tolerate conditions in the human gastric system.

Materials and methods

Investigated yeast strains

Table 1 lists the yeast strains with the corresponding abbreviations that were investigated in this study. With the exception of the yeast strain C. sat CSa1, which is a proprietary strain of a specific brewery (provided for this study and stored at the Research Center Weihenstephan, TUM), all other yeast strains can be purchased commercially. The collection site from which the yeast strains were obtained is indicated in the footnote of Table 1.

Determination of vitamin B

A total of four selected non-*Saccharomyces* yeast strains from Table 1, namely C. sat 247, C. sat CSa1, K. mar 653, and S. lud SL17, were inoculated from wort slant agars under sterile conditions into 500 ml flasks each containing 250 ml of unhopped wort (7.0°P, pH 5.5) that was prepared from malt extract (Weyermann®, Bamberg, Germany). After 72 h propagation at 20°C on a WiseShake orbital shaker (Witeg Labortechnik GmbH, Wertheim, Germany) at 80 rpm, the yeast suspensions were transferred to sterile 2500 ml flasks containing 1800 ml of similar unhopped wort and propagated for an additional 72 h. After propagation, the cell counts were determined using the Cellometer® Vision (Nexcelom Bioscience LLC, Lawrence, MA, USA).

The four yeast strains were selected as they were able to form particularly positive flavor properties during fermentation

Table 1. Yeast species and strain numbers with corresponding abbreviations used in this study.

Yeast strain number	Yeast strain abbreviation	Yeast species
CBS 5640	C. fab 5640	*C. fabianii*[a]
CBS 5650	C. fab 5650	*C. fabianii*[a]
TUM 238	C. mis 238	*C. misumaiensis*[b]
YH837A-3D4	C. mis CM1	*C. misumaiensis*[b]
TUM 247	C. sat 247	*C. saturnus*[b]
CBS 4549	C. sat 4549	*C. saturnus*[a]
YHMH22AA-3H1	C. sat CSa1	*C. saturnus*[c]
YHMH47B-3C4	K. ser 3C4	*K. servazzii*[b]
TUM G9K	K. lac G9K	*K. lactis*[b]
TUM 653	K. mar 653	*K. marxianus*[b]
CBS 3082T	L. klu 3082	*L. kluyveri*[a]
YHAK1A-3I1	P. klu PK1	*P. kluyveri*[b]
TUM 68	S. cer 68	*S. cerevisiae*[b]
TUM SL17	S. lud SL17	*S. ludwigii*[b]
PI S 6; Lu27	S. fib Lu27	*S. fibuligera*[b]
TUM G10S	S. pom G10S	*S. pombe*[b]
YH824A-1I6	T. del 1I6	*T. delbrueckii*[b]

[a]Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; [b]Research Center Weihenstephan (BLQ), Freising, Germany; and [c]Private collection.

(Methner et al. 2022), and were then used to produce nonalcoholic beers. In small-scale fermentations performed in triplicate, 1800 ml unhopped wort (7.0°P, pH 5.5) in 2000 ml sterile Duran glass bottles (Schott AG, Mainz, Germany) were pitched each at 15×10^6 cells/ml ($\pm\ \sigma = 1 \times 10^6$ cells/ml) and closed with glass fermentation airlocks on top. To produce the wort, malt extract (Weyermann®) was blended with distilled water in a wort kettle to 7°P original gravity, boiled for 5 min and then cooled down to the pitching temperature of 20°C using a plate cooler. A volume of 50 l wort was pumped into a cylindroconical tank before 1800 ml was filled into each of the 2000 ml bottles for the fermentations. Propagation yeasts were centrifuged (Roto Super 40, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) in sterilized 500 ml PPCO centrifuge bottles (Nalgene, Thermo Fisher Scientific, Waltham, MA, USA) at 750 × *g* for 5 min and the supernatant was discarded before pitching. The samples were incubated at 20°C for fermentation. The fermentation time of 144 h was chosen based on previous experiments (Methner et al. 2022). Subsequently, the samples were then transferred to cold storage at 2°C for another 96 h before performing the analytical analyses described in Table 2.

From the results obtained by the analytical methods shown in Table 2, one-sample *t*-tests were performed using OriginPro 2020 as statistical software to evaluate whether the triplicates of each beer were insignificantly different from each other. If there was no significant difference in the one-sample *t*-test, it may be assumed that a composite sample from the respective triplicates would reveal a statistically significant result in the vitamin B analysis. Therefore, unless significant differences were found, composite samples from the triplicates were used for vitamin B analysis.

The four produced nonalcoholic beers and the corresponding wort were measured for B vitamins, whereas the propagated yeast and the sedimented yeast after fermentation were only quantified in case of the yeast strain S. lud SL17. SGS Institut Fresenius GmbH analyzed the 7°P wort, propagated and sedimented yeast of the yeast strain S. lud SL17 as well as its nonalcoholic beer for all B vitamins except folate using the methods listed in Table 3. Additionally, pantothenic acid, biotin, and cobalamin of the three nonalcoholic beers fermented with the yeast strains C. sat 247, C. sat CSa1, and K. mar 653 were determined by SGS Institut Fresenius GmbH according to the methods in Table 3. Since SGS Institut Fresenius GmbH, Freiburg, Germany is accredited according to DIN EN ISO/IEC 17025, it was scientifically permissible to perform single determinations of the selected B vitamins. Due to the accreditation, the coefficients of variation were calculated from the respective standardized methods and were used for the statistical evaluation.

Prior to the quantification of B vitamins for the propagated and the sedimented yeast, the samples were centrifuged for 10 min at 750 × *g* (centrifuge Z 366 K, HERMLE, Wehingen, Germany). The supernatant was discarded and the yeast was resuspended with 20 ml sterile physiological NaCl solution to eliminate the influence of wort or beer. The washing procedure was repeated three times before the analyses were performed.

Folate was analyzed in triplicate in the beer SL17, in the three experimental beers 247, CSa1, and 653, and in the propagated and sedimented yeast of the yeast strain S. lud SL17 as follows. The folate extraction of the samples was performed under dimmed light as described in Striegel et al. (2018). A total of 100–150 mg of the yeast and 500 mg of the liquid samples were used for extraction. For the quantification $^{13}C_5$-PteGlu, $^{13}C_5$-H_4folate, $^{13}C_5$-5-CH_3-H_4folate, $^{13}C_5$-5-CHO-H_4folate, and $^{13}C_5$-10-CHO-PteGlu were added in an equal amount of the expected concentration of the unlabeled analytes in the sample. For deconjugation, 900 ml chicken pancreas solution and 400 ml rat serum were added to the sample. The preparation of the solutions for deconjugation was performed according to Striegel et al. (2018). After overnight incubation at 37°C, the samples were boiled in a water bath at 100°C and 10 ml acetonitrile was added once the samples had cooled down. The samples were purified by solid phase extraction (SPE) before being measured by using LC-MS/MS. The LC-MS/MS analysis was carried out on a Shimadzu Nexera X2 UHPLC system (Shimadzu, Kyoto, Japan) with a Raptor ARC-18 column (2.7 µm, 100 × 2.1 mm, Restek, Bad Homburg, Germany) and a Raptor ARC-18 precolumn (2.7 µm, 5 × 2.1 mm, Restek) as a stationary phase. The mobile phase for the binary gradient consisted of (A) distilled water with 0.1% (v/v) formic acid and (B) acetonitrile with 0.1% (v/v) formic acid at a flow rate of 0.4 ml/min. The LC-system was coupled with a triple quadrupole mass spectrometer (LCMS-8050, Shimadzu) and operated in positive ESI mode for all analytes. The specified settings as well as method validation have been described previously (Striegel et al. 2018).

Table 2. Analytical methods of the wort and the nonalcoholic beers according to MEBAK[a].

Analysis	Method	Device
Original gravity, apparent attenuation, and ethanol content	MEBAK WBBM 2.9.6.3	Bending vibration and NIR spectroscopy, Alcolyzer Plus with DMA 5000 X sample 122 (Anton-Paar GmbH, Ostfildern, Germany)
pH	MEBAK WBBM 2.13	pH meter with pH electrode, ProfiLine pH3210 pH meter (Xylem Inc., New York, NY, USA)

[a]MEBAK® (2012), Editor: Dr F. Jacob: The MEBAK collection of brewing analysis methods: Wort, beer, and beer-based beverages. Collection of methods of the Mitteleuropäischen Brautechnischen Analysenkommission. Self-published by MEBAK.

Table 3. Analytical methods of vitamin B used by SGS Institut Fresenius GmbH, Freiburg, Germany, (accreditation according to DIN EN ISO/IEC 17025) for wort, beer, and propagated as well as sedimented yeast.

Analysis	Method—wort/beer	Method—yeast
B_1–Thiamine	DIN EN 14122, HPLC/Fl	DIN EN 14122, HPLC/Fl
B_2–Riboflavin	DIN EN 14152, HPLC/Fl	DIN EN 14152, HPLC/Fl
B_3–Niacin	AOAC 944.13, microbiological	AOAC 944.13, microbiological
B_5–Pantothenic acid	AOAC 945.74, microbiological	AOAC 945.74, microbiological
B_6–Pyridoxine	DIN EN 14663, HPLC/Fl	DIN EN 14663, HPLC/Fl
B_7–Biotin	SOP M 3532, LC-MS/MS	SOP M 655, microbiological, *L. plantarum*
B_{12}–Cobalamin	AOAC 952.20/986.23, microbiological	AOAC 952.20/986.23, microbiological

Determination of BAs

For the analysis of BAs, yeast strains from slant agars were inoculated into 50 ml of sterilized wort made from unhopped malt extract (Weyermann®) at 12.0°P and pH 5.46. Prior to propagation, the wort was sterilized at 100°C for 45 min. After 72 h propagation, the yeast cells were counted using the Cellometer® Vision (Nexcelom Bioscience LLC). For fermentation, each yeast was pitched in triplicate at a rate of 30×10^6 cells/ml in 300 ml of 12.0°P wort from malt extract (Weyermann®) into 500 ml Duran glass bottles (Schott AG). In order to keep the thermal influence on the wort as low as possible for the subsequent measurement of BAs, the malt extract was only diluted with boiling distilled water (100°C) for the fermentation trials while the Duran glass bottles (Schott AG) and glass fermentation airlocks were previously autoclaved at 121°C for 15 min. The fermentations were incubated at 20°C for 8 days before being analyzed for BAs. To ensure that fermentation occurred and for statistical evaluation, the original gravity, apparent attenuation, ethanol content, and pH of the finished beers were determined according to the methods in Table 2. As already described for statistical evaluation of B vitamins, one-sample *t*-tests were performed using OriginPro 2020 as statistical software to evaluate whether there was any significant difference between the triplicates of each beer. If there was no significant difference in the one-sample *t*-test, it may be assumed here too, that a composite sample from the respective triplicates would reveal statistically significant results in the analysis of BAs. Therefore, unless significant differences were found, composite samples from the triplicates were used for the analysis. Moreover, the samples were checked microscopically for microbiological purity. In addition to the 16 investigated yeast strains and the additional reference yeast strain S. cer 68 from Table 1, a wort sample was analyzed as a negative control. The positive control consisted of three pale barley malt kernels, each from three different malt batches added to the wort at the beginning of the fermentation. To determine which microorganisms led to the positive finding of BAs, the beer was fractionally streaked on Wallerstein Nutrient (WLN) agar (Sigma-Aldrich, St. Louis, MO, USA) as well as wort agar and incubated for 48 h at 28°C. Morphologically different individual colonies were identified by a IVD MALDI Biotyper® system solution based on the microflex® LT/SH MALDI-TOF mass spectrometer (Bruker Daltonics GmbH & Co. KG, Bremen, Germany). The corresponding Bruker standard operating procedures were applied for the sample preparations for bacteria and yeasts. The direct transfer method (DT) was used for bacteria and the extended direct transfer method (eDT) for yeasts. SGS Institut Fresenius GmbH, Freiburg, Germany, (accreditation according to DIN EN ISO/IEC 17025) performed the analysis of the BAs histamine, tryptamine, phenylethylamine, isopentylamine, putrescine, cadaverine, tyramine, spermidine, and spermine using HPLC SLMB 1391.1 (Var. 1). The limit of determination was at least 5 mg/l for all BAs analyzed. Since the majority of the measured concentrations were below the limit of determination but above the limit of detection, trends of the individual BAs are presented as result ranges for all 17 of the analyzed beers. The positive control is shown separately.

Stress tolerance tests

As a preliminary test, the physiological growth of the yeast strains from Table 1 was investigated at 37°C by inoculating each yeast as a pure culture from slant agar into 1 ml of sterile physiological sodium chloride solution and counting them using the Cellometer® Vision (Nexcelom Bioscience LLC). The solutions were adjusted to 1×10^6 cells/ml with sterile physiological sodium chloride solution and fractions streaked in triplicate onto WLN agar (Sigma-Aldrich) plates, which had been incubated for 4 h at 37°C. Subsequently, the agar plates were kept for 7 days at 37°C and were checked for growth at the end of the incubation period. Here, the reference yeast strain S. cer TUM 68 was included as a positive control as the yeast species *S. cerevisiae* as a top-fermenting yeast is known for its ability to grow at 37°C (Lodolo et al. 2008, Hutzler et al. 2015).

The yeast strains that showed growth at the end of the incubation period at a temperature of 37°C were further investigated *in vitro* for human gastric juice tolerance. One yeast strain (S. lud SL17) that did not grow at 37°C was added as a negative control. The selected pure yeast cultures were first propagated for

Table 4. Composition of synthetic gastric juice according to DIN 19738:2000–05.

Substance	Concentration [mg/100 ml]
KCl	290
NaCl	70
KH_2PO_4	27
Pepsin	100
Mucin	300
10% HCl (for pH adjustment)	As required for pH 2

72 h on a WiseShake orbital shaker (Witeg Labortechnik GmbH) at 80 rpm and 20°C in 50 ml of sterilized 9°P wort prepared from unhopped malt extract (Weyermann®). After propagation, the yeast cells were counted and their viability was determined with the aid of Cellometer ViaStainTM AOPI Staining Solution (Nexcelom Bioscience LLC) using the Cellometer® Vision (Nexcelom Bioscience LLC). The volumes of yeast cell suspensions were calculated and adjusted to 15×10^6 viable cells per ml using fresh, sterile 9°P unhopped wort for the adjustments. A volume of 10 ml of each yeast solution was pipetted into sterile 15 ml Sarstedt tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany). The synthetic gastric juice was prepared according to the instructions of DIN 19738:2000–05 and the formulation is listed in Table 4.

While the inorganic components were prepared as a 10-fold concentrated stock solution and were rediluted with double-distilled water, pepsin from porcine gastric mucosa, lyophilized powder, ≥ 2500 units/mg protein (Sigma-Aldrich), and mucin from porcine stomach (Typ II M1778, Sigma-Aldrich) were stirred in just before adding the yeast sample. The experiments were carried out in duplicate.

Each 100 ml of synthetic gastric juice was temperature-controlled at 37°C in sterile 300 ml wide-neck flasks in an automatically temperature-controlled water bath (heating bath B-490, Büchi Labortechnik AG, Flawil, Switzerland). Once the gastric juice reached the target temperature, 10 ml yeast suspension was added by stirring and the pH was adjusted to 2.0 with 10% hydrochloric acid. The suspension was kept at 37°C in a shaking water bath (Gesellschaft für Labortechnik mbH, Burgwedel, Germany) with an integrated thermostat at a frequency of 200/min for 10 min before being adjusted to a pH of 7.5 with solid sodium bicarbonate. Fine adjustment was performed with saturated $NaHCO_3$ solution. The solution was shaken until the pH value was stable at 7.5 before 10 ml of the solution was transferred to a sterile 15 ml Sarstedt tube (Sarstedt AG & Co. KG). Excess sample material was discarded. A volume of 10 ml of each sample material was held at 37°C for 2 h before being centrifuged at 750 × *g* (centrifuge Z366K, Hermle Labortechnik, Wehingen, Germany) for 5 min, washed with sterile physiological sodium chloride solution, and resuspended to 10 ml total volume. A small amount was fractionally streaked on YM agar [0.3% malt extract (Merck, Darmstadt, Germany), 0.3% yeast extract (Sigma-Aldrich), 0.5% peptone from casein, pancreatic digest (Sigma-Aldrich), 1.0% (+)-glucose anhydrous (Carl Roth, Karlsruhe, Germany), 2.0% agar (sifin diagnostics gmbh, Berlin, Germany), 95.9% double-distilled water], preincubated for 4 h at 37°C with an inoculation loop under sterile conditions and incubated at 37°C for 7 days. If yeast colonies grew on the YM agar, they were examined microscopically under a Zeiss bright field microscope. Moreover, the viability of the yeasts was investigated as a percentage right after the 2 h incubation at 37°C and subsequent washing procedure. For this purpose, the Cellometer® Vision (Nexcelom Bioscience LLC) was initially used and sample preparation using Cellometer ViaStain™ AOPI Staining Solution (Nexcelom Bioscience LLC) according to the manufacturer's instructions. This method was supplemented by counting the cells with a Zeiss brightfield microscope in a hemocytometer (Thoma, depth of 0.100 mm) by diluting the samples 1:1 with methylene blue solution beforehand (Herrmann and Schindler 2022).

Results and discussion

Vitamin B concentrations in selected nonalcoholic beers and yeast strain S. lud SL17

Initially, the one-sample *t*-test was performed as described in the materials and methods section. Without exception, the mean population of each triplicate of the beers and the corresponding wort was not significantly different from the test mean at the 0.05 level (cf. Table S1, Supporting Information). Consequently, composite samples could be produced from the respective triplicates to gain statistically significant results.

In Fig. 1, the concentrations of the eight B vitamins in the wort as well as in the corresponding beers are depicted. Concentrations of B vitamins are shown in μg/l and are compared to the Recommended Nutrient Intake (RNI) published by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO; Food and Agriculture Organization (FAO)/World Health Organization (WHO) Nutrition Division 2001). The RNI only serves as an indicator as different studies make different recommendations in some cases. For example, a study from 2019 raised recommended levels of vitamin B12 intake for adults to 4.0 μg/day (Ströhle et al. 2019). Moreover, the RNI indicated applies to adults over the age of 18. In case there are different values for women and men, the mean value is shown. Raw data can be found in Table S2 (Supporting Information). The selected focus on the B vitamins pantothenic acid, biotin, folate, and cobalamin for the four nonalcoholic beers was due to the fact that biotin, folate, and cobalamin play a significant nutritional role in human metabolism, which will be explained in more detail. Pantothenic acid was additionally determined in all beers, as this vitamin is known to be essential for yeast growth of the species *S. cerevisiae* (Williams et al. 1940). All other B vitamins were analyzed only for the beer SL17, since the yeast strain S. lud SL17 is already commercially used in practice to produce nonalcoholic beers.

The diagrams in Fig. 1 generally show that none of the beers exceeded the RNI according to FAO/WHO. However, it is noticeable that the biotin concentration in beer SL17 was approximately twice as high as in the initial wort, which was not the case for any other B vitamin and is depicted in diagram F. While only 1.53 μg/l (CV ± 0.156) biotin was measured in the wort used for fermentation, the beer SL17 contained 3.17 μg/l (CV ± 0.323) biotin. Bamforth (2002), who reviewed the vitamin B concentration ranges in beer, found a common range from 2 to 15 μg/l. Accordingly, the biotin concentration is within the expected range. The RNI is 30 μg/l, and so approximately 10% of the daily requirement would be covered by consuming 1 l of the nonalcoholic beer. Based on the low biotin concentration in the initial wort, it could be assumed that the yeast strain *S. ludwigii* SL17 had the ability to synthesize and release biotin or that biotin reserves stored in the yeast cell were released during fermentation. It is noticeable that the two *C. saturnus* yeast strains 247 and CSa1 as well as the *K. marxianus* yeast strain 653 were unable to release biotin. They might have utilized it for their own metabolic process since no more biotin could be detected in these three beers. The fact that biotin is present in

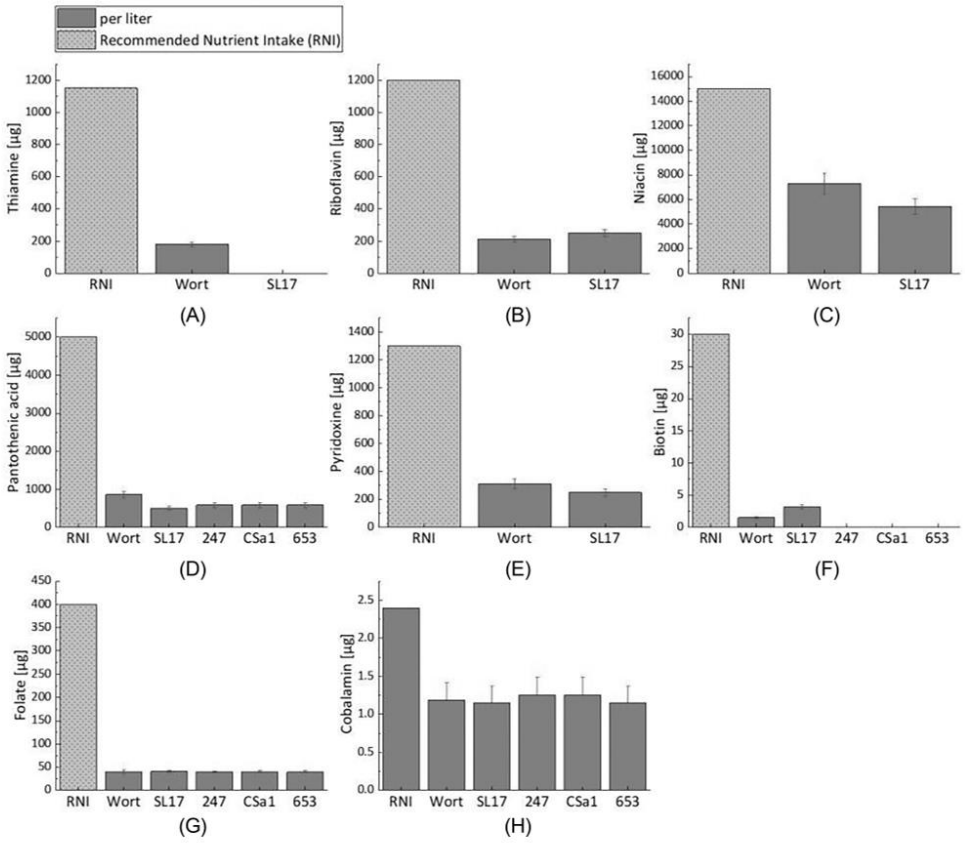


Figure 1. Concentrations in µg/l of the B vitamins thiamine (A), riboflavin (B), niacin (C), pantothenic acid (D), pyridoxine (E), biotin (F), folate (G), and cobalamin (H) analyzed in wort as well as in nonalcoholic beers fermented with the yeast strains *S. ludwigii* SL17, *C. saturnus* 247, *C. saturnus* CSa1, and *K. marxianus* 653. Thiamine, riboflavin, niacin, and pyridoxine analyses were only conducted for wort and nonalcoholic beers fermented with the yeast strain *S. ludwigii* SL17. RNI according to FAO and WHO (Food and Agriculture Organization (FAO)/World Health Organization (WHO) Nutrition Division 2001). Enlarged representations of the individual diagrams can be found in Figure S1 (Supporting Information).

the raw materials of brewer's wort and is taken up by the yeasts as an important growth factor was already described by Lynes in 1948 (Lynes and Norms 1948). Still, Wu et al. (2005) assumed that some yeasts might be able to synthesize biotin themselves. In their study, sake yeasts were able to produce biotin so they speculated that specific genes needed to be present to allow biotin synthesis. Hall and Dietrich (2007) described that the functional gene cluster *BIO1–BIO6* is required for yeasts to be biotin-prototrophic and to produce the vitamin accordingly. Many *S. cerevisiae* yeast strains can partially synthesize biotin, but *de novo* synthesis is not possible. Partial synthesis requires certain precursors to be present in the culture medium from which biotin can be formed (Stolz 2009). It cannot be completely excluded that S. lud SL17 could use these precursors from the wort to synthesize biotin or that the yeast strain carries the specific gene cluster. Still, this would have to be investigated in future research. Nevertheless, in the further course of the study, in addition to the beer, the yeast strain S. lud SL17 was also examined for biotin in order to be able to draw further conclusions.

Concentrations of around 40 µg/l folate were quantified in both the wort and the four beers (cf. Fig. 1, diagram G). Therefore, the yeasts do not appear to utilize significant amounts of folate during their fermentation metabolism nor do they seem to synthesize this vitamin. Thus, the folate most likely originated from the raw material malt and was responsible for the concentration found in the beer. A study by Koren et al. (2021) revealed that barley is a natural source of folate and, depending on the quality of the malt and the malting process, barley malt is also an adequate source of this vitamin. Moreover, Narziss et al. (2017) stated that 70–100 µg/l folate in beer can originate from the raw material malt. Based on the results, only around 10% of the RNI for folate would be achieved by consuming 1 l of any of the four investigated nonalcoholic beers. Folate could still supplement a balanced diet and it can be generally concluded that the nonalcoholic beers represent

a valuable source of this B vitamin. A common folate range in European beers is between 30 and 64 μg/l, which is consistent with the measured values in this study (Owens et al. 2007). The range can be wide since, in addition to folate from the raw material malt, some yeast species like the traditional brewing yeast *S. cerevisiae* are known to synthesize folate (Jacob et al. 2019). Both folate and biotin are essential B vitamins involved in catalytic properties in the human metabolism due to their function as obligatory cofactors of enzymes (Baumgartner 2013). A folate deficiency can, e.g. lead to cardiovascular diseases, cancer or cognitive dysfunctions (Ebara 2017), whereas a biotin deficiency can lead to negative effects on the immune system, cell proliferation and lipid metabolism among other things (Zempleni et al. 2009). Moreover, biotin most likely plays a role in regulating gene expression. Deficiency may also be associated with neurological diseases (León-Del-Río 2019).

Based on the results of Fig. 1, the B vitamins thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, and cobalamin are derived from the raw material barley malt, since none of these B vitamins in the nonalcoholic beers were notably above the concentration in the initial brewer's wort. While diagram A shows that thiamine was completely utilized by the yeast strain S. lud SL17 for its own metabolism, riboflavin stayed unaltered within the scope of the measurement variations (Fig. 1, diagram B) and on average around 20% of pyridoxine (Fig. 1, diagram E) was taken up by the yeast strain. For niacin (Fig. 1, diagram C), the yeast strain S. lud SL17 required on average about 25% of this vitamin present in the wort for its own metabolic processes. Pantothenic acid (Fig. 1, diagram D) was absorbed to a small extent from the wort by all yeast strains studied during fermentation. Although this B vitamin is essential for yeast growth, the pantothenic acid present in the wort was sufficient to meet the demand of the investigated yeasts. Between 10% and 35% of the RNI of riboflavin, niacin, pantothenic acid, and pyridoxine could be quantified in the beers per liter. Although these amounts were not sufficient to meet the RNI, the beers could still serve as a supplement to meet the daily vitamin B requirements. The same could apply to cobalamin (diagram H), where similar concentrations were measured in both the wort and the beers. Cobalamin appeared to remain unaffected in the wort during fermentation by the four yeast strains within the limits of measurement inaccuracies. Consumption of 1 l of one of the four nonalcoholic beers per day could cover around 50% of the RNI according to the FAO/WHO. However, it must be considered that, e.g. with regard to cobalamin, there are more recent studies that recommend even 4 μg per day (Ströhle et al. 2019). It is known that beer naturally contains cobalamin in a concentration range between 3 and 30 μg/l (Bamforth 2002, Heimpel 2003). Most probably it originates from the raw material barley malt as besides the chemical production pathway, only prokaryotes are able to synthesize cobalamin (Friedmann and Cagen 1970, Eschenmoser 1974, Lawrence and Roth 1996). Plants do not seem to be able to produce cobalamin (Duda et al. 1967). Therefore, microbiological contamination on the plants is most likely responsible for the formation of this vitamin (Watanabe et al. 1991). In a work by Krahl (2010), it was revealed that microorganisms grew to varying degrees during germination on the grains. This caused the differences in the vitamin B12 concentration in the beers as the quantity of malt was the decisive influencing factor. This could explain why only approximately 1.2 μg/l cobalamin was measured in the wort as well as in the nonalcoholic beers as the original gravity was adjusted to 7°P. The quantity of barley malt used was therefore significantly lower than in a regular beer.

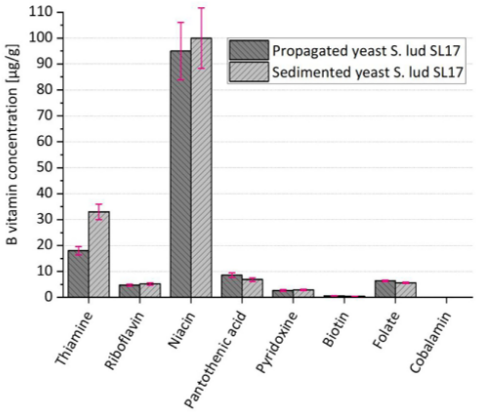


Figure 2. Concentrations in μg/g of the B vitamins thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, biotin, folate, and cobalamin analyzed in the propagated yeast *S. ludwigii* S. lud SL17 as well as the sedimented yeast S. lud SL17.

It was not clarified in the previous investigations whether the yeasts store the B vitamins absorbed from the wort or immediately consumed them during their own yeast cell metabolism. Based on the complete B vitamin analysis of the yeast strain S. lud SL17, both the propagated yeast before fermentation and the sedimented yeast after fermentation were analyzed for all eight B vitamins. The results are depicted in Fig. 2. Raw data can be found in Table S3 (Supporting Information).

Thiamine, which was completely taken up from the wort by the yeast strain S. lud SL17 during fermentation (cf. Fig. 1, diagram A), was either adsorbed or absorbed by the yeast. Although on average 18 μg/g thiamine was already quantified in the propagated yeast, a value almost twice as high (around 33 μg/g) was present in the sedimented yeast after fermentation. Therefore, approximately 15 μg/g thiamine accumulated in the yeast. The yeast mass after fermentation was 16.4 g on average (± 0.4). Accordingly, approximately 240 μg/l thiamine was absorbed from the wort. Since a total of around 330 μg thiamine was originally present in the 1.8 l wort sample, it could be assumed that part of the vitamin B_1 from the wort was already utilized for yeast cell metabolism, while the rest was stored inside the cells. The yeast cell, therefore, appeared to have a storage function for this B vitamin. Riboflavin, niacin, pyridoxine, and cobalamin remained unaltered in the yeast by considering the coefficients of variation. However, since Fig. 1 showed that niacin and pyridoxine were partially reduced during fermentation, these two B vitamins appeared to have been utilized immediately. For riboflavin and cobalamin, it could be assumed that S. lud SL17 did not require these B vitamins for its metabolism. Since no cobalamin could be measured in either the propagated or the sedimented yeast, it was possible to confirm the assumption that the cobalamin quantified in the beers originated from the wort, which fits the findings from existing literature (Krahl 2010). Pantothenic acid was reduced by about 20% during fermentation (cf. Fig. 1). Therefore, it could be deduced that S. lud SL17 immediately metabolized the pantothenic acid taken up from the wort and, in addition, used its own reserves. The folate concentration in the sedimented yeast decreased by approximately 10%, showing a slight consumption by the yeast

during fermentation. Biotin decreased by 30% during fermentation in the yeast strain S. lud SL17. At a yeast mass of 16.4 g on average, around 3.1 μg biotin was released into the wort or utilized by the yeast for its own metabolism. Since there was already approximately 2.8 μg of biotin in 1.8 l of wort, it could be assumed that the yeast released a small amount of biotin during fermentation and needed the larger share for its own cell metabolism. Based on this additional investigation, it is to be expected that the yeast strain S. lud SL17 did not possess the ability to synthesize biotin itself and it had a storage function inside the cell.

Concentrations of BAs in selected nonalcoholic beers

Prior to the measurement of BAs, to ensure that fermentations with the 17 yeast strains from Table 1 proceeded, the beers were analyzed for original gravity, ethanol, apparent attenuation, and pH after fermentation. The individual results can be found in Table S4 (Supporting Information). In summary, the fermentations proceeded as expected. The four maltose- and sucrose-negative yeast strains C. mis 238, C. mis CM1, K. ser 3C4, and P. klu PK1 showed the lowest apparent attenuation as well as the lowest ethanol concentrations. As a maltose-positive yeast, the reference yeast strain S. cer 68 exhibited by far the highest apparent attenuation and ethanol concentration, whereas the maltose-negative yeasts showed values in between. The original gravity was deliberately selected at approximately 12°P so that the ethanol content of all beers exceeded 0.50% (v/v; cf. Table S4, Supporting Information). In the event that BAs were detected, it was necessary to ensure that the limit value of 50 mg/kg total BAs (Loret et al. 2005) in the beers was not exceeded within the nonalcoholic range ≤ 0.50% (v/v).

Regarding the one-sample *t*-tests, the *t*-tests were also initially performed for BAs as described in the "Materials and methods" section. Since, without exception, the mean population of each triplicate of the beers did not differ significantly at the 0.05 level from the test mean (cf. Table S5, Supporting Information), composite samples from the respective triplicates were permitted in order to gain statistically significant results with regard to the selected BAs. As mentioned in the "Materials and methods" section, the majority of the quantified results of the BAs were below the limit of determination but above the limit of detection. The limit of determination is set by SGS Institut Fresenius GmbH at ≥ 5 mg/l. Without exception, all beers produced with the 17 experimental yeast strains from Table 1 were below the limit of determination and only the positive control exhibited concentrations above this threshold. Therefore, only the result ranges of the 17 yeast strains are shown as a summary in Fig. 3, whereas the positive control is shown individually.

While histamine and spermine were below the limit of detection for all 17 yeast strains without exception, the other seven BAs were formed in very small amounts or were already present in the wort sample. Minor amounts of putrescine, cadaverin, tyramine, and spermidine were, therefore, quantified in the brewer's wort. Nevertheless, the concentrations of the analyzed nine BAs were, without exception, below the limit of determination (5.0 mg/l) in the wort. It is known from previous studies that besides microbial formation during fermentation, BAs can also be formed during malting (Gasarasi et al. 2003). While putrescine and cadaverine remained almost unaltered in the beer samples, some of the yeast strains seemed to be able to reduce tyramine and spermidine either partially or even completely during fermentation as is already discussed in the literature (Benito et al. 2015, Ivit et al. 2018).

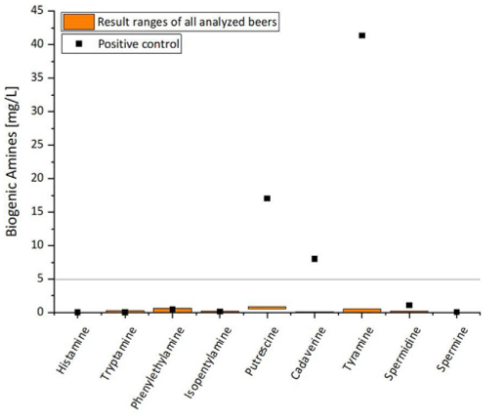


Figure 3. BAs histamine, tryptamine, phenylethylamine, isopentylamine, putrescine, cadaverine, tyramine, spermidine, and spermine in mg/l of 17 different yeast strains (cf. Table 1) presented as result ranges due to concentrations below the limit of determination (limit of determination ≥ 5.0 mg/l) as well as a positive control shown individually.

Isopentylamine was formed in small amounts by almost all the yeast strains examined, while only two yeast strains could synthesize marginal amounts of tryptamine and phenylethylamine. These results give a first indication that the investigated yeast strains, without exception, could not build up notable amounts of BAs from wort precursors during fermentation. In total, the highest concentration of BAs in the beers produced was below 3 mg/l. This is significantly lower than the average concentration of regular uncontaminated beers, which are reported in literature to be between 8 and 30 mg/l (Narziss et al. 2017). In beers with 12°P original gravity, the histamine content is usually 0.15–0.20 mg/l, while the histamine concentration for the investigated beers was so low that it could not be detected. Accordingly, no adverse health effects are suspected from BAs in the nonalcoholic beers produced with the non-*Saccharomyces* yeasts investigated. In addition, the BA results of the beer fermented with the *Saccharomyces* yeast strain S. cer 68 showed no significant differences from the non-*Saccharomyces* yeasts.

The positive control, which was composed of three pale barley malt kernels each from three different batches added to the brewer's wort at the beginning of the fermentation, demonstrated that the three BAs cadaverine, putrescine, and tyramine could be detected at concentrations significantly above the limit of determination of 5 mg/l. In Fig. 3, cadaverine is just under 8 mg/l, while a concentration of 17 mg/l was measured for putrescine and about 41 mg/l for tyramine. The exact concentrations can be viewed in Table S6 (Supporting Information). For all other BAs of the positive control, the concentrations were below the limit of determination. Using MALDI-TOF, it was found that the wort contained microorganisms including *Lactobacillus plantarum*, *Candida krusei*, and *Acetobacter* sp., which originated from the pale malt grains. BAs are mainly formed by microbial decarboxylation of amino acids and the formation is often observed from lactic acid bacteria (Brink et al. 1990, Alcaide-Hidalgo et al. 2007, Spano et al. 2010, Sumby et al. 2014). *Lactobacillus plantarum* is known from the literature for its ability to produce putrescine from the amino acid arginine which

Table 5. Growth of the investigated yeast strains at 37°C on WLN agar after 7 days of incubation.

Yeast strain abbreviation	Growth at 37°C
C. fab 5640	++
C. fab 5650	++
C. mis 238	–
C. mis CM1	–
C. sat 247	–
C. sat 4549	–
C. sat CSa1	–
K. ser 3C4	–
K. lac G9K	–
K. mar 653	++
L. klu 3082	++
P. klu PK1	++
S. cer 68	++
S. lud SL17	–
S. fib Lu27	++
S. pom G10S	++
T. del 1I6	–

(++) growth observed, (-) no growth.

is found in brewer's wort (Arena and Manca de Nadra 2001, Ferreira and Guido 2018). Besides putrescine, cadaverine, and tyramine can be formed by *L. plantarum* (Bonnin-Jusserand et al. 2012, Alan et al. 2018). The BAs tyramine and cadaverine are formed from the amino acids tyrosine and lysine, which are also present in brewer's wort (Ferreira and Guido 2018, Vicente et al. 2021). *Candida krusei* (*Pichia kudriavzevii*) possess decarboxylase activity against the amino acids tyrosine and lysine (Delgado-Ospina et al. 2020) so it could be speculated that this yeast species might have been responsible for the elevated tyramine and cadaverine concentrations in the positive control. However, further research would need to be conducted in the future to prove this speculation. There is no literature to date about the formation of BAs by acetic acid bacteria such as *Acetobacter* sp. On the contrary, studies may indicate that BAs could be degraded during acetic fermentation (Landete et al. 2007, Ordóñez et al. 2013, 2015).

Stress tolerance tests

Before the 17 yeast strains listed in Table 1 were investigated *in vitro* for their tolerance to synthetic human gastric juice, a pretest was performed to first elucidate the ability of the individual yeast strains to grow at 37°C, based on human body temperature. Table 5 lists the results after 7 days of incubation.

A total of eight of the total 17 yeast strains from Table 5 showed growth on WLN agar within 7 days at an incubation temperature of 37°C. While S. cer 68 used as a positive control showed growth, the two strains of the yeast species *C. fabianii*, K. mar 653, L. klu 3082, P. klu PK1, S. fib Lu27, and S. pom G10S were also able to proliferate at the selected incubation temperature. In case yeasts are not viable at 37°C, potential pathogenicity to the human organism is largely excluded. Therefore, only the previously mentioned eight yeast strains were considered for the following experiment in the synthetic gastric juice environment. The yeast strain S. lud SL17 was included as a negative control. Figure 4 shows whether the selected nine yeast strains survived the synthetic gastric juice treatment at 37°C as described in the methods section. The viabilities of the yeast cells were analyzed before and 2 h after the treatment to study the resistance of the yeast strains to the synthetic gastric juice and to draw a direct comparison.

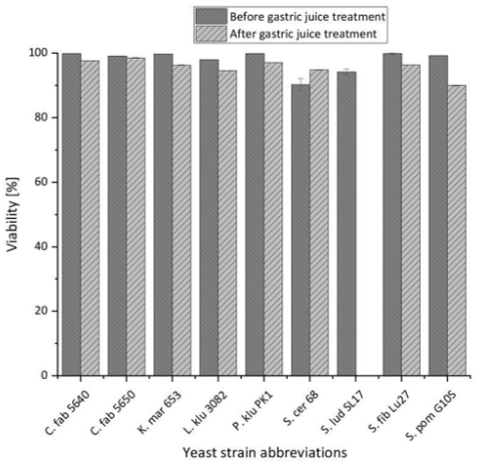


Figure 4. Viability (%) by counting cells with a Zeiss bright-field microscope in a Thoma counting chamber with methylene blue before and after synthetic gastric juice treatment of the selected yeast strains C. fab 5640, C. fab. 5650, K. mar 653, L. klu 3082, P. klu PK1, S. fib Lu27, S. pom G10S, and the positive control S. cer 68 as well as negative control S. lud SL17.

Figure 4 reveals that the viability of all yeast strains examined before synthetic gastric juice treatment was > 90%. The yeast strains C. fab 5640, C. fab. 5650, K. mar 653, P. klu PK1, S. fib Lu27, and S. pom G10S even showed viabilities > 99%. The viability of the yeast strain L. klu 3082 was close to 98%, while the two reference yeast strains S. lud SL17 and S. cer 68 were between 90% and 95% viability (± 2.0%). Besides the methylene blue method, viability was also determined by the cell counting method described in the methods chapter using the AOPI staining solution containing propidium iodide. Both methods gave similar results before the synthetic gastric juice treatment. However, following synthetic gastric juice treatment for 10 min at 37°C, components from the synthetic gastric juice fluoresced as well as the living yeast cells, giving a consistent viability of 100%. Consequently, only the method using methylene blue was suitable to measure accurate viability in this case.

Looking at the viability decreases in Fig. 4, in addition to the decrease in viability for the negative control, only the yeast strain S. pom G10S showed a decrease in viability of just over 10%. For the other investigated yeast strains, the viability decreased by only 0.5%–3.6%, showing that the influence of the synthetic gastric juice treatment had a minor effect on the viability of the yeast cells. The positive control S. cer 68 increased in viability by almost 5%. Therefore, the yeast cells were presumably able to proliferate after the 10-min synthetic gastric juice treatment and the 2-h regeneration at a pH of 7.5 and a temperature of 37°C. This was likely because 10 ml of the yeast cell suspension in the propagation wort was added to the synthetic gastric juice, thus still providing nutrient substrate for the yeasts to grow on. The fact that the negative control S. lud SL17 showed no viability at all after the treatment with synthetic gastric juice could be attributed to a higher sensitivity to the low pH of 2,which may have led to a denaturation of functional proteins in the cell membrane, causing the yeast cells to lose functionality (Vilgis 2020).

Table 6. Growth at 37°C on YM agar of the nine selected yeast strains (including S. cer 68 as positive and S. lud SL17 as negative control) after synthetic gastric juice treatment and additional incubation for 7 days.

Yeast strain abbreviation	Growth at 37°C after gastric juice treatment
C. fab 5640	++
C. fab 5650	++
K. mar 653	++
L. klu 3082	++
P. klu PK1	++
S. cer 68	++
S. lud SL17	–
S. fib Lu27	++
S. pom G10S	++

(+) growth observed, (-) no growth.

The retention time of the yeast suspensions in the synthetic gastric juice was deliberately limited to 10 min, since previous studies revealed that liquids have a short retention time in the stomach. For example, Best and Conheim described in 1910 that liquids leave the stomach rapidly in both empty and filled states (Best and Cohnheim. 1910). This statement was later substantiated by Minami and Maccallum. They found that liquids leave the empty stomach according to first-order kinetics with a half-life of about 10–20 min (Minami and Mccallum 1984), whereby no gender-specific difference could be observed (Bennink et al. 1998). Nevertheless, it was found that the speed of gastric emptying depends on its filling state with food pulp as an empty stomach behaves differently to a stomach filled with food pulp (Minami and Mccallum 1984). For this study, an empty stomach with the shortest half-life of 10 min was assumed as in case any of the investigated yeast strains could be harmful for human health, a short gastric emptying speed should be considered.

In addition to investigating the short-term response to the gastric environment, the investigation examined whether the selected yeast strains were able to proliferate after the synthetic gastric juice treatment. For this purpose, the yeast suspensions were streaked on YM agar as described in the methods chapter. Table 6 presents the results.

The results reveal that, apart from the negative control S. lud SL17, the other eight yeast strains were able to continue growing at 37°C after synthetic gastric juice treatment. Microbiological examination using brightfield microscopy clearly identified the yeast cells, which showed that the formed colonies were not caused by bacterial contamination. Consequently, the yeast strains C. fab 5640, C. fab 5650, K. mar 653, L. klu 3082, P. klu PK1, S. fib Lu27, and S. pom G10S were most likely to survive in the gastric environment and subsequently proliferated at 37°C. Certainly, further studies would need to be performed to confirm this statement. However, the results provide an initial indication of this potential. Additionally, further experiments would also be needed to establish whether the yeast strains could also survive and proliferate in the human intestine. Since seven out of the 16 investigated non-*Saccharomyces* yeasts showed high viability at 37°C and after a 10-min *in vitro* treatment in synthetic gastric juice, further investigations into safety considerations of the yeasts should be conducted. These would include, e.g. studies of toxin expression and virulence genes. With regard to the two yeast strains K. mar 653 and S. pom G10S, it could be assumed that they do not cause any harm to the human organism, as they have QPS status according to EFSA. They could even potentially serve as probiotic microbes. However, extensive investigations would be necessary to evaluate this. In 2006, the FAO published a comprehensive systematic guideline for the evaluation of probiotic microorganisms (Food and Agriculture Organization of the United Nations (FAO)—Nutrition Division 2006). Staniszewski and Kordowska-Wiater (2021) summarized the characteristics that probiotic strains need to have in order to be labeled as such. Non-*Saccharomyces* yeast species such as *K. marxianus* (Fonseca et al. 2008, Hatoum et al. 2012, Cissé et al. 2019, Motey et al. 2020), *K. lactis* (Fadda et al. 2017, Oliveira et al. 2019), *K. servazzii* (Ben Taheur et al. 2021), *S. fibuligera* (Lakshmi Ragavan and Das 2017), *S. pombe* (Gil-Rodríguez et al. 2015), and *T. delbrueckii* (Pereira Andrade et al. 2019, Agarbati et al. 2020) appear increasingly in the context of probiotic or potentially probiotic yeasts. There are currently no study results on this subject relating to *L. kluyveri* and *C. fabianii*. Furthermore, it must be kept in mind that probiotic properties are strain specific (Agarbati et al. 2020).

Conclusion

In this study, the metabolic properties of selected non-*Saccharomyces* yeasts suitable for producing nonalcoholic beers were investigated for B vitamins, BAs and tolerance to synthetic human gastric juice *in vitro*. With the exception of thiamine, all other analyzed B vitamins, which derived most likely from the raw material malt, were detected in the beer fermented with the yeast strain *S. ludwigii* TUM SL17, covering at least 10% of the RNI per liter. Cobalamin, folate, and pantothenic acid were also measured in nutritionally relevant amounts in the nonalcoholic beers 247, CSa1, and 653 in concentrations comparable to those in the beer SL17. Therefore, B vitamins in nonalcoholic beers fermented with non-*Saccharomyces* yeasts could serve as a supplement for a balanced diet as they represent a valuable source of B vitamins. The selected BAs stayed far below the limit of determination of 5 mg/l in all investigated beers without exception, while histamine was even below the limit of detection and could not be detected. Consequently, the yeast strains investigated are not capable of synthesizing relevant amounts of the nine analyzed BAs during fermentation, which means that no negative health impact of the beers can be traced back to these BAs. Furthermore, out of the 16 experimental yeasts, seven strains, namely *C. fabianii* 5640, *C. fabianii* 5650, *K. marxianus* 653, *L. kluyveri* 3082, *P. kluyveri* PK1, *S. fibuligera* Lu27, and *S. pombe* G10S, revealed they were capable of proliferating at 37°C and tolerating the conditions of synthetic human gastric juice during *in vitro* experiments. As a precaution, and to eliminate any potential safety concerns for consumers, these yeast strains should be investigated further regarding their use to produce nonalcoholic beers.

Supplementary data

Supplementary data are available at FEMSYR online.

Conflict of interest statement. None declared.

Funding

This work was funded by the BMWK (German Ministry of Economic Affairs and Climate Action), Forschungsvereinigung Wifoe (Association for the Promotion of Science of the German Brewing Industry, Berlin). The IGF Project (grant number AiF 20658 N) of the Wifoe is supported via AiF within the program for promoting

the Industrial Collective Research (IGF) of the BMWK, based on a decision by the German Bundestag. Y.M. was in receipt of grants.

References

Agarbati A, Canonico L, Marini E. *et al.* Potential probiotic yeasts sourced from natural environmental and spontaneous processed foods. *Foods* 2020;**9**:32143376.

Ahuja K, Rawat A. *Market Size of Non-Alcoholic Beer Worldwide from 2016 to 2024 (in billion U.S. dollars)*. Global Market Insights. 2020. https://www.gminsights.com/industry-analysis/non-alcoholic-beer-market (17 June 2021, date last accessed).

Alan Y, Topalcengiz Z, Dığrak M. Biogenic amine and fermentation metabolite production assessments of *Lactobacillus plantarum* isolates for naturally fermented pickles. *LWT* 2018;**98**:322–8.

Alcaide-Hidalgo JM, Moreno-Arribas MV, Martín-Álvarez PJ. *et al.* Influence of malolactic fermentation, postfermentative treatments and ageing with lees on nitrogen compounds of red wines. *Food Chem* 2007;**103**:572–81.

Arena ME, Manca de Nadra MC. Biogenic amine production by *lactobacillu s*. *J Appl Microbiol* 2001;**90**:158–62.

Bamforth CW. Nutritional aspects of beer—a review. *Nutr Res* 2002;**22**:227–37.

Baumgartner MR. Vitamin-responsive disorders: cobalamin, folate, biotin, vitamins B1 and E. *Handb Clin Neurol* 2013;**113**:1799–810.

Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit Amine - eine wichtige stoffklasse in der amtlichen lebensmittelüberwachung. 2020. Bavaria. updated on 4/9/2020. (27 January 2022, date last accessed).

Bellut K, Arendt EK. Chance and challenge: non-*Saccharomyces* yeasts in nonalcoholic and low alcohol beer brewing – a review. *J Am Soc Brew Chem* 2019;**77**:77–91.

Ben Taheur F, Mansour C, Chaieb K. Application of kefir probiotics strains as aflatoxin B1 binder in culture medium, milk and simulated gastrointestinal conditions. In: *Proceedings of the MOL2NET'21, Conference on Molecular, Biomedical and Computational Sciences and Engineering*. 2021. 10307. Paris. doi: 10.3390/mol2net-07-10307.

Beneduce L, Romano A, Capozzi V. *et al.* Biogenic amine in wines. *Ann Microbiol* 2010;**60**:573–8.

Benito Á, Calderón F, Palomero F. *et al.* Combine use of selected *Schizosaccharomyces pombe* and *Lachancea thermotolerans* yeast strains as an alternative to the traditional malolactic fermentation in red wine production. *Molecules* 2015;**20**:9510–23.

Benito S. The impact of *Torulaspora delbrueckii* yeast in winemaking. *Appl Microbiol Biotechnol* 2018;**102**:3081–94.

Bennink R, Peeters M, van den Maegdenbergh V. *et al.* Comparison of total and compartmental gastric emptying and antral motility between healthy men and women. *Eur J Nucl Med Mol Imaging* 1998;**25**:1293–9.

Best F, Cohnheim. O. Über die verweildauer von flüssigkeiten im magen. *Hoppe-SeylerÂ´s Zeitschrift FÃ¼R Physiologische Chemie* 1910;**69**:117–9.

Bonnin-Jusserand M, Grandvalet C, Rieu A. *et al.* Tyrosine-containing peptides are precursors of tyramine produced by *Lactobacillus plantarum* strain IR BL0076 isolated from wine. *BMC Microbiol* 2012;**12**:199.

Brink B, Damink C, Joosten HMLJ. *et al.* Occurrence and formation of biologically active amines in foods. *Int J Food Microbiol* 1990;**11**:73–84.

Buiatti S, Boschelle O, Mozzon M. *et al.* Determination of biogenic amines in alcoholic and non-alcoholic beers by HPLC. *Food Chem* 1995;**52**:199–202.

Caruso M, Fiore C, Contursi M. *et al.* Formation of biogenic amines as criteria for the selection of wine yeasts. *World J Microbiol Biotechnol* 2002;**18**:159–63.

Cissé H, Kagambèga B, Sawadogo A. *et al.* Molecular characterization of *Bacillus*, lactic acid bacteria and yeast as potential probiotic isolated from fermented food. *Sci Afr* 2019;**6**:e00175.

Delgado-Ospina J, Acquaticci L, Molina-Hernandez JB. *et al.* Exploring the capability of yeasts isolated from Colombian fermented cocoa beans to form and degrade biogenic amines in a lab-scale model system for cocoa fermentation. *Microorganisms* 2020;**9**:28.

Duda J, Pedziwilk Z, Zodrow K. Studies on the vitamin B12 content of the leguminous plants. *Acta Microbiologica Polska* 1967;**6**:233–8.

Ebara S. Nutritional role of folate. *Congenit Anom* 2017;**57**:138–41.

EFSA-BIOHAZ Panel Introduction of a qualified presumption of safety (QPS) approach for assessment of selected microorganisms referred to EFSA - opinion of the scientific committee. *EFSA J* 2007;**5**:587.

EFSA-BIOHAZ Panel Updated list of QPS status recommended biological agents in support of EFSA 987 risk assessments. *EFSA J* 2021;**19**:6689.

Eschenmoser A. Organische naturstoffsynthese heute. Vitamin B_{12} als Beispiel. *Naturwissenschaften* 1974;**61**:513–25.

European Food Safety Authority Scientific opinion on risk based control of biogenic amine formation in fermented foods. *EFSA J* 2011;**9**:2393.

Fadda ME, Mossa V, Deplano M. *et al.* In vitro screening of *Kluyveromyces strains* isolated from Fiore Sardo cheese for potential use as probiotics. *LWT* 2017;**75**:100–6.

Ferreira I, Guido L. Impact of wort amino acids on beer flavour: a review. *Fermentation* 2018;**4**:23.

Ferreira IMPLVO, Pinho O, Vieira E. *et al.* Brewer's *Saccharomyces* yeast biomass: characteristics and potential applications. *Trends Food Sci Technol* 2010;**21**:77–84.

Fonseca GG, Heinzle E, Wittmann C. *et al.* The yeast *Kluyveromyces marxianus* and its biotechnological potential. *Appl Microbiol Biotechnol* 2008;**79**:339–54.

Food and Agriculture Organization (FAO)/World Health Organization (WHO) Nutrition Division. *Human Vitamin and Mineral Requirements*. Rome: FAO. 2001. p. 014–9228. https://www.fao.org/documents/card/en/c/ceec621b-1396-57bb-8b35-48a60d7faaed/ (7 February 2022, date last accessed).

Food and Agriculture Organization of the United Nations (FAO) - Nutrition Division. *Probiotics in Food. Health and Nutritional Properties and Guidelines for Evaluation*. Report of a Joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food, including powder milk with live lactic acid bacteria: Cordoba, Argentina, 1-4 October 2001: report of a Joint FAO/WHO Working Group on drafting guidelines for the evaluation of probiotics in food. Rome: Food and Agriculture Organization of the United Nations. 2006. p. 0254–4725.

Friedmann HC, Cagen LM. Microbial biosynthesis of B12-like compounds. *Annu Rev Microbiol* 1970;**24**:159–208.

Gasarasi G, Kelgtermans M, Verstrepen K. *et al.* Occurrence of biogenic amines in beer: causes and proposals of remedies. *Monatsschrift Für Brauwissenschaft* 2003;**56**:58–63. https://lirias.kuleuven.be/1758314?limo=0 (24 March 2022, date last accessed).

Gil-Rodríguez AM, Carrascosa AV, Requena T. Yeasts in foods and beverages: in vitro characterisation of probiotic traits. *LWT Food Sci Technol* 2015;**64**:1156–62.

Granchi L, Romano P, Mangani S. *et al.* Production of biogenic amines by wine microorganisms. *In Bulletin de l'OIV-Office International de la Vigne et du Vin* 2005; **78**:895–6. https://www.researchgate.net/profile/lisa-granchi/publicati

on/285097576_production_of_biogenic_amines_by_wine_microorganisms/links/565ff7e008aeafc2aaca9b16/production-of-biogenic-amines-by-wine-microorganisms.pdf.
Habschied K, Živković A, Krstanović V. *et al.* Functional beer—a review on possibilities. *Beverages* 2020;**6**:51.
Hall C, Dietrich FS. The reacquisition of biotin prototrophy in *Saccharomyces cerevisiae* involved horizontal gene transfer, gene duplication and gene clustering. *Genetics* 2007;**177**:2293–307.
Hatoum R, Labrie S, Fliss I. Antimicrobial and probiotic properties of yeasts: from fundamental to novel applications. *Front Microbiol* 2012;**3**:421. https://scholar.google.de/citations?user=qpt4fr0aaaaj&hl=de&oi=sra (4 March 2022, date last accessed).
Heimpel H. Pathogenese des Vitamin-B12- und des folsäuremangels. 2003. http://www.onkodin.de/e8/e12933/e13040/index_ger.html, updated on 8/14/2003. (3 April 2022, date last accessed).
Herrmann JV, Schindler E. VOENMILEI - Diagnostischer Leitfaden zur mikroskopischen gärkontrolle. Herstellung eines präparats zur methylenblaufärbung. Edited by bayerische landesanstalt für weinbau und gartenbau (LWG). Veitshöchheim. 2022. https://www.lwg.bayern.de/analytik/087446/index.php (3 September 2022, date last accessed).
Hutzler M, Koob J, Riedl R. *et al.* Yeast identification and characterization. In: Chapter 6 Hill AE. (ed.), *Brewing Microbiology. Managing Microbes, Ensuring Quality and Valorising Waste*. London: Woodhead Publ./Elsevier, Woodhead Publishing Series in Food Science, Technology and Nutrition. 2015. p. 289.
Ivit NN, Loira I, Morata A. *et al.* Making natural sparkling wines with non-*Saccharomyces* yeasts. *Eur Food Res Technol* 2018;**244**:925–35.
Izquierdo-Pulido M, Hernández-Jover T, Mariné-Font A. *et al.* Biogenic amines in European beers. *J Agric Food Chem* 1996;**44**:3159–63.
Jacob FF, Striegel L, Rychlik M. *et al.* Yeast extract production using spent yeast from beer manufacture: influence of industrially applicable disruption methods on selected substance groups with biotechnological relevance. *Eur Food Res Technol* 2019;**245**:1169–82.
Kalac P, Krízek M. A review of biogenic amines and polyamines in beer. *J Inst Brew* 2003;**109**:123–8.
Kennedy DO. B vitamins and the brain: mechanisms, dose and Efficacy–a review. *Nutrients* 2016;**8**:68.
Kokole D, Jané Llopis E, Anderson P. Non-alcoholic beer in the European Union and UK: availability and apparent consumption. *Drug Alcohol Rev*. 2021;**41**:550–560.
Koren D, Hegyesné Vecseri B, Kun-Farkas G. Evolution of folate content during barley malt production. *Acta Alimentaria* 2021;**49**:238–46.
Krahl M. *Funktionelle Getränke auf Basis vermälzter Zerealien und Pseudozerealien*. München: Technische Universität München. 2010. https://mediatum.ub.tum.de/972948 (24 March 2022, date last accessed).
Lakshmi Ragavan M, Das N. Molecular identification of probiotic yeast strains and their characterization. *Asian J Pharm Clin Res* 2017;**10**:339.
Landete JM, Ferrer S, Pardo I. Biogenic amine production by lactic acid bacteria, acetic bacteria and yeast isolated from wine. *Food Control* 2007;**18**:1569–74.
Lara-Hidalgo CE, Hernández-Sánchez H, Hernández-Rodríguez C *et al.* Yeasts in Fermented Foods and their Probiotic Potential. *Austin J Nutr Metab.* 2017;**4**:1045.
Laulund S, Wind A, Derkx PMF. *et al.* Regulatory and safety requirements for food cultures. *Microorganisms* 2017;**5**:28.
Lawrence JG, Roth JR Evolution of coenzyme B12 synthesis among enteric bacteria: evidence for loss and reacquisition of a multigene complex. *Genetics* 1996;**142**:11–24.
León-Del-Río A. Biotin in metabolism, gene expression, and human disease. *J Inherit Metab Dis* 2019;**42**:647–54.
Lodolo EJ, Kock JLF, Axcell BC. *et al.* The yeast *Saccharomyces cerevisiae* - the main character in beer brewing. *FEMS Yeast Res* 2008;**8**:1018–36.
Loret S, Deloyer P, Dandrifosse G. Levels of biogenic amines as a measure of the quality of the beer fermentation process: data from Belgian samples. *Food Chem* 2005;**89**:519–25.
Lynes KJ, Norms FW. Biotin in the materials and process of brewing. *J Inst Brew* 1948;**54**:150–7.
Marten R, Amul GGH, Casswell S. Alcohol: global health's blind spot. *Lancet Glob Health* 2020;**8**:e329–30.
Mayer O, Simon J, Rosolová H. A population study of the influence of beer consumption on folate and homocysteine concentrations. *Eur J Clin Nutr* 2001;**55**:605–9.
Methner Y, Hutzler M, Zarnkow M. *et al.* Investigation of non-*Saccharomyces* yeast strains for their suitability for the production of non-alcoholic beers with novel flavor profiles. *In J Am Soc Brew Chem* 2022;**80**:341–55.
Minami H, Mccallum RW. The physiology and pathophysiology of gastric emptying in humans. *Gastroenterology* 1984;**86**:1592–610.
Motey GA, Johansen PG, Owusu-Kwarteng J. *et al.* Probiotic potential of *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* isolated from West African spontaneously fermented cereal and milk products. *Yeast* 2020;**37**:403–12.
Narziss L, Back W, Gastl M. *et al. Abriss der Bierbrauerei*. 8th ed. Newark: John Wiley & Sons Incorporated. 2017. https://ebookcentral.proquest.com/lib/gbv/detail.action?docID=4783858 (1 March 2022, date last accessed).
Oliveira DR, Lopes ACA, Pereira RA. *et al.* Selection of potentially probiotic *Kluyveromyces lactis* for the fermentation of cheese whey–based beverage. *Ann Microbiol* 2019;**69**:1361–72.
Ordóñez JL, Callejón RM, Morales ML. *et al.* A survey of biogenic amines in vinegars. *Food Chem* 2013;**141**:2713–9.
Ordóñez JL, Sainz F, Callejón RM. *et al.* Impact of gluconic fermentation of strawberry using acetic acid bacteria on amino acids and biogenic amines profile. *Food Chem* 2015;**178**:221–8.
Owens JE, Clifford AJ, Bamforth CW. Folate in beer. *J Inst Brew* 2007;**113**:243–8.
Pereira Andrade R, Resende Oliveira D, Alencar Lopes AC. *et al.* Survival of *Kluyveromyces lactis* and *Torulaspora delbrueckii* to simulated gastrointestinal conditions and their use as single and mixed inoculum for cheese production: food research international (125). 2019;**125**:108620. https://www.sciencedirect.com/science/article/pii/s0963996919304983 (6 March 2022, date last accessed).
Ricci A, Allende A, Bolton D. *et al.* Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 8: suitability of taxonomic units notified to EFSA until march 2018. *EFSA J* 2018;**16**:e05315.
Salanță LC, Coldea TE, Ignat MV. *et al.* Non-alcoholic and craft beer production and challenges. *Processes* 2020;**8**:1382.
Shalaby AR. Significance of biogenic amines to food safety and human health. *Food Res Int* 1996;**29**:675–90.
Spano G, Russo P, Lonvaud-Funel A. *et al.* Biogenic amines in fermented foods. *Eur J Clin Nutr* 2010;**64**:S95–100.
Staniszewski A, Kordowska-Wiater M. Probiotic and potentially probiotic yeasts-characteristics and food application. *Foods* 2021;**10**:1306.
Steensels J, Verstrepen KJ. Taming wild yeast: potential of conventional and nonconventional yeasts in industrial fermentations. *Annu Rev Microbiol* 2014;**68**:61–80.

Stolz J. *Biotin - Das Vitamin für Haut, Haar und Hefe*. BIOspektrum (Springer Nature Switzerland AG). 2009;**15**:132–5. https://www.researchgate.net/profile/j-stolz/publication/242200296_biotin_wurde_durch_arbeiten_an_der_backerhefe_saccharomyces_cerevisiae_entdeckt_trotz_mehr_als_100_jahren_forschung_sind_immer_noch_viele_fragen_zum_biotinmetabolismus_der_hefe_unbeantwortet_the_vitam/links/572865dc08ae586b21e388ab/biotin-wurde-durch-arbeiten-an-der-baeckerhefe-saccharomyces-cerevisiae-entdeckt-trotz-mehr-als-100-jahren-forschung-sind-immer-noch-viele-fragen-zum-biotinmetabolismus-der-hefe-unbeantwortet-the-vita.pdf (23 March 2022, date last accessed).

Striegel L, Chebib S, Netzel ME. *et al.* Improved stable isotope dilution assay for dietary folates using LC-MS/MS and its application to strawberries. *Front Chem*. 2018;**6**:11.

Ströhle A, Richter M, González-Gross M. *et al.* The revised D-A-CH-reference values for the intake of vitamin B12: prevention of deficiency and beyond. *Mol Nutr Food Res* 2019;**63**:e1801178.

Sumby KM, Grbin PR, Jiranek V. Implications of new research and technologies for malolactic fermentation in wine. *Appl Microbiol Biotechnol* 2014;**98**:8111–32.

Ternes W. (ed.), *Lexikon der Lebensmittel und der Lebensmittelchemie*. Stuttgart: Wissenschaftliche Verlagsgesellschaft. 2007. Unveränd. Nachdr. Stuttgart: Wiss. Verl.-Ges.

Torrea D, Ancín C. Content of biogenic amines in a chardonnay wine obtained through spontaneous and inoculated fermentations. *J Agric Food Chem* 2002;**50**:4895–9.

U.S. Food and Drug Administration Microorganisms and microbial-derived ingredients used in food (Partial list). 2018. updated on 4/1/201. https://www.fda.gov/food/generally-recognized-safe-gras/microorganisms-microbial-derived-ingredients-used-food-partial-list (15 July 2022, date last accessed).

U.S. Food and Drug Administration Microorganisms and microbial-derived ingredients used in food. 2017. www.fda.gov (31 January 2020, date last accessed).

Vicente J, Calderón F, Santos A. *et al.* High potential of *Pichia kluyveri* and other *Pichia* species in wine technology. *Int J Mol Sci* 2021;**22**:1196.

Vieira EF, Carvalho J, Pinto E. *et al.* Nutritive value, antioxidant activity and phenolic compounds profile of brewer's spent yeast extract. *J Food Compos Anal* 2016;**52**:44–51.

Vilgis TA. Biologische grundlagen unserer ernährung. In : *Biophysik der Ernährung*. Berlin, Heidelberg: Springer Spektrum, 2020. p. 1–54. https://link.springer.com/chapter/10.1007/978-3-662-61151-7_1 (30 March 2022, date last accessed).

Wang B, Tan F, Chu R. *et al.* The effect of non-*Saccharomyces* yeasts on biogenic amines in wine. *Trends Food Sci Technol* 2021;**116**:1029–40.

Watanabe F, Nakano Y, Tamura Y. *et al.* Vitamin B12 metabolism in a photosynthesizing green alga, *Chlamydomonas reinhardtii*. *Biochim Biophys Acta Gen Sub* 1991;**1075**:36–41.

Williams RJ, Eakin RE, Snell EE. The relationship of inositol, thiamin, biotin, pantothenic acid and vitamin B6 to the growth of yeasts. *J Am Chem Soc* 1940;**62**:1204–7.

Wu H, Ito K, Shimoi H. Identification and characterization of a novel biotin biosynthesis gene in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 2005;**71**:6845–55.

Zempleni J, Wijeratne SSK, Hassan YI. Biotin. *Biofactors* 2009;**35**:36–46.

3 Discussion

For the consumer, the choice of beer is largely dependent on its flavor. To date, little research has been conducted on the use of non-*Saccharomyces* yeasts to diversify beer flavor. A majority of the literature references on this topic listed in the introduction of this thesis (section 1) were not published before 2015 and most of them even start from 2019 (cf. Table 3), which confirms the novelty of the research interest dealing with the investigations on the flavor formation by fermenting brewer's wort with non-*Saccharomyces* yeasts. As already mentioned in the introduction, the yeast has the greatest influence on the beer flavor during fermentation above that of hops and malt [145, 260, 262]. Both regular alcoholic beers as well as NABs can be produced by using non-*Saccharomyces* yeasts to generate novel and promising beer flavors. Particularly in the field of NABs there is a need for optimization with regard to flavor in order to reduce wort off-flavors. Non-*Saccharomyces* yeasts have great potential to reduce wort aldehydes during the fermentation process. The wort aldehydes are generally perceived as undesirable off-flavors by the consumer and can often be detected in LABs produced by arrested fermentation [57, 278]. Although the flavor formation is strain-dependent, for many years, the purpose of using the yeast species *S. ludwigii* for fermentation has been to degrade wort off-flavors in the brewing industry [57, 103]. In numerous studies, *S. ludwigii* is used as a control strain due to its commercial application and to compare fermentation characteristics of new alternative non-*Saccharomyces* yeasts with a known and well-studied non-*Saccharomyces* yeast species [27, 31, 43, 118]. Therefore, also in the context of this thesis, *S. ludwigii* was used as a well-known reference yeast species for the production of NABs. Yeast species as candidates for the production of alcoholic beers were compared either to a well-known *S. cerevisiae* or *S. pastorianus* reference yeast strain for beer production depending on the research background.

In this thesis, two main research topics were set: Flavor formation and carbohydrate metabolism. The carbohydrate metabolism is significantly related to the differentiation for the production of regular alcoholic beers, LABs or NABs. Both main topics will be discussed in the following, while further research approaches such as hop and ethanol tolerance as well as flocculation behavior are discussed where relevant. In addition, the topics of food safety and health effects, which were focused on in publication 5, are included in the discussion as they are highly relevant to the entire field of non-*Saccharomyces* yeast research.

Due to a lack of knowledge about the metabolic properties of a multitude of non-*Saccharomyces* yeasts in brewer's wort, a large-scale screening was conducted in publication 1. A new method was developed in 96-well microtiter plates to test a relatively large number of yeasts for brewing-relevant metabolic functions such as sugar metabolism (can be strain-specific, cf. Table 2), tolerance to hop acids and ethanol in a relatively short period of time. The general outcome was that hop as well as ethanol tolerance are basically given for most of the 110 investigated yeast strains to be suitable for the production of regular beers containing alcohol. Due to the relatively large number of yeast strains screened, it is most likely that this statement is transferable to further non-*Saccharomyces* yeast strains of the investigated species.

In conventional Pils beers, up to 38 mg iso-α-acids/L can be measured, while some IPAs reach up to 100 mg iso-α-acids/L [19]. Therefore, 50 mg iso-α-acids/L and 100 mg iso-α-acids/L were selected for the 96-well plate screening. All 110 screened yeast strains tolerated 50 mg iso-α-acids/L and were thus suitable for the production of Pils beers. Only one *D. hansenii* yeast strain of the total screened yeast strains, which accounted for less than 1%, did not show growth at 100 mg iso-α-acids/L. This proves

that most of the non-*Saccharomyces* yeast strains can even be used to ferment IPAs. Regarding hop *β*-acids, only 3% of the 110 yeast strains did not tolerate concentrations of 100–200 ppm while a mix of iso-*α* and *β*-acids was not tolerated by 7% of the investigated yeast strains. This leads to the assumption that the combination of both hop acids enhances the inhibitory effect. As mentioned in section 1.4.1, *β*-acids have poor solubility in beer as they are not isomerized during wort boiling [279]. Accordingly, *β*-acids can only impair yeast activity during cold hopping. However, since cold hopping usually takes place after the main fermentation, the impairment by *β*-acids is rather negligible. Nevertheless, cold hopping can be advantageous for inhibiting bacterial spoilage in beer due to the antimicrobial properties of *β*-acids [89].

A sufficient ethanol tolerance of suitable non-*Saccharomyces* yeasts is necessary for the production of regular alcoholic beers as ethanol acts as one of the greatest stress factors for the yeasts (cf. section 1.4.1) [85]. Additionally, it is relevant for the application in co-fermentations, provided that non-*Saccharomyces* yeast are added simultaneously to the fermentation with *Saccharomyces* brewing yeasts. If non-*Saccharomyces* yeasts are inoculated at the beginning of the fermentation only for the purpose of flavor formation, whereas a common *Saccharomyces* yeast is added at a later stage for ethanol production, insufficient ethanol tolerance could be advantageous in order to prevent competition between the different yeast species. Insufficient ethanol tolerance would inevitably stop the yeast cell metabolism of the non-*Saccharomyces* yeast, allowing the *Saccharomyces* yeast to prevail. The results from publication 1 show that a large number of yeast strains tolerate ethanol concentrations of 5% (*v*/*v*). Consequently, they could be applied to produce regular beers containing alcohol. The ethanol tolerance decreases significantly above an ethanol concentration of 8% (*v*/*v*), as around a quarter of the total of 110 yeast strains investigated no longer showed any growth. An ethanol concentration of 10% (*v*/*v*) inhibited growth of almost half of the examined strains. Only one of the 110 yeast strains did not show growth at 5% (*v*/*v*) ethanol, namely *C. misumaiensis* CM1. Since this strain is maltose-negative, it can still be used without restrictions for the production of NAB. However, it needs to be taken into consideration that in the case of co-fermentation with a *Saccharomyces* yeast, the latter would prevail due to its much stronger ethanol tolerance.

With regard to sugar utilization in the 96-well plate screening, it was noticeable that around 65% of the yeasts were able to metabolize sucrose, while maltose was only utilized by around 30% of the yeast strains. Maltotriose was metabolized by merely 25% of the investigated strains. As already explained in section 1.4.2, the yeasts, which can only take up the monosaccharides glucose and fructose, lack the corresponding gene segments to form an extracellular invertase as well as intercellular permeases and intracellular α-glucosidases [191, 315, 341]. One possible reason why relatively few yeasts have the ability to metabolize maltose can be traced back to the evolutionary history. The gene segments designated as MAL loci for the utilization of maltose (cf. Section 1.4.2) could be duplicated or deleted as a result of yeast domestication. This would explain why MAL genes are more abundant in beer and sake-related strains, while they are often absent in wine strains [58, 111]. Since less than one third of the investigated yeast strains were able to utilize maltose, which accounts for almost half of the available sugars in brewer's wort, there is – at least based on sugar utilization – greater potential to produce NABs using non-*Saccharomyces* yeasts. Moreover, a large proportion of the maltose-negative yeast strains formed fruity odorants in the sensory odor test of publication 1 and there is a greater need for sensory improvement in the area of NABs. Therefore, the focus in the publications 2 to 4 was on NABs and LABs. It may also be advantageous that 35% of the total of 110 investigated yeast strains cannot metabolize sucrose either. For these yeast strains, the amount of fermentable sugars is even lower

due to the lack of an extracellular invertase [341]. In order to stay below the 0.5% (*v*/*v*) ethanol required by German law for NABs (cf. section 1.3) [233, 346], the original gravity for sucrose- and maltose-negative yeasts can even be slightly increased although sucrose plays a minor role in the wort sugar composition with only around 3% related to a pale brewer's wort [240]. The resulting higher proportion of residual sugars is beneficial as this leads to an improved mouthfeel [179].

In the following, the sugar utilization results from the 96-well microtiter plate tests are compared with literature data based on Kurtzman [175] (cf. Table 2) to evaluate and discuss the significance of the screening method. According to Kurtzman [175], the species *C. misumaiensis*, *H. uvarum*, *K. servazzii*, *M. pulcherrima*, *N. holstii* and *P. kluyveri* can only metabolize monosaccharides. A total of 31 yeast strains of these six species were screened and just over 90% were found to be as described in literature. The *K. servazzii* yeast strain KS2 metabolized all tested sugars in the 96-well plate screening. Consequently, no measurement inaccuracy can be assumed. Nevertheless, the result would have to be verified in future studies. On the other hand, two *Metschnikowia* sp. were found to metabolize maltose, whereas no sucrose could be utilized. In these two cases, measurement inaccuracies most likely occured although the strain levels for the investigated *Meschnikowia* yeasts have not been clarified (cf. section 1.2.1).

Furthermore, Kurtzman described that the yeast species *C. saturnus*, *K. marxianus*, *L. kluyveri*, *S. ludwigii* and *T. microellipsoides* are able to ferment sucrose in addition to the monosaccharides glucose and fructose [175]. The statement from literature was true for all 13 yeast strains studied. *L. kluyveri* and *T. microellipsoides* could additionally utilize melibiose apart from glucose, fructose and sucrose. A clear differentiation between aerobic and anaerobic fermentation should be considered due a possible Kluyver effect (cf. section 1.4.2), as it was established in publication 2 that the Kluyver effect occurs, for example, in the yeast strain *L. kluyveri* 3082 during aerobic fermentation. This enables the strain to metabolize maltose apart from glucose, fructose and sucrose. Although *S. ludwigii* was not included in the 96-well plate screening, sugar utilization for this yeast strain (SL17) had been previously researched [207]. A yeast strain-dependent sucrose utilization is present in *K. lactis*, *T. delbrueckii*, and *Z. rouxii*. The ten investigated *K. lactis* yeast strains could, without exception, metabolize sucrose based on the results of the 96-well plate screening, while one of the eight *T. delbrueckii* yeast strains was unable to metabolize sucrose. Of the 14 *Z. rouxii* yeast strains, four strains appeared to lack invertase activity.

According to Kurtzman [175], *C. fabianii*, *S. fibuligera*, *Z. rouxii* and *Z. florentina* have the ability to metabolize glucose, sucrose and maltose. *C. fabianii* was not considered in the 96-well plate screening yet. However, the studies with maltose and maltotriose as selective media in publication 2 revealed that the two *C. fabianii* strains could metabolize maltose and maltotriose. In brewer's wort, metabolization was weak and slow, allowing the production of NABs. This is consistent with the statements made in literature [175]. Of the eight *S. fibuligera* yeasts investigated in the 96-well plate test, all strains metabolized maltose, without exception. However, the strain SF3/Lu27 stood out as it showed no growth in the selective media containing fructose and sucrose. The sugar utilization of this yeast strain was studied in greater depth in publications 2 and 3. In publication 3, the sugar transport systems for maltose and maltotriose were elucidated for *S. fibuligera*. The results from the two publications revealed that there were measurement inaccuracies in the screening, as the yeast strain SF3/Lu27 is able to utilize both fructose and sucrose. Furthermore, it was found that SF3/Lu27 as well as *S. fibuligera* SF4 possess a maltose permease to actively transport maltose into the cell and that both yeast strains can metabolize small amounts of maltotriose due to an extracellular amylase system, which could not be found in the 96-well plate screening [63, 110, 141, 176]. Possible reasons for the fact that

maltotriose could only be metabolized in small amounts were highlighted in publication 3. Photometric measurement proved challenging, especially for the yeast species *S. fibuligera* and will therefore be taken up later in the discussion. Of the total of 14 *Z. rouxii* yeast strains screened, five did not metabolize maltose, though this may have been due to a weak metabolism as indicated in the literature (cf. Table 2) [175]. *Z. florentina* grew only in glucose and fructose media in the microtiter plate. It would need to be verified whether this was due to measurement inaccuracies or whether the strain deviates from the literature data [175].

For *K. lactis*, *S. pombe*, *T. delbrueckii* and *W. anomalus*, maltose metabolism is variable and thus dependent on the yeast strain [175]. Of the nine *K. lactis* and eight *T. delbrueckii* strains screened, none had the ability to metabolize maltose. In contrast, all six *W. anomalus* yeast strains and seven out of the ten *S. pombe* strains metabolized maltose.

D. hansenii can metabolize glucose, sucrose and maltose either only weakly or not at all based on Kurtzman [175]. While all twelve strains investigated metabolized the monosaccharides glucose and fructose, two strains were unable to utilize sucrose and seven strains were unable to utilize maltose.

In summary, it can be concluded that, with few exceptions, the 96-well microtiter plate test yielded results with regard to sugar utilization that are consistent with previous findings from the literature [175]. 15 yeast strains were selected for publication 2, which were fundamentally suitable for the production of NABs due to their sugar utilization, ethanol and hop tolerance in the 96-well plate screening. In addition, the selection was based on a POF-negative as well as positive sensory odor test result from publication 1. Based on publication 2, it could indeed be proven that the 15 yeast strains are suitable for producing NABs with exceptional, novel flavors, confirming the successful application of the 96-well plate screening method. Moreover, the additional sugar utilization tests in selective media with maltose and maltotriose conducted in publication 2 led to comparable results to the 96-well plate screening. Accordingly, the results from the screening methods from publication 1 are significant and highly suitable for a screening of the suitability of new non-*Saccharomyces* yeast strains for beer brewing.

Although the 96-well microtiter plate test revealed a high suitability to investigate a large number of different yeast strains for brewing-relevant properties in a relatively short period of time, it needs to be taken into account that it is an initial screening method. Sensory odor tests of all yeast strains are inevitable and further brewing trials would still need to be conducted with the most promising yeast strains to investigate the flavor formation and metabolic properties in brewer's wort. Depending on the morphology of the yeast strain, growth irregularities can occasionally occur in the 96-well plate, which can cause measurement inaccuracies. Two different cases occurred that could lead to inaccuracies in the photometric measurement. The first case was a wreath-like growth on the outer wall of the well, which indicated a lower optical density (OD) and consequently weaker growth than had actually taken place, especially during the first two days of the test. This growth behavior is depicted in Figure 5A. The second case concerned the flocculation behavior of some yeast strains, which led to clump or sphere-like formation as shown in Figure 5B. In particular, the *S. fibuligera* yeast strains revealed a sphere-like growth in the selective media, which resulted in fluctuating or low OD values and were studied more in depth in the publications 2 and 3. Therefore, a daily visual control of the microtiter plates is recommended by applying the screening method, as these measurement inaccuracies can be easily comprehended.

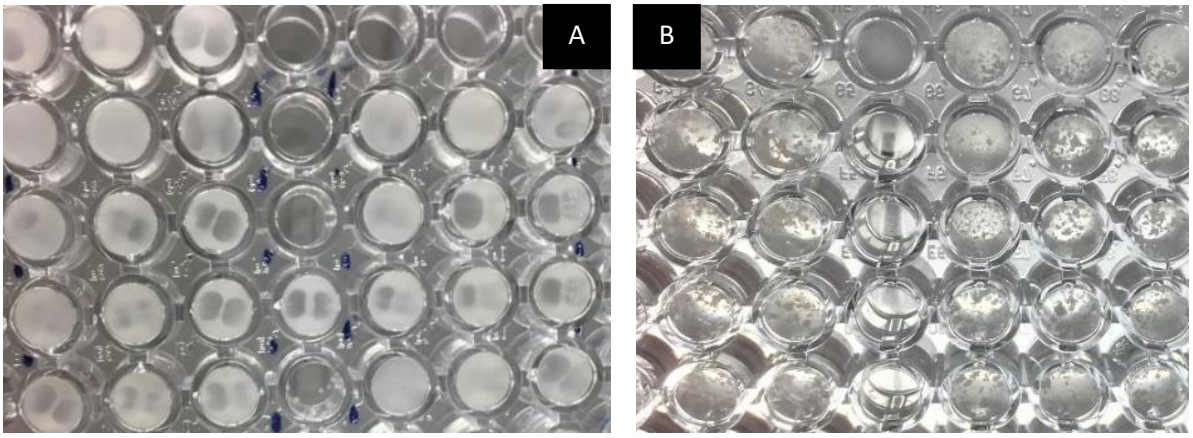


Figure 5. Cutouts of 96-well microtiter plates with irregular growth of selected yeast strains: A) showing partly wreath-like growth on the outer walls of the wells and B) showing the formation of flocculation and partly sphere-like growth.

As already addressed in the discussion and highlighted in publication 2, the morphology of *S. fibuligera* plays a crucial role during the photometric measurements as it differs depending on the media composition. In Yeast Peptone (YP) media, the morphology of *S. fibuligera* manifested itself in a macroscopically spherical growth (small gelatinous yeast balls), which influenced the measurement precision of the photometer negatively due to an inhomogeneous distribution of the yeast spheres. Figure 6 depicts the *S. fibuligera* yeast strain SF3/Lu27 compared to the *S. cerevisiae* reference yeast strain TUM 177 in cuvettes for measurement in a photometer.

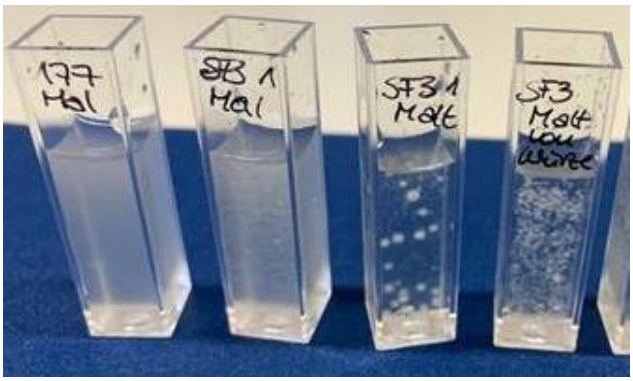


Figure 6. Morphology of the *Saccharomycopsis fibuligera* yeast strain SF3/Lu27 in 1:10 dilution (yeast:0.9% NaCl solution) in comparison to the reference yeast strain *Saccharomyces cerevisiae* TUM 177. 177 Mal: *S. cerevisiae* grown in Yeast Peptone (YP) medium + 1% maltose from wort slant agar; SF3 1 Mal: *S. fibuligera* SF3 grown in YP medium + 1% maltose from YM agar; SF3 1 Malt: *S. fibuligera* SF3 in YP medium + 1% maltotriose from YM agar; SF3 Malt from wort: SF3 strain from a fresh wort slant agar grown in YP medium.

Figure 6 shows the inhomogenous distribution of SF3 yeast spheres in a 1:10 solution consisting of one part of yeast cell suspension and nine parts 0.9% NaCl solution. While *S. cerevisiae* TUM 177, chosen as reference yeast strain, is homogeneously distributed in the solution, the morphology of *S. fibuligera* SF3 varies depending on the composition of the cultivation medium and whether a single colony of YM agar or yeast cell material from a wort slant agar was chosen for cultivation. Depending on the intensity of the spherical growth of *S. fibuligera*, the measurement accuracy in the photometer varies. In a complex medium, such as brewer's wort, a mycelium rather than spheres forms during the initial growth phase. Nevertheless, mycelial growth can also pose challenges for the flocculation and filtration of industrial beer production. For this reason, the flocculation behavior was investigated in greater detail in publication 3. A first successful approach to significantly reduce mycelial formation could be achieved by micromanipulating single cells and cultivating them for fermentations in 2-L small-scale

trials. However, no trials have been carried out on a pilot or industrial scale, nor have multiple consecutive trials been tested yet. These should be carried out in order to confirm an actual suitability for an industrial scale. The reduced mycelia growth had a negligible influence on the flavor formation, which is explained in publication 3. The reason why the morphology of *S. fibuligera* alters in different growth media could not be conclusively explained. Nevertheless, the flocculation behavior of the yeast species was discussed in detail in publication 3.

It was also not conclusively clarified why *S. fibuligera* can metabolize maltose and maltotriose in selective media within a short period of time, while sugar utilization from media with complex sugar composition, such as brewer's wort, proceeds only very slowly. This issue was already discussed in depth in publication 3. In summary, there were presumed interactions between glucose and maltose transporters, which can inhibit each other, or glucose was constantly available due to extracellular amylase activity, which in turn inhibited maltose uptake [99, 124, 164, 265]. A different behavior in selective medium and brewer's wort could also be observed for other yeast species. For example, the yeast strain *Z. rouxii* ZR9 metabolized maltose and maltotriose in the 96-well plate test, while the yeast strain *S. pombe* S11 showed only maltose activity. Nevertheless, in publication 1, *S. pombe* S11 revealed a significantly higher fermentative activity in brewer's wort than *Z. rouxii* ZR9, although a reverse behavior could have been assumed on the basis of the results. As a general finding, it can be stated that the carbohydrate metabolism of this yeast species in selective medium is not transferable to a complex medium like brewer's wort. In the case of *S. fibuligera*, the brewer can take advantage of the behavior in terms of slow sugar metabolism to produce either NABs or LABs, although in principle the yeast can also be used to produce beers containing alcohol by increasing the original gravity. It should be mentioned though, that the yeast strain *S. fibuligera* SF4 achieved a final ethanol concentration of 3.10% (*v*/*v*) during the fermentation of a 12.5 °P wort in publication 1. In contrast, in publication 3, only ethanol concentrations between 0.83 and 1.20% (*v*/*v*) were reached during the fermentation of a 10 °P wort at a comparable fermentation temperature of 27-28 °C, depending on whether it was the mother culture or the micromanipulated culture. This corresponded to a final apparent attenuation of almost 47% in publication 1, whereas in publication 3, apparent attenuations of around 16–23% were reached. Although the results from publication 3 show that there is already a 7% difference in apparent attenuation between the use of the mother culture and the micromanupulated culture, it could not be conclusively clarified why a significantly higher apparent attenuation was achieved in publication 1. A contamination by a *Saccharomyces* yeast cannot be completely excluded in publication 1 despite adherence to a hygienic working method. If contamination did occur, it did not affect the flavor of the beer as the profiles from publication 1 and 3 are within a comparable range. It would be interesting to test co-fermentations with *S. fibuligera* and *S. cerevisiae* in future studies. It might be possible that the fruity, plum and berry-like flavors produced by *S. fibuligera* during wort fermentation will remain and dominate, while the ethanol content in the beer could be increased by the *Saccharomyces* yeast.

In order to obtain an initial impression of the flavor formation of the yeast strains, a sensory odor test was carried out in brewer's wort in publication 1. As the test is simple and quick to perform, it is ideally suited to this purpose. However, it must be considered that the test was carried out under aerobic conditions. Although the wort is aerated before fermentation to ensure an initial yeast cell proliferation, the yeasts shift into anaerobic metabolism as soon as the oxygen is consumed. Without an oxygen supply, anaerobic fermentation activates different metabolic pathways than aerobic fermentation, which can lead to different flavor formations. This becomes apparent by comparing the results of selected yeast strains from the descriptive sensory odor impressions in 75 mL wort from publication 1

with the flavor impressions in the beers produced in 2-L fermentations from publications 1 and 2. Since a 7 °P wort was used in publication 2 and a 12.5 °P wort was chosen in publication 1, the odor impressions from publication 1 are compared in Table 4 only with the corresponding evaluation for 7 °P or 12 °P.

Table 4. Comparison of sensory odor impressions in wort (aerobic fermentation) against flavor impressions in beer (anaerobic fermentation).

Yeast strain	Strain Abbr.	Sensory odor impression	Flavor impression in beer
C. misumaiensis	CM1	isoamyl acetate	fruits, honey, caramel
C. misumaiensis	CM3/238	fruits	neutral, kiwi, gooseberry, citrus, honey
C. saturnus	CSa1	isoamyl acetate	cool mint sweets, pear, banana
K. servazzii	KS3/3C4	fruits, floral	floral, honey, red berries
K. lactis	K9/G9K	fruits	fruits, apple
K. marxianus	Km4/653	fruits	apple, red berry, honey, stone fruit, butter
L. kluyveri	LK1/3082	neutral, acidic	red berry, stone fruit, apple, honey, butter
P. kluyveri	PK1	yeast, isoamyl acetate	neutral, banana, cool mint sweets, solvent
S. fibuligera	SF3/Lu27	fruits	stone fruit, plum, red berry, apple, honey, butter
S. fibuligera	SF4	fruits, plum, honey	fruits, plum, strawberry
S. pombe	G10S	fruits, sulfurous	apple, red berry, stone fruit
S. pombe	S11	fruits, honey	floral, grape, honey, clove
T. delbrueckii	T26/1I6	neutral	neutral, marzipan, wort
Z. rouxii	ZR9	fruits	wheat beer, fruits, floral, clove

In general, the flavor impressions in beer, which were evaluated by smelling and tasting, are more precise than only the odor impressions in wort. The comparison in Table 4 shows that there were indeed strain-dependent differences between the aerobically fermented odor samples and the anaerobically fermented beers in terms of flavor formation. While the yeast strain *C. misumaiensis* CM1 formed isoamyl acetate under the influence of oxygen, which can be manifested as banana, pear or cool mint sweets, none of these odor components could be detected in the beer. Although the beer was rated as generally fruity, honey and caramel were also perceived. To preserve the flavor substance isoamyl acetate in the beer, it could be investigated in future trials whether a controlled, continuous aeration with oxygen during fermentation would lead to the desired result. However, before the end of fermentation, it must be ensured that the oxygen is completely consumed by the yeasts to avoid oxidation of the beer which would negatively affect the beer flavor [125]. The yeast strain *C. misumaiensis* CM3/238 also lost fruitiness during anaerobic fermentation. Although a general fruitiness remained, it was not dominant, allowing a more neutral beer to be produced with this yeast strain. In case the flavor formation is significantly enhanced with an aerobic fermentation, a possible Kluyver effect (cf. section 1.4.2) of the yeast should be taken into account when oxygen is added. If the Kluyver effect occurs, the yeast is Kluyver-effect positive and has the ability to metabolize further sugars, depending on the species, and an increased ethanol formation may occur [109].

The yeast strain *C. saturnus* CSa1 produced clearly perceptible amounts of isoamyl acetate during both aerobic and anaerobic fermentation, and *K. servazzii* KS3/3C4 showed no noticeable differences either. Similar results were obtained with the yeast strains *K. lactis* K9/G9K, *K. marxianus* Km4/653, *P. kluyveri* PK1, *S. fibuligera* SF3/Lu27, *S. fibuligera* SF4 and *T. delbrueckii* T26/1I6. Although, with the exception of *P. kluyveri*, no specific odor components could be detected in the sensory odor test which were described as generally fruity or neutral. However, the neutral flavor of the *T. delbrueckii* yeast strain was defined as wort-like in the beer. Thus, the yeast strain is not ideally suitable for producing beers with promising flavors. In the sensory odor test, *L. kluyveri* LK1/3082 produced a neutral and acidic aroma. A broader spectrum of flavors could be perceived in the beers during the tastings. Fruity flavors were detected, as well as honey and buttery notes. The yeast strain *S. pombe* G10S produced fruity, but also sulfurous notes in the sensory odor test. Sulfurous flavor compounds could not be recognized in the beers. Instead, fruity flavors dominated. In this case, the yeast strain is more suitable for anaerobic fermentation and can therefore be ideally used to produce NABs. *S. pombe* S11 and *Z. rouxii* ZR9 produced fundamentally similar flavor components during aerobic and anaerobic fermentation. However, floral and clove-like flavors were additionally perceived after anaerobic fermentation with both yeast strains. Since both yeasts were POF-negative, the clove-like flavor could either be traced back to eugenol, which was not analyzed, or complex interactions of the different flavor compounds could have triggered the wheat beer flavor [212]. Contamination, for example by a POF-positive *S. cerevisiae* [206], cannot be completely excluded either although care was taken to work hygienically. In general, yeasts capable of producing phenolic flavors during fermentation of brewer's wort were excluded for further investigations. The reason was that phenolic flavors are not novel flavors in beer, as they can already be found in many wheat beers above the threshold level and lead to a clove-like flavor [206]. Therefore, POF-positive yeasts were not within the objective of this thesis. Nevertheless, maltose-negative yeast strains with POF-positive properties could be interesting for future research as non-alcoholic wheat beers are occasonally described as having wort off-flavors depending on the production method, which can potentially be eliminated by using suitable POF-positive non-*Saccharomyces* yeast strains. For example, in the research work from publication 1, a *D. hansenii* (DH1), a *N. holstii* (NH2) and a *Z. florentina* (ZF1) yeast strain were found which were positively rated in the odor test, but maltose-negative and POF-positive at the same time. These strains could be tested in future studies for their suitability in the production of non-alcoholic wheat beers. Additionally, co-fermentations with POF-positive and POF-negative, maltose-negative non-*Saccharomyces* yeasts offer potential to combine clove-like and fruity flavors. Sensory tastings would be essential to determine which combinations of yeasts and pitching rates form a harmonious flavor profile.

Since this thesis specifically focused on yeast species that produced promising novel flavors during the fermentation of brewer's wort, a literature research was conducted beforehand and was continued in the course of this work. The results of the research are summarized in Table 3. Subsequently, it will be discussed whether similar flavor properties can be expected within a yeast species during wort fermentation and whether the findings of this thesis are in line with those from the literature. For *C. saturnus*, *D. hansenii*, *L. kluyveri*, *N. holstii*, *S. fibuligera* and *T. microellipsoides* no conspicuous flavor substances are described in literature during fermentation of brewer’s wort as already mentioned in Table 3. Consequently, a comparison and discussion are impossible. Furthermore, no significant comparison can be conducted for *H. uvarum*, *Meschnikowia* sp., *W. anomalus* and *Z. florentina* as these species were only examined for their flavor formation in the odor test within the scope of this thesis, while no fermentations were carried out.

The flavor formation of *C. fabianii* has already been described in literature as variable, as van Rijswick et al. found high levels of esters resulting in fruity flavors, while Bellut et al. describes unpleasant flavors in the beer [30, 330, 331]. In publication 2, two different *C. fabianii* yeast strains were therefore investigated, both of which produced a rather non-specific fruity flavor during fermentation. Therefore, the flavor impressions are closer to the study results of van Rijswick et al. Apple-like and buttery flavors dominated, with the buttery note being perceived as quite harmonious, particularly in the beer fermented with the yeast strain C. fab 5650, as the beer was awarded a good DLG score of 4.44. The *C. misumaiensis* yeast strain studied by Bellut et al. also produced unpleasant flavors due to high concentrations of ethyl acetate (65.7 mg/L). Isoamyl acetate was measured at concentrations around the flavor threshold of 0.90 mg/L (cf. Table 3) [30]. The two *C. misumaiensis* yeast strains investigated in publication 2 did not reveal isoamyl acetate or ethyl acetate concentrations above the thresholds. Ethyl acetate ranged from 10.5–15.9 mg/L in the two beers, while isoamyl acetate ranged from 0.23–0.33 mg/L. Moreover, both beers were rated as predominantly neutral with a slightly fruity note and no noticeable off-flavors. Consequently, the selection of the yeast strain seems to have a significant influence on the beer flavor for both *C. fabianii* and *C. misumaiensis*.

Based on previous studies, *K. servazzii* was described as being able to increase fruitiness and to produce pear and apple notes [119, 156]. Although pear and apple flavors were not found in the beer produced with *K. servazzii* KS3/3C4, fruity flavors were detected. It can therefore be assumed that the species generally increases the fruitiness of beers. In addition, the yeast strain KS3/3C4 produced floral notes, which not every *K. servazzii* strain appears able to synthesize. In contrast, *K. lactis* was described in literature to produce mainly floral and honey flavors during wort fermentation and also in synthetic glucose wort media [119, 127]. On a glucose wort plate, it synthesized a strawberry fragrance. The fermentations in publication 2 resulted in more fruity than floral flavors, most reminiscent of apple. Literature describes rose-like flavors during wort fermentation also by *K. marxianus* which can be traced back to 2-phenylethyl acetate in concentrations of 3.9 mg/L [119, 156]. Although honey-like flavors could be found after fermentation with the *K. marxianus* yeast strain 653 in publication 2, which can possibly arise from 2-phenylethyl acetate, only small amounts of 0.35 mg/L could be measured in the beer. The fruity, apple and red berry flavors were more dominant in the beer fermented with *K. marxianus* 653. Consequently, both *Kluyveromyces* yeast strains investigated in this thesis produced more pronounced fruity than floral flavors even though the floral flavors are known from literature.

The yeast species *P. kluyveri* does not seem to have strong deviations in its basic flavor formation in brewer's wort. From existing literature, it is known that the species releases high concentrations of esters, especially isoamyl acetate, and thus produces a distinct fruitiness and banana flavor [139, 274]. Nevertheless, there appear to be strain-dependent differences, as the *P. kluyveri* yeast strain PK1, which was investigated in this thesis, increased the fruitiness only slightly with isoamyl acetate concentrations above the odor threshold. Ethyl acetate led to solvent-like flavors similar to those reported by Holt et al., while no further significant concentrations of esters could be detected as discovered by Saerens and Swiegers [139, 274].

For the four yeast species *S. ludwigii*, *S. pombe*, *T. delbrueckii* and *Z. rouxii*, more frequent research has already been conducted in relation to beer production than for the other yeasts investigated in this thesis. Regarding the flavor formation, the four species all have varying results, which can be found in Table 3. Therefore, it can be concluded, that the flavor formation is strongly strain-dependent for these species and a suitable yeast strain can be selected depending on the desired target flavor of the beer. Since all four yeast species tend to create beers with diacetyl concentrations above the odor threshold

value depending on the selected strain, further research could focus on this topic [31, 43, 50, 103, 241, 304]. It is not only possible to search for suitable strains that release less diacetyl during fermentation, but also technologically, a longer maturation of the beer could lead to improved diacetyl degradation [170].

The comparison of the flavor formation by the yeast strains investigated in this thesis with the results found in literature generally leads to the conclusion that there is a significant strain dependence. This seems slightly less pronounced for the species *P. kluyveri*, which forms isoamyl acetate as the main flavor component. Also, between the *C. saturnus* strains investigated in publication 2, only relatively small differences in flavor expression were found within the species and the main flavor component was isoamyl acetate. The formation of isoamyl acetate is enzyme-catalyzed by AATases and these are localized in specific gene segments (cf. section 1.4.3) [106, 196, 355]. It has not yet been clarified whether the yeast species *C. saturnus* and *P. kluyveri* possess the same gene segments for the formation of isoamyl acetate as *S. cerevisiae*. However, the results from this thesis indicate that the gene segments are species-specific. Nevertheless, technological factors play a decisive role regarding the flavor formation. In publication 4, the influence of technological factors such as temperature, original gravity and pitching rate, was demonstrated and discussed in detail using the example of the yeast strain *C. saturnus* 247. Depending on the settings of these factors, *C. saturnus* produced variable flavor characteristics. Furthermore, in publication 1 the sensory odor test showed that even a difference in original gravity between 7 and 12 °P can lead to deviating odor impressions depending on the yeast strain. Therefore, not only can the beer flavor be influenced by the yeast strain selection, but it can also be influenced by specifically adjusting the fermentation parameters. Optimization with regard to the desired flavor profile is individual for each yeast strain and can be carried out by applying Response Surface Methodology (RSM). The suitability of this method has been demonstrated by the results of publication 4. Moreover, earlier studies stated a successful optimization based on RSM [30, 221, 263]. The Design of Experiment can be customized depending on the desired flavor of the target application. In addition, raw materials and brewing processes can be varied to further alter the beer flavor and the flavor stability. For example, significant flavor variation can be achieved by using aroma hops or specialty malts, and depending on the mashing and wort boiling process, the beer flavor can also be influenced [34, 142, 226, 239]. In publication 4, the influence of the thermal load on the beer flavor was already mentioned and is known to promote instability in beer flavor [209, 248]. De Schutter dealt intensively with the thermal influence during wort boiling on the flavor stability of beer and summarized that most of the key flavor components in wort are based on dimethylsulfide (DMS) formation, lipid oxidation and Maillard reactions which includes Strecker degradation [294]. The thermal load causes the accumulation of Maillard intermediates. Although some of these intermediates can be reduced by yeasts during fermentation, the remaining amount can lead to off-flavors during beer aging [291]. For publications 1 to 5, it needs to be mentioned with regard to the flavor formation that malt extract was used for the wort preparation. As a result, the thermal input was increased compared to a conventionally produced wort. The use of malt extract has the advantage of wort standardization. However, since it is a natural product, it is not possible to maintain an identical quality between the individual production batches. To ensure an identical product, a synthetic medium would need to be selected. Approaches with synthetic wort medium have, for example, been conducted by Saerens et. al or Weigert [276, 345]. A disadvantage, however, would be that brewer's wort has a complex composition and the metabolism of the yeasts may therefore work differently. If non-*Saccharomyces* yeasts that only partially metabolize the wort sugars during fermentation due to their physiological properties are used to produce NABs or LABs, thermal stabilization of the beer is essential. Due to the

residual sugars remaining in the beer, there is a risk that other yeasts, which have the ability to utilize these residual sugars from the beer, could contaminate and proceed with the fermentation process. Consequently, the ethanol content in the beer would increase and the flavor of the beer would most likely be modified. Additionally, microbiological safety could no longer be guaranteed. The use of sour wort or sour malt is recommended if the non-*Saccharomyces* yeasts cannot induce a sufficient pH drop to 4.2–4.6 in the beer as already mentioned in section 1.3. The yeast strains investigated in publication 2 were only partially able to induce a sufficient pH drop and fluctuated in the range between pH 4.6–5.1. A sufficient pH drop impacts the taste of the beer positively and produces a safer product from a microbiological point of view as the growth of pathogenic microbes is inhibited in beer at a pH below 4.6 [13]. Although ethanol also plays a crucial role in inhibiting the growth and survival of pathogens, a decreasing pH has a beneficial effect on their inhibition. Moreover, there is a synergistic antimicrobial effect between pH and hop *iso*-α-acids [213]. A specific selection of the non-*Saccharomyces* yeast strain can contribute to avoiding additional acidification. While it has already been found in the publications 2 and 3 that *S. fibuligera*, *K. marxianus* and *C. misumaiensis* are able to release a sufficient amount of acids despite weak fermentation performance, further yeast species are known from literature for their acid formation [28]. *L. thermotolerans*, for example, has already been mentioned in publication 3 to form high amounts of lactic acid at the expense of ethanol during fermentation [298]. *H. vineae*, *L. fermentati*, *Schizosaccharomyces japonicus* and *W. anomalus* also appear in literature in the context of sour beer production due to their ability to produce lactic acid during fermentation [28, 55, 86, 254, 270]. Moreover, co-fermentations could be considered to combine a flavor-neutral, acid-releasing non-*Saccharomyces* yeast with a flavor-producing species to use potential synergies. However, it would have to be tested in practical trials whether the co-fermentation leads to the desired synergies.

In general, with regard to flavor formation, it must be considered that the wort composition varies due to the variability of the raw materials [211]. The amino acid composition of the wort was not considered in any of the publications 1 to 5 and would therefore be interesting for future research work. There is evidence that variation in the wort spectrum can trigger major changes in the final beer flavor in this respect. Worts with high percentages of glucose and fructose have higher ester levels than maltose-rich worts as the two monosaccharides presumably stimulate the glycolytic pathway. This, in turn, leads to higher levels of acetyl CoA which is necessary for the formation of esters as described in section 1.4.3 [46, 184]. Furthermore, with regard to flavor formation by non-*Saccharomyces* yeasts, the odor thresholds should be redefined, especially for NABs. This was already discussed in publication 2, since flavors that remained below the instrumental analytics threshold values from literature references could be perceived during the sensory evaluations. A possible approach for NABs could be the use of thresholds based on water instead of regular beers due to the low ethanol content. In addition, synergistic effects of different flavor-active substances could play a crucial role. The flavor analysis of beers produced with non-*Saccharomyces* yeasts would have to be extended in order to detect novel beer flavors, which are currently not analyzed as standard in beers. By applying a full flavor analysis, as exemplified in publication 4 for the yeast strain *C. saturnus* 247, characteristic flavor-active substances could be detected to gain further insights into the flavor formation of non-*Saccharomyces* yeasts during wort fermentation.

The topics of microbial safety and health effects are relevant to all publications in this thesis and were focused on in publication 5. In the introduction (cf. Section 1.4.4), instances were described that investigate and discuss the safe use of microbes, including *Saccharomyces* and non-*Saccharomyces* yeasts.

A safe and harmless use of yeasts for food fermentations, and in this case specifically for brewing, must be ensured. This includes ensuring that the yeasts do not produce any substances of concern during fermentation. If the yeasts are inactivated after fermentation, for example by pasteurization or sterile filtration, they can no longer influence the final product via their own metabolic activities. Nevertheless, it is possible that the microorganisms synthesize and release substances during fermentation which may be harmful to health and which are not eliminated during pasteurization or sterile filtration, such as biogenic amines.

In regular uncontaminated beers, the average concentration of biogenic amines is between 8–30 mg/L which is considered safe [192, 240]. Several studies have been conducted in the past to measure biogenic amines in beers [47, 150, 271] and were already summarized in a review by Kalac and Krízek [159]. Details on biogenic amines in beer have already been highlighted and discussed in publication 5 and should definitely be considered in the context of using novel non-*Saccharomyces* yeasts on a strain-specific base for the production of beers although only very low concentrations of biogenic amines could be measured for the 16 non-*Saccharomyces* yeast strains studied in this thesis. Apart from biogenic amines, it was already mentioned in the introduction (cf. section 1.4.4) that methanol (from pectin) or urea as precursor of ethyl carbamate can also be potentially harmful substances in beer [307, 314]. These substances could therefore also be analyzed in future studies. Furthermore, nitrosamines detected in beers were formerly discussed as having carcinogenic properties, generated during the malt kilning process [33]. These concerns could be mitigated by changes in production technology although this does not necessarily apply to developing countries [177]. In a more recent publication, Fan and Lin also recommend further studies on nitrosamines in beer for a better risk assessment [98]. Although nitrosamines do not appear to be produced by metabolic properties of yeasts, all potentially harmful substances should be considered in order to sell beers that are completely harmless to the customer's health.

However, since both substances which arise during the beer production process and the microorganisms themselves may pose potential risks for consumption, only species that are considered absolutely safe should be used for beer brewing. In the introduction in section 1.4.4. the yeast species that have been granted QPS status by EFSA were listed, among them, *D. hansenii*, *H. uvarum*, *K. lactis*, *K. marxianus*, *S. pombe* and *W. anomalus*, which were tested for their suitability for beer brewing in this thesis [267]. Additionally, *Z. rouxii* was proposed to be included on the QPS list [166]. In the context of publication 5, it was found that in addition to the yeast species considered safe by EFSA, *C. misumaiensis*, *C. saturnus*, *K. servazzii*, *S. ludwigii*, and *T. delbreuckii* showed no growth at 37 °C. This is consistent with data from literature, which indicate that *C. misumaiensis* stops growing at 35 °C, *C. saturnus* and *S. ludwigii* do not grow at 37 °C, whereas growth at 37 °C is variable for *K. servazzii* and *T. delbreuckii* [38]. For the yeast strains investigated in publication 5, the risk of harming the human organism is significantly reduced, since they are not able to proliferate at human body temperature of 37 °C. *C. fabianii*, *L. kluyveri* and *S. fibuligera* showed significant growth at 37 °C. Therefore, particular caution is required when using them to ferment beers. While the three yeast species are known from other food fermentations as stated in Table 1 [3, 30, 156, 157, 183, 187, 330, 331, 350, 352] and *S. fibuligera* has even been mentioned as possessing probiotic potential [178], any health risks must be ruled out before the yeast species can be considered safe. Moreover, for the three species, the findings from publication 5 are in line with existing knowledge, as *C. fabianii* and *S. fibuligera* are able to grow at 37 °C according to literature references, while growth for *L. kluyveri* is variable at 37 °C [38]. With regard to *P. kluyveri*, it is known that the species can variably grow at 37 °C depending on the yeast strain

[38]. This corresponds to the strain investigated in publication 5, which grew at 37 °C. For a specific *P. kluyveri* yeast strain from Chr. Hansen A/S (Denmark), a "no objection letter" already exists from the FDA supporting the strain for beer application with GRAS status [222]. Based on existing literature, *M. pulcherrima* and *N. holstii* can grow strain-dependently at 37 °C, whereas *T. microellipsoides* shows no growth above a temperature of 35 °C [38]. Thus, if *M. pulcherrima* or *N. holstii* strains are considered for beer production, growth behavior at 37 °C should be investigated as fundamental research. According to literature, *Z. florentina* does not possess the ability to grow at 37 °C [38]. The yeast species has further been investigated in several studies for its suitability for beer brewing [55, 139, 244].

In summary, it can be concluded that the *C. fabianii*, *L. kluyveri*, *M. pulcherrima* and *N. holstii* yeast strains investigated in this thesis should be given particularly greater attention with regard to a safe and harmless application in beer brewing. Nevertheless, all yeast species that were not granted QPS or GRAS status must also be used with caution. A potential risk can be minimized by filtering the beer and subsequent pasteurization to remove the yeast cells from the product and inactivate any residues. Moreover, the yeast strains should be further investigated for antifungal resistance, potential allergic reactions, and pathogenic potential by genome sequencing [222, 244].

Although the consideration of health concerns is mandatory, positive health benefits are summarized in the introduction in section 1.4.5. Of the mentioned health-promoting substances, some B vitamins, such as biotin and folate, can be synthesized by yeasts and were therefore of relevance for the investigations into non-*Saccharomyces* yeasts [153, 349]. Bamforth summarized relevant B vitamin concentrations in beers in a review [18]. For thiamine (B_1) he indicated a range of 3–80 µg/L, for riboflavin (B_2) 20–800 µg/L. For niacin (B3) 3000–8000 µg/L were indicated, while for pyridoxine (B_6) 70–1700 µg/L were listed. Biotin (B_7) was indicated at 2–15 µg/L, folate (B_9) at 40–600 µg/L, and cobalamin (B_{12}) at 3–30 µg/L. For pantothenic acid (B_5), no values were provided [18]. In the beers examined from publication 5, which were produced by using *S. ludwigii* SL17, *C. saturnus* 247 and CSa1 and *K. marxianus* 653, thiamine was found in concentrations below the limit of determination of < 100 µg/L. Around 250 µg/L riboflavin, around 5300 µg/L niacin, between 500–580 µg/L pantothenic acid, around 250 µg/L pyridoxine, between 0–3.2 µg/L biotin, approx. 40 µg/L folate and between 1.15–1.25 µg/L cobalamin were analyzed. Although Bamforth's data are based on regular beers containing ethanol and the beers analyzed in publication 5 were NABs, most B vitamins were in the ranges reported by Bamforth [18]. Folate was at the lower minimum at about 40 µg/L, while biotin was in the target range of 2–15 µg/L depending on the applied yeast strain. The beer fermented with the yeast strain *S. ludwigii* SL17 was just below 3.2 µg/L, while biotin in the other three beers was below the limit of determination (< 1.0 µg/L). Cobalamin was determined in all beers below the reference range given by Bamforth [18]. This may be due to the fact that cobalamin cannot be synthesized by yeasts and is carried over into the beer by the raw material barley malt depending on the strength of prokaryote growth during germination on the grains [167]. The selected low original gravity of 7 °P in the NABs in publication 5 may therefore be decisive for the correspondingly lower cobalamin concentrations.

In general, the database for B vitamins in NABs is weak. Moreover, in commercially produced beers it is often not transparent which production process was chosen and whether non-*Saccharomyces* yeasts were used. This poses a challenge for a direct comparison with the beers from publication 5. Zabihpour et al. published some B vitamin concentrations of NABs from the Iranian market and measured concentrations between 19.630–68.470 µg/kg for thiamine, 20–80 µg/kg for riboflavin and 9.020–43.110 µg/kg for pyridoxine (Zabihpour et al., 2021). With the exception of riboflavin, the published concentrations are extremely high and raise the question of whether a vitamin addition occurred in

the NABs that would not be permitted in compliance with the German Purity Law. While in publication 5, no yeast strains were found that could synthesize significant amounts of B vitamins during brewer's wort fermentation, there is still extensive research potential in this area.

To summarize, an equally extensive research potential exists for the investigation of further non-*Saccharomyces* yeasts for beer brewing to diversify beer flavor. Since flavor variations can already be achieved on a strain level, which has been established in numerous studies (cf. Table 3) as well as in the context of this thesis, the potential variety of beers is almost inexhaustible. Furthermore, co-fermentations offer great potential, which was not investigated in this thesis. Research in this area could be intensified in order to further increase the flavor variety and to extend the final apparent attenuations. The application of *Saccharomyces* yeasts in co-fermentations with non-*Saccharomyces* yeasts offers great potential for diversifying beer flavor while also achieving higher ethanol concentrations.

It is also essential that the experiments from the 2-L small-scale fermentations mainly applied in this thesis, will be transferred to a pilot scale and later to an industrial scale. Adjustments may be necessary in the course of upscaling, which can often already be made at the pilot stage. The best results can be implemented on an industrial scale. It might vary, depending on the yeast strain, whether a yeast can be transferred from small to large scale without extensive adjustments. Existing studies state that upscaling of experimental beers using non-*Saccharomyces* yeasts can be successful [30, 221].

Since the focus of this thesis was on the production of NABs with novel flavor profiles, some options will be presented below to further enhance the sensory profile of NABs produced by biological process in compliance with the German Purity Law. These can also be regarded as an outlook for further research. Ethanol is a flavor carrier, the absence of which can no longer compensate for possible off-flavors in the beer [227, 303]. High-gravity brewing increases the formation of higher alcohols and esters [7, 43]. Even after dilution with brewing water, the fruity character is retained [240, 275]. To counteract an unbalanced, overly fruity or solvent-like flavor impression caused by high-gravity brewing, the oxygen supply and fermentation temperature can be adjusted accordingly [4, 234]. Furthermore, CO_2 stripping of wort or beer can intensify the interaction between the yeast and medium and expel undesirable wort flavor compounds [7, 227]. Another option for the improvement of the sensory profile is an addition of aroma hops at a higher dosage at the end of wort boiling or by placing the hops in the whirlpool in advance to mask off-flavors [102]. Furthermore, cold hopping after fermentation offers an opportunity to introduce additional hop flavor compounds into the beer. The type of hop, the blending, temperature and contact time are parameters that can be adjusted to influence an effective transfer of hop flavor substances into the beer [233, 252].

4 References

[1] Adamenko, K., Kawa-Rygielska, J., and Kucharska, A. Z. 2020. Characteristics of Cornelian cherry sour non-alcoholic beers brewed with the special yeast *Saccharomycodes ludwigii*. *Food Chem.* 312, 125968.

[2] Agarbati, A., Canonico, L., Marini, E., Zannini, E., Ciani, M., and Comitini, F. 2020. Potential Probiotic Yeasts Sourced from Natural Environmental and Spontaneous Processed Foods. *Foods (Basel, Switzerland)* 9, 3, 287.

[3] Aidoo, K. E., Nout, M. J. R., and Sarkar, P. K. 2006. Occurrence and function of yeasts in Asian indigenous fermented foods. *FEMS Yeast Res.* 6, 1, 30–39.

[4] Anderson, R. G. and Kirsop, B. H. 1974. The Control of Volatile Ester Synthesis during the Fermentation of Wort of High Specific Gravity. *J. Inst. Brew.* 80, 1, 48–55.

[5] Andorrà, I., Berradre, M., Rozès, N., Mas, A., Guillamón, J. M., and Esteve-Zarzoso, B. 2010. Effect of pure and mixed cultures of the main wine yeast species on grape must fermentations. *Eur. Food Res. Technol.* 231, 2, 215–224.

[6] Anfang, N., Brajkovich, M., and Goddard, M. R. 2009. Co-fermentation with *Pichia kluyveri* increases varietal thiol concentrations in Sauvignon Blanc. *Aust. J. Grape Wine Res.* 15, 1, 1–8.

[7] Annemüller, G. and Manger, H. J. 2013. *Gärung und Reifung des Bieres*. VLB, Deutschland: Berlin.

[8] Anness, B. J. and Bamforth, C. W. 1982. Dimethyl sulphide—a review. *J. Inst. Brew.* 88, 4, 244–252.

[9] Arruda Moura Pietrowski, G. de, dos Santos, C. M. E., Sauer, E., Wosiacki, G., and Nogueira, A. 2012. Influence of fermentation with *Hanseniaspora* sp. yeast on the volatile profile of fermented apple. *J. Agric. Food Chem.* 60, 39, 9815–9821.

[10] Axcell, B. C. 2007. R&D in brewing: the importance of underpinning knowledge. *Proc. Eur. Brew. Conv.* 31, 173.

[11] Azzolini, M., Tosi, E., Lorenzini, M., Finato, F., and Zapparoli, G. 2015. Contribution to the aroma of white wines by controlled *Torulaspora delbrueckii* cultures in association with *Saccharomyces cerevisiae*. *World J. Microbiol. Biotechnol.* 31, 2, 277–293.

[12] Back, W. 2005. *Ausgewählte Kapitel der Brauereitechnologie*. Hans Carl Verlag, Nürnberg.

[13] Back, W. and Bohak, I. 2008. *Ausgewählte Kapitel der Brauereitechnologie*. Hans Carl Verlag, Nürnberg.

[14] Baert, J. J., Clippeleer, J. de, Hughes, P. S., Cooman, L. de, and Aerts, G. 2012. On the origin of free and bound staling aldehydes in beer. *J. Agric. Food Chem.* 60, 46, 11449–11472.

[15] Baiano, A. 2021. Craft beer: An overview. *Compr. Rev. Food Sci. Food Saf.* 20, 2, 1829–1856.

[16] Baiano, A., Conte, A., and Alessandro Del Nobile, M. 2012. Beer: Advances in Processing and Preservation. *Recent Patents on Engineering* 6, 2, 83–95.

[17] Baiano, A. and Petruzzi, L. 2017. The role of starter cultures and spontaneous fermentation in traditional and innovative beer production. In *Starter Cultures in Food Production*. John Wiley & Sons, Ltd, 231–254. https://doi.org/10.1002/9781118933794.ch12.

[18] Bamforth, C. W. 2002. Nutritional aspects of beer—a review. *Nutr. Res.* 22, 1-2, 227–237.

[19] Bamforth, C. W. 2003. Wort Composition and Beer Quality. In *Brewing yeast fermentation performance*, K. Smart, Ed. Blackwell Science, Oxford, UK, Malden, MA, 75–85. https://doi.org/10.1002/9780470696040.ch7.

[20] Bamforth, C. W. 2006. *Scientific principles of malting and brewing*. American Society of Brewing Chemists, St. Paul, Minn.

[21] Barbosa, C., Lage, P., Esteves, M., Chambel, L., Mendes-Faia, A., and Mendes-Ferreira, A. 2018. Molecular and phenotypic characterization of *Metschnikowia pulcherrima* strains from Douro wine region. *Fermentation* 4, 1, 8.

[22] Barnett, J. A. and Lichtenthaler, F. W. 2001. A history of research on yeasts 3: Emil Fischer, Eduard Buchner and their contemporaries, 1880-1900. *Yeast* 18, 4, 363–388.

[23] Basso, R. F., Alcarde, A. R., and Portugal, C. B. 2016. Could non-*Saccharomyces* yeasts contribute on innovative brewing fermentations? *Food Res. Int.* 86, 112–120.

[24] Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit. 2020. *Amine - eine wichtige Stoffklasse in der amtlichen Lebensmittelüberwachung*. https://www.lgl.bayern.de/lebensmittel/chemie/toxische_reaktionsprodukte/amine.html. Accessed January 27, 2022.

[25] Behr, J. and Vogel, R. F. 2009. Mechanisms of hop inhibition: hop ionophores. *J. Agric. Food Chem.* 57, 14, 6074–6081.

[26] Belitz, H.-D., Grosch, W., and Schieberle, P. 2009. *Food Chem.* Springer.

[27] Bellut, K. and Arendt, E. K. 2019. Chance and Challenge: Non-*Saccharomyces* Yeasts in Nonalcoholic and Low Alcohol Beer Brewing – A Review. *J. Am. Soc. Brew. Chem.* 77, 2, 77–91.

[28] Bellut, K., Krogerus, K., and Arendt, E. K. 2020. *Lachancea fermentati* Strains Isolated From Kombucha: Fundamental Insights, and Practical Application in Low Alcohol Beer Brewing. *Front. Microbiol.* 11, 764.

[29] Bellut, K., Michel, M., Hutzler, M., Zarnkow, M., Jacob, F., Schutter, D. P. de, Daenen, L., Lynch, K. M., Zannini, E., and Arendt, E. K. 2019. Investigation into the Potential of *Lachancea fermentati* Strain KBI 12.1 for Low Alcohol Beer Brewing. *J. Am. Soc. Brew. Chem.* 77, 3, 157–169.

[30] Bellut, K., Michel, M., Zarnkow, M., Hutzler, M., Jacob, F., Atzler, J. J., Hoehnel, A., Lynch, K. M., and Arendt, E. K. 2019. Screening and Application of *Cyberlindnera* Yeasts to Produce a Fruity, Non-Alcoholic Beer. *Fermentation* 5, 4, 103.

[31] Bellut, K., Michel, M., Zarnkow, M., Hutzler, M., Jacob, F., Schutter, D. de, Daenen, L., Lynch, K., Zannini, E., and Arendt, E. 2018. Application of Non-*Saccharomyces* Yeasts Isolated from Kombucha in the Production of Alcohol-Free Beer. *Fermentation* 4, 3, 66.

[32] Benito, S., Palomero, F., Morata, A., Calderón, F., and Suárez-Lepe, J. A. 2012. New applications for *Schizosaccharomyces pombe* in the alcoholic fermentation of red wines. *Int. J. Food Sci. Technol.* 47, 10, 2101–2108.

[33] Berger, A. 1998. Science commentary: Why wine might be less harmful than beer and spirits. *BMJ* 317, 7162, 844.

[34] Bettenhausen, H. M., Barr, L., Broeckling, C. D., Chaparro, J. M., Holbrook, C., Sedin, D., and Heuberger, A. L. 2018. Influence of malt source on beer chemistry, flavor, and flavor stability. *Food Res. Int.* 113, 487–504.

[35] Bhally, H. S., Jain, S., Shields, C., Halsey, N., Cristofalo, E., and Merz, W. G. 2006. Infection in a neonate caused by *Pichia fabianii*: importance of molecular identification. *Med. Mycol.* 44, 2, 185–187.

[36] Bisson, L. F., Coons, D. M., Kruckeberg, A. L., and Lewis, D. A. 1993. Yeast sugar transporters. *Crit. Rev. Biochem. Mol. Biol.* 28, 4, 259–308.

[37] Blackwell, M. 2011. The fungi: 1, 2, 3 … 5.1 million species? *Am. J. Bot.* 98, 3, 426–438.

[38] Boekhout, T., Bai, F. Y., Daniel, H. M., Groenewald, M., Robert, V., Tan, S., and Yurkov, A., Eds. 2022. *The Yeasts Database*. https://theyeasts.org/. Accessed August 17, 2022.

[39] Bokulich, N. A. and Bamforth, C. W. 2013. The Microbiology of Malting and Brewing. *Microbiol. Mol. Biol. Rev.* 77, 2, 157–172.

[40] Bokulich, N. A., Bamforth, C. W., and Mills, D. A. 2012. Brewhouse-resident microbiota are responsible for multi-stage fermentation of American coolship ale. *PloS one* 7, 4, e35507.

[41] Boundy-Mills, K., Stratford, M., and Miller, M. W. 2011. Chapter 62 - *Saccharomycodes* E.C. Hansen (1904). In *The yeasts*. *A taxonomic study*, C. P. Kurtzman, Ed. Elsevier, Amsterdam, 747–750.

[42] Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., Harnett, J., Huys, G., Laulund, S., Ouwehand, A., Powell, I. B., Prajapati, J. B., Seto, Y., Ter Schure, E., van

Boven, A., Vankerckhoven, V., Zgoda, A., Tuijtelaars, S., and Hansen, E. B. 2012. Food fermentations: microorganisms with technological beneficial use. *Int. J. Food Microbiol.* 154, 3, 87–97.

[43] Brányik, T., Silva, D. P., Baszczyňski, M., Lehnert, R., and e Silva, Joao B Almeida. 2012. A review of methods of low alcohol and alcohol-free beer production. *J. Food Eng.* 108, 4, 493–506.

[44] Breuer, U. and Harms, H. 2006. *Debaryomyces hansenii*—an extremophilic yeast with biotechnological potential. *Yeast* 23, 6, 415–437.

[45] Brewers Association. 2022. *2021 Annual Craft Brewing Industry Production Report*. *Craft brewers sales rebound to realize 8% growth*. https://www.brewersassociation.org/press-releases/brewers-association-releases-annual-craft-brewing-industry-production-report-and-top-50-producing-craft-brewing-companies-for-2021/. Accessed 16 August 2022.

[46] Budroni, M., Zara, G., Ciani, M., and Comitini, F. 2017. *Saccharomyces* and Non-*Saccharomyces* Starter Yeasts. In *Brewing Technology*, M. Kanauchi, Ed. InTech. http://dx.doi.org/10.5772/intechopen.68792.

[47] Buiatti, S., Boschelle, O., Mozzon, M., and Battistutta, F. 1995. Determination of biogenic amines in alcoholic and non-alcoholic beers by HPLC. *Food Chem.* 52, 2, 199–202.

[48] Bundesanstalt für Arbeitsschutz und Arbeitsmedizin (BAuA), Ausschuss für Biologische Arbeitsstoffe. 2016. *TRBA 460 „Einstufung von Pilzen in Risikogruppen“*. *Technische Regel für Biologische Arbeitsstoffe*. https://www.baua.de/DE/Angebote/Rechtstexte-und-Technische-Regeln/Regelwerk/TRBA/TRBA-460.html. Accessed April 2, 2023.

[49] Calderbank, J. and Hammond, J. R. M. 1994. Influence of Higher Alcohol Availability on Ester Formation by Yeast. *J. Am. Soc. Brew. Chem* 52, 2, 84–90.

[50] Callejo, M. J., García Navas, J. J., Alba, R., Escott, C., Loira, I., González, M. C., and Morata, A. 2019. Wort fermentation and beer conditioning with selected non-*Saccharomyces* yeasts in craft beers. *Eur. Food Res. Technol.* 245, 6, 1229–1238.

[51] Callejo, M. J., Tesfaye, W., González, M. C., and Morata, A. 2020. Chapter 5: Craft Beers: Current situation and future trends. In *New advances on fermentation processes*, R. M. Martinez-Espinosa, Ed. IntechOpen, London, 83–94.

[52] Cañas, P. M. I., García-Romero, E., Manso, J. M. H., and Fernández-González, M. 2014. Influence of sequential inoculation of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* in the quality of red wines. *Eur. Food Res. Technol.* 239, 2, 279–286.

[53] Canonico, L., Agarbati, A., Comitini, F., and Ciani, M. 2016. *Torulaspora delbrueckii* in the brewing process: A new approach to enhance bioflavour and to reduce ethanol content. *Food Microbiol.* 56, 45–51.

[54] Canonico, L., Ciani, E., Galli, E., Comitini, F., and Ciani, M. 2020. Evolution of Aromatic Profile of *Torulaspora delbrueckii* Mixed Fermentation at Microbrewery Plant. *Fermentation* 6, 1, 7.

[55] Canonico, L., Galli, E., Ciani, E., Comitini, F., and Ciani, M. 2019. Exploitation of Three Non-Conventional Yeast Species in the Brewing Process. *Microorganisms* 7, 1, 11.

[56] Capece, A., Fusco, D. de, Pietrafesa, R., Siesto, G., and Romano, P. 2021. Performance of Wild Non-Conventional Yeasts in Fermentation of Wort Based on Different Malt Extracts to Select Novel Starters for Low-Alcohol Beers. *Appl. Sci.* 11, 2, 801.

[57] Capece, A., Romaniello, R., Siesto, G., and Romano, P. 2018. Conventional and Non-Conventional Yeasts in Beer Production. *Fermentation* 4, 2, 38.

[58] Carbonetto, B., Ramsayer, J., Nidelet, T., Legrand, J., and Sicard, D. 2018. Bakery yeasts, a new model for studies in ecology and evolution. *Yeast* 35, 11, 591–603.

[59] Catallo, M., Iattici, F., Randazzo, C. L., Caggia, C., Krogerus, K., Magalhães, F., Gibson, B., and Solieri, L. 2021. Hybridization of *Saccharomyces cerevisiae* Sourdough Strains with Cryotolerant *Saccharomyces bayanus* NBRC1948 as a Strategy to Increase Diversity of Strains Available for Lager Beer Fermentation. *Microorganisms* 9, 3, 514.

[60] Chambers, P. J. and Pretorius, I. S. 2010. Fermenting knowledge: the history of winemaking, science and yeast research. *EMBO reports* 11, 12, 914–920.

[61] Chaves Batista, A., Rego Vieira, J., and Pessoa Coelho, R. 1959. *Schwanniomyces hominis* n. sp. as the cause of an epidermal lesion in the newborn. *Revista ecuatoriana de higiene y medicina tropical* 16, 136–141.

[62] Chen, E. C.-H. 1978. The Relative Contribution of Ehrlich and Biosynthetic Pathways to the Formation of Fusel Alcohols. *J. Am. Soc. Brew. Chem.* 36, 1, 39–43.

[63] Chen, L., Chi, Z.-M., Chi, Z., and Li, M. 2010. Amylase production by *Saccharomycopsis fibuligera* A11 in solid-state fermentation for hydrolysis of Cassava starch. *Appl. Biochem. Biotechnol.* 162, 1, 252–263.

[64] Chevance, F., Guyot-Declerck, C., Dupont, J., and Collin, S. 2002. Investigation of the beta-damascenone level in fresh and aged commercial beers. *J. Agric. Food Chem.* 50, 13, 3818–3821.

[65] Christiaens, J. F., van Mulders, S. E., Duitama, J., Brown, C. A., Ghequire, M. G., Meester, L. de, Michiels, J., Wenseleers, T., Voordeckers, K., and Verstrepen, K. J. 2012. Functional divergence of gene duplicates through ectopic recombination. *EMBO reports* 13, 12, 1145–1151.

[66] Cioch-Skoneczny, M., Klimczak, K., Satora, P., Skoneczny, S., Zdaniewicz, M., and Pater, A. 2020. The Use of non-*Saccharomyces* Yeast and Enzymes in Beer Production. *Sciendo* 24, 2, 223–246.

[67] Cissé, H., Kagambèga, B., Sawadogo, A., Tankoano, A., Sangaré, G., Traoré, Y., Ouoba, I. I. L., and Savadogo, A. 2019. Molecular characterization of *Bacillus*, lactic acid bacteria and yeast as potential probiotic isolated from fermented food. *Scientific African* 6, e00175.

[68] Clausen, M., Lamb, C. J., Megnet, R., and Doerner, P. W. 1994. PAD1 encodes phenylacrylic acid decarboxylase which confers resistance to cinnamic acid in *Saccharomyces cerevisiae*. *Gene* 142, 1, 107–112.

[69] Coghe, S., Benoot, K., Delvaux, F., Vanderhaegen, B., and Delvaux, F. R. 2004. Ferulic acid release and 4-vinylguaiacol formation during brewing and fermentation: indications for feruloyl esterase activity in *Saccharomyces cerevisiae*. *J. Agric. Food Chem.* 52, 3, 602–608.

[70] Collin, S., Jerkovic, V., Bröhan, M., and Callemien, D. 2013. Polyphenols and Beer Quality. In *Natural Products*. Springer Berlin Heidelberg, Berlin, Heidelberg, 2333–2359. http://dx.doi.org/10.1007/978-3-642-22144-6_78.

[71] Coton, E., Coton, M., Levert, D., Casaregola, S., and Sohier, D. 2006. Yeast ecology in French cider and black olive natural fermentations. *Int. J. Food Microbiol.* 108, 1, 130–135.

[72] Cousseau, F. E. M., Alves, S. L., Trichez, D., and Stambuk, B. U. 2013. Characterization of maltotriose transporters from the *Saccharomyces eubayanus* subgenome of the hybrid *Saccharomyces pastorianus* lager brewing yeast strain Weihenstephan 34/70. *Lett. Appl. Microbiol.* 56, 1, 21–29.

[73] Crafack, M., Mikkelsen, M. B., Saerens, S., Knudsen, M., Blennow, A., Lowor, S., Takrama, J., Swiegers, J. H., Petersen, G. B., and Heimdal, H. 2013. Influencing cocoa flavour using *Pichia kluyveri* and *Kluyveromyces marxianus* in a defined mixed starter culture for cocoa fermentation. *Int. J. Food Microbiol.* 167, 1, 103–116.

[74] Crauwels, S., Steensels, J., Aerts, G., Willems, K., Verstrepen, K., and Lievens, B. 2015. *Brettanomyces bruxellensis*, essential contributor in spontaneous beer fermentations providing novel opportunities for the brewing industry. *BrewingScience* 68, 9, 110–121.

[75] Crumplen, R. M., Slaughter, J. C., and Stewart, G. G. 1996. Characteristics of maltose transporter activity in an ale and large strain of the yeast *Saccharomyces cerevisiae*. *Lett. Appl. Microbiol.* 23, 6, 448–452.

[76] Daenen, L., Saison, D., Sterckx, F., Delvaux, F. R., Verachtert, H., and Derdelinckx, G. 2008. Screening and evaluation of the glucoside hydrolase activity in *Saccharomyces* and *Brettanomyces* brewing yeasts. *Journal of Applied Bacteriology* 104, 2, 478–488.

[77] D'Amore, T., Crumplen, R., and Stewart, G. G. 1991. The involvement of trehalose in yeast stress tolerance. *J. Ind. Microbiol.* 7, 3, 191–195.

[78] Dashko, S., Zhou, N., Tinta, T., Sivilotti, P., Lemut, M. S., Trost, K., Gamero, A., Boekhout, T., Butinar, L., Vrhovsek, U., and Piskur, J. 2015. Use of non-conventional yeast improves the wine aroma profile of Ribolla Gialla. *J. Ind. Microbiol. Biotechnol.* 42, 7, 997–1010.

[79] Day, R. E., Rogers, P. J., Dawes, I. W., and Higgins, V. J. 2002. Molecular analysis of maltotriose transport and utilization by *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 68, 11, 5326–5335.

[80] Dequin, S. and Casaregola, S. 2011. The genomes of fermentative *Saccharomyces*. *C. R. Biol.* 334, 8-9, 687–693.

[81] Deutsche Apotheker Zeitung. 2002. Weniger Alkohol – mehr gesundheitsfördernde Inhaltsstoffe 10, 143.

[82] Deutsche BierAkademie. 2022. *BierTalk - Interview mit den Hefejägern von der TU München, Martin Zarnkow und Mathias Hutzler* 95, Bamberg, Berlin, München.

[83] Díaz, C., Molina, A. M., Nähring, J., and Fischer, R. 2013. Characterization and dynamic behavior of wild yeast during spontaneous wine fermentation in steel tanks and amphorae. *BioMed Res. Int.* 2013, 540465.

[84] Dickenson, C. J. 1983. Cambride Prize Lecture Dimethyl Sulphide-Its Origin and Control in Brewing. *J. Inst. Brew.* 89, 1, 41–46.

[85] Ding, J., Huang, X., Zhang, L., Zhao, N., Yang, D., and Zhang, K. 2009. Tolerance and stress response to ethanol in the yeast *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 85, 2, 253.

[86] Domizio, P., House, J. F., Joseph, C. M. L., Bisson, L. F., and Bamforth, C. W. 2016. *Lachancea thermotolerans* as an alternative yeast for the production of beer. *J. Inst. Brew.* 122, 4, 599–604.

[87] Domizio, P., Romani, C., Lencioni, L., Comitini, F., Gobbi, M., Mannazzu, I., and Ciani, M. 2011. Outlining a future for non-*Saccharomyces* yeasts: selection of putative spoilage wine strains to be used in association with *Saccharomyces cerevisiae* for grape juice fermentation. *Int. J. Food Microbiol.* 147, 3, 170–180.

[88] Dufour, J.-P., Llorente, B., Dujon, B., Kumara, S., and Verstrepen, K. J. 2002. A three member gene family is responsible for the synthesis of short chain fatty acid esters during fermentation. *Brew. Dig* 77, 36–37.

[89] Dušek, M., Olšovská, J., Krofta, K., Jurková, M., and Mikyška, A. 2014. Qualitative determination of β-acids and their transformation products in beer and hop using HR/AM-LC-MS/MS. *J. Agric. Food Chem.* 62, 31, 7690–7697.

[90] Dvořák, J., Dostálek, P., Štěrba, K., Čejka, P., Kellner, V., Čulík, J., and Beinrohr, E. 2006. Determination of Total Sulphur Dioxide in Beer Samples by Flow-Through Chronopotentiometry. *J. Inst. Brew.* 112, 4, 308–313.

[91] Dzialo, M. C., Park, R., Steensels, J., Lievens, B., and Verstrepen, K. J. 2017. Physiology, ecology and industrial applications of aroma formation in yeast. *FEMS Microbiol. Rev.* 41, Supp_1, S95-S128.

[92] EFSA-BIOHAZ Panel. 2007. Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA - Opinion of the Scientific Committee. *EFSA Journal* 5, 12, 587.

[93] Einfalt, D. 2021. Barley-sorghum craft beer production with *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* yeast strains. *Eur. Food Res. Technol.* 247, 2, 385–393.

[94] Erten, H. and Tanguler, H. 2010. Influence of *Williopsis saturnus* yeasts in combination with *Saccharomyces cerevisiae* on wine fermentation. *Lett. Appl. Microbiol.* 50, 5, 474–479.

[95] Esteves, M., Barbosa, C., Vasconcelos, I., Tavares, M. J., Mendes-Faia, A., Mira, N. P., and Mendes-Ferreira, A. 2019. Characterizing the Potential of the Non-Conventional Yeast *Saccharomycodes ludwigii* UTAD17 in Winemaking. *Microorganisms* 7, 11, 478.

[96] European Food Safety Authority. 2011. Scientific Opinion on risk based control of biogenic amine formation in fermented foods. *EFSA Journal* 9, 10, 2393.

[97] Fabre, C. E., Duviau, V. J., Blanc, P. J., and Goma, G. 1995. Identification of volatile flavour compounds obtained in culture of *Kluyveromyces marxianus*. *Biotechnol. Lett.* 17, 11, 1207–1212.

[98] Fan, C.-C. and Lin, T.-F. 2018. N-nitrosamines in drinking water and beer: Detection and risk assessment. *Chemosphere* 200, 48–56.

[99] Federoff, H. J., Eccleshall, T. R., and Marmur, J. 1983. Carbon catabolite repression of maltase synthesis in *Saccharomyces carlsbergensis*. *J. Bacteriol.* 156, 1, 301–307.

[100] Fijan, S. 2014. Microorganisms with claimed probiotic properties: an overview of recent literature. *Int. J. Environ. Res. Public Health* 11, 5, 4745–4767.

[101] Fonseca, G. G., Heinzle, E., Wittmann, C., and Gombert, A. K. 2008. The yeast *Kluyveromyces marxianus* and its biotechnological potential. *Appl. Microbiol. Biotechnol.* 79, 3, 339–354.

[102] Forster, A. and Gahr, A. 2012. Hopping of low alcohol beers. *BrewingScience* 65, 72–82.

[103] Francesco, G. de, Turchetti, B., Sileoni, V., Marconi, O., and Perretti, G. 2015. Screening of new strains of *Saccharomycodes ludwigii* and *Zygosaccharomyces rouxii* to produce low-alcohol beer. *J. Inst. Brew.* 121, 1, 113–121.

[104] Fröhlich-Wyder, M.-T. 2003. Yeasts in dairy products. In *Yeasts in food*. Elsevier, 209–237.

[105] Fröhlich-Wyder, M.-T., Arias-Roth, E., and Jakob, E. 2019. Cheese yeasts. *Yeast* 36, 3, 129–141.

[106] Fujii, T., Kobayashi, O., Yoshimoto, H., Furukawa, S., and Tamai, Y. 1997. Effect of aeration and unsaturated fatty acids on expression of the *Saccharomyces cerevisiae* alcohol acetyltransferase gene. *Appl. Environ. Microbiol.* 63, 3, 910–915.

[107] Fujii, T., Nagasawa, N., Iwamatsu, A., Bogaki, T., and Hamachi, M. 1994. Molecular cloning, sequence analysis and expression of the yeast alcohol acetyltransferase gene. *Appl. Environ. Microbiol* 60, 2786–2792.

[108] Fujii, T., Yoshimoto, H., Nagasawa, N., Bogaki, T., Tamai, Y., and Hamachi, M. 1996. Nucleotide sequences of alcohol acetyltransferase genes from lager brewing yeast, *Saccharomyces carlsbergensis*. *Yeast* 12, 6, 593–598.

[109] Fukuhara, H. 2003. The Kluyver effect revisited. *FEMS Yeast Res.* 3, 4, 327–331.

[110] Futatsugi, M., Ogawa, T., and Fukuda, H. 1993. Purification and properties of two forms of glucoamylase from *Saccharomycopsis fibuligera*. *Journal of Fermentation and Bioengineering* 76, 6, 521–523.

[111] Gallone, B., Steensels, J., Prahl, T., Soriaga, L., Saels, V., Herrera-Malaver, B., Merlevede, A., Roncoroni, M., Voordeckers, K., and Miraglia, L. 2016. Domestication and divergence of *Saccharomyces cerevisiae* beer yeasts. *Cell* 166, 6, 1397-1410. e16.

[112] Gamero, A., Dijkstra, A., Smit, B., and Jong, C. de. 2020. Aromatic Potential of Diverse Non-Conventional Yeast Species for Winemaking and Brewing. *Fermentation* 6, 2, 50.

[113] Gamero, A., Quintilla, R., Groenewald, M., Alkema, W., Boekhout, T., and Hazelwood, L. 2016. High-throughput screening of a large collection of non-conventional yeasts reveals their potential for aroma formation in food fermentation. *Food Microbiol.* 60, 147–159.

[114] Gamero, A., Ren, X., Lamboni, Y., Jong, C. de, Smid, E. J., and Linnemann, A. R. 2019. Development of A Low-Alcoholic Fermented Beverage Employing Cashew Apple Juice and Non-Conventional Yeasts. *Fermentation* 5, 3, 71.

[115] Garavaglia, C. and Swinnen, J. F. M. 2018. *Economic perspectives on craft beer*. *A revolution in the global beer industry*, C. Garavaglia, and J. Swinnen, Ed. Palgrave Macmillan, Basingstoke, Hampshire.

[116] García-Martos, P., Hernández-Molina, J. M., Galán, F., Ruiz-Henestrosa, J. R., García-Agudo, R., Palomo, M. J., and Mira, J. 1999. Isolation of *Hanseniaspora uvarum* (*Kloeckera apiculata*) in humans. *Mycopathology* 144, 73–75.

[117] García-Martos, P., Mira, J., Galán, F., and Hernández, J. M. 1996. Sexual forms of yeasts in clinical samples. *Mycopathologia et Mycologia Applicata* 136, 2, 67–70.

[118] Gibson, B., Geertman, J.-M. A., Hittinger, C. T., Krogerus, K., Libkind, D., Louis, E. J., Magalhães, F., and Sampaio, J. P. 2017. New yeasts-new brews: modern approaches to brewing yeast design and development. *FEMS Yeast Res.* 17, 4.

[119] Gibson, B., Krogerus, K., Ekberg, J., Mikkelson, A., Pentikäinen, S., Wilpola, A., and Vidgren, V., Eds. 2015. Non-conventional yeast as a new tool for beer flavour modification. *Proceedings of the EBC Congress*. Porto, Portugal. 24-28.

[120] Gibson, B. and Liti, G. 2015. *Saccharomyces pastorianus*: genomic insights inspiring innovation for industry. *Yeast* 32, 1, 17–27.

[121] Gibson, B. R., Lawrence, S. J., Leclaire, J. P. R., Powell, C. D., and Smart, K. A. 2007. Yeast responses to stresses associated with industrial brewery handling. *FEMS Microbiol. Rev.* 31, 5, 535–569.

[122] Gijs, L., Chevance, F., Jerkovic, V., and Collin, S. 2002. How low pH can intensify beta-damascenone and dimethyl trisulfide production through beer aging. *J. Agric. Food Chem.* 50, 20, 5612–5616.

[123] Gil-Rodríguez, A. M., Carrascosa, A. V., and Requena, T. 2015. Yeasts in foods and beverages: In vitro characterisation of probiotic traits. *LWT* 64, 2, 1156–1162.

[124] Görts, C.P.M. 1969. Effect of glucose on the activity and the kinetics of the maltose-uptake system and of α-glucosidase in *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta - General Subjects* 184, 2, 299–305.

[125] Gray, P. P., Stone, I., and Atkin, L. 1947. Systemetic Study of the Influence of Oxidation on Beer Flavor. *Proceedings. Annual meeting - American Society of Brewing Chemists* 5, 1, 101–112.

[126] Guido, L. F., Carneiro, JR, Santos, JR, Almeida, P. J., Rodrigues, J. A., and Barros, A. A. 2004. Simultaneous determination of E-2-nonenal and β-damascenone in beer by reversed-phase liquid chromatography with UV detection. *J. Chrom. A* 1032, 1-2, 17–22.

[127] Gutiérrez, A., Boekhout, T., Gojkovic, Z., and Katz, M. 2018. Evaluation of non-*Saccharomyces* yeasts in the fermentation of wine, beer and cider for the development of new beverages. *J. Inst. Brew.* 124, 4, 389–402.

[128] Haehn, H. and Glaubitz, M. 1933. *Beer manufacture*. Patent US1898047 A.

[129] Hahn, P. 2012. Alkoholfreies Bier – zulässige Verkehrsbezeichnung. *Brauwelt* 39, 1130–1132.

[130] Haile, M. and Kang, W. H. 2019. Isolation, Identification, and Characterization of Pectinolytic Yeasts for Starter Culture in Coffee Fermentation. *Microorganisms* 7, 10.

[131] Haile, M. and Kang, W. H. 2020. Antioxidant Properties of Fermented Green Coffee Beans with *Wickerhamomyces anomalus* (Strain KNU18Y3). *Fermentation* 6, 1, 18.

[132] Hammond, J. R.M. 1993. *Brewing yeast. The Yeasts*. Academic Press, London, 5–67.

[133] Hansen, J. and Piškur, J. 2004. Fungi in brewing: Biodiversity and Biotechnology Perspectives. In *Handbook of Fungal Biotechnology*, D. K. Arora, Ed. Marcel Dekker, New York, U.S.A, 233–248.

[134] Hatoum, R., Labrie, S., and Fliss, I. 2012. Antimicrobial and probiotic properties of yeasts: from fundamental to novel applications. *Front. Microbiol.* 3, 421.

[135] Hazelwood, L. A., Walsh, M. C., Pronk, J. T., and Daran, J.-M. 2010. Involvement of vacuolar sequestration and active transport in tolerance of *Saccharomyces cerevisiae* to hop iso-alpha-acids. *Appl. Environ. Microbiol.* 76, 1, 318–328.

[136] Hesseltine, C. W. and Kurtzman, C. P. 1990. Yeasts in amylolytic food starters. *Anales del Instituto de Biología. Serie Botánica* 60, 1, 1–7.

[137] Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., and Sanders, M. E. 2014. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 8, 506–514.

[138] Hiralal, L., Olaniran, A. O., and Pillay, B. 2014. Aroma-active ester profile of ale beer produced under different fermentation and nutritional conditions. *J. Biosci. Bioeng.* 117, 1, 57–64.

[139] Holt, S., Mukherjee, V., Lievens, B., Verstrepen, K. J., and Thevelein, J. M. 2018. Bioflavoring by non-conventional yeasts in sequential beer fermentations. *Food microbiol.* 72, 55–66.

[140] Horák, J. 2013. Regulations of sugar transporters: insights from yeast. *Curr. Genet.* 59, 1-2, 1–31.

[141] Hostinová, E. 2002. Amylolytic enzymes produced by the yeast *Saccharomycopsis fibuligera*. *Biologia-Bratislava* 57, SUP/2, 247–252.

[142] Hudson, J. R. and Birtwistle, S. E. 1966. Wort-Boiling in Relation to Beer Quality. *J. Inst. Brew.* 72, 1, 46–50.

[143] Hutzler, M. 2021. *Hefebiodiversität traditioneller und moderner hopfenhaltiger Bierfermentationen und deren gezielte Erweiterung über entwickelte Hefejagdmethoden*. Habilitationsschrift, Technische Universität Berlin.

[144] Hutzler, M., Koob, J., Riedl, R., Schneiderbanger, H., Mueller-Auffermann, K., and Jacob, F. 2015. Yeast identification and characterization. In *Brewing microbiology*. *Managing microbes, ensuring quality and valorising waste*, A. E. Hill, Ed. Woodhead Publishing series in food science, technology and nutrition 289. Woodhead Publ./Elsevier, London, 65–104. https://doi.org/10.1016/B978-1-78242-331-7.00005-8.

[145] Hutzler, M., Stretz, D., Meier-Dörnberg, T., Michel, M., and Schneiderbanger, H. 2015. Großes Aromapotenzial - die vielfältige Aromenwelt der Hefen. *Brauindustrie* 4, 28–31.

[146] Hutzler, M., Zarnkow, M., Hans, S., Stretz, D., Meier-Dörnberg, T., Methner, Y., Schneiderbanger, H., and Jacob, F. 2020. New Yeasts - New Beers. *Brewing and Beverage Industry International*, 16–23.

[147] Ianieva, O. and Podgorsky, V. 2020. Enological potential of non-*Saccharomyces* yeast strains of enological and brewery origin from Ukrainian Collection of Microorganisms. *Mycology*, 12, 3, 1–13.

[148] Iattici, F., Catallo, M., and Solieri, L. 2020. Designing New Yeasts for Craft Brewing: When Natural Biodiversity Meets Biotechnology. *Beverages* 6, 1, 3.

[149] Ilet, D. R. 1995. Aspects of the analysis, role, and fate of sulphur dioxide in beer - A review. *MBAA TQ* 32, 213–221.

[150] Izquierdo-Pulido, M., Hernández-Jover, T., Mariné-Font, A., and Vidal-Carou, M. C. 1996. Biogenic Amines in European Beers. *J. Agric. Food Chem.* 44, 10, 3159–3163.

[151] Jackowski, M. and Trusek, A. 2018. Non-alcoholic beer production – an overview. *Polish J. Chem. Technol.* 20, 4, 32–38.

[152] Jacob, F., Ed. 2012. *The MEBAK collection of brewing analysis methods: Wort, beer and beer-based beverages. Collection of methods of the Mitteleuropäischen Brauchtechnischen Analysenkommission.* Self-published by MEBAK.

[153] Jacob, F. F., Striegel, L., Rychlik, M., Hutzler, M., and Methner, F.-J. 2019. Yeast extract production using spent yeast from beer manufacture: influence of industrially applicable disruption methods on selected substance groups with biotechnological relevance. *Eur. Food Res. Technol.* 245, 6, 1169–1182.

[154] Jentsch, M. 2007. Top-fermented beer specialities in focus. *Brauwelt Int.* 5, 332–334.

[155] Jiang, J. 1993. Identification of flavour volatile compounds produced by *Kluyveromyces lactis*. *Biotechnology techniques* 7, 12, 863–866.

[156] Johansson, L., Nikulin, J., Juvonen, R., Krogerus, K., Magalhães, F., Mikkelson, A., Nuppunen-Puputti, M., Sohlberg, E., Francesco, G. de, and Perretti, G. 2021. Sourdough cultures as reservoirs of maltose-negative yeasts for low-alcohol beer brewing. *Food microbiol.* 94, 103629.

[157] Jolly, N. P., Varela, C., and Pretorius, I. S. 2014. Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res.* 14, 2, 215–237.

[158] Jung, H., Lee, S.-J., Lim, J. H., Kim, B. K., and Park, K. J. 2014. Chemical and sensory profiles of makgeolli, Korean commercial rice wine, from descriptive, chemical, and volatile compound analyses. *Food Chem.* 152, 624–632.

[159] Kalac, P. and Krízek, M. 2003. A Review of Biogenic Amines and Polyamines in Beer. *J. Inst. Brew.* 109, 2, 123–128.

[160] Kamoshita, M., Matsumoto, Y., Nishimura, K., Katono, Y., Murata, M., Ozawa, Y., Shimmura, S., and Tsubota, K. 2015. *Wickerhamomyces anomalus* fungal keratitis responds to topical treatment with antifungal micafungin. *Journal of infection and chemotherapy: official journal of the Japan Society of Chemotherapy* 21, 2, 141–143.

[161] Karabín, M., Hudcová, T., Jelínek, L., and Dostálek, P. 2016. Biologically Active Compounds from Hops and Prospects for Their Use. *Compr. Rev. Food Sci. Food Saf.* 15, 3, 542–567.

[162] Kaygusuz, I., Mulazimoglu, L., Cerikcioglu, N., Toprak, A., Oktay, A., and Korten, V. 2003. An unusual native tricuspid valve endocarditis caused by *Candida colliculosa*. *Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 9, 4, 319–322.

[163] King, A. and Richard Dickinson, J. 2000. Biotransformation of monoterpene alcohols by *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Kluyveromyces lactis*. *Yeast* 16, 6, 499–506.

[164] Klein, C. J., Olsson, L., Rønnow, B., Mikkelsen, J. D., and Nielsen, J. 1996. Alleviation of glucose repression of maltose metabolism by MIG1 disruption in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 62, 12, 4441–4449.

[165] Koné, M. K., Guéhi, S. T., Durand, N., Ban-Koffi, L., Berthiot, L., Tachon, A. F., Brou, K., Boulanger, R., and Montet, D. 2016. Contribution of predominant yeasts to the occurrence of aroma compounds during cocoa bean fermentation. *Food Res. Int.* 89, 2, 910–917.

[166] Koutsoumanis, K., Allende, A., Alvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., Davies, R., Cesare, A. de, Hilbert, F., Lindqvist, R., Nauta, M., Peixe, L., Ru, G., Simmons, M., Skandamis, P., Suffredini, E., Cocconcelli, P. S., Fernández Escámez, P. S., Maradona, M. P., Querol, A., Suarez, J. E., Sundh, I., Vlak, J., Barizzone, F., Correia, S., and Herman, L. 2020. Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA (2017-2019). *EFSA Journal* 18, 2, e05966.

[167] Krahl, M. 2010. *Funktionelle Getränke auf Basis vermälzter Zerealien und Pseudozerealien*. Dissertation, Technische Universität München.

[168] Kreissl, J., Mall, V., Steinhaus, P., and Steinhaus, M. *Leibniz-LSB@TUM Odorant Database Version 1.2.* Leibniz Institute for Food Systems Biology at the Technical University of Munich: Freising, Germany, 2022 (https://www.leibniz-lsb.de/en/databases/leibniz-lsbtum-odorant-database).

[169] Krogerus, K., Eerikäinen, R., Aisala, H., and Gibson, B. 2022. Repurposing brewery contaminant yeast as production strains for low-alcohol beer fermentation. *Yeast* 39, 1-2, 156–169.

[170] Krogerus, K. and Gibson, B. R. 2013. 125th Anniversary Review: Diacetyl and its control during brewery fermentation. *J. Inst. Brew.* 119, 86-97.

[171] Krogerus, K., Holmström, S., and Gibson, B. 2018. Enhanced wort fermentation with de novo lager hybrids adapted to high-ethanol environments. *Appl. Environ. Microbiol.* 84, 4, e02302-17.

[172] Kunz, T. and Methner, F.-J. 2009. *Verfahren zur Herstellung eines Getränkes*. Patent EP2382303A1.

[173] Kurtzman, C. P. 2000. Four new yeasts in the *Pichia anomala* clade. *Int. J. Syst. Evol. Microbiol.* 50, 1, 395–404.

[174] Kurtzman, C. P. 2011. Chapter 42 - *Lindnera* Kurtzman, Robnett & Basehoar-Powers (2008). In *The yeasts*. *A taxonomic study*, C. P. Kurtzman, Ed. Elsevier, Amsterdam, 521–543. https://doi.org/10.1016/B978-0-444-52149-1.00042-2.

[175] Kurtzman, C. P., Ed. 2011. *The yeasts*. *A taxonomic study*. Elsevier, Amsterdam.

[176] Kurtzman, C. P. and Smith, M. T. 2011. Chapter 63 - *Saccharomycopsis* Schiönning (1903). *Saccharomycopsis fibuligera* (Lindner) Klöcker (1924). In *The yeasts*. *A taxonomic study*, C. P. Kurtzman, Ed. Elsevier, Amsterdam, 755–756. https://doi.org/10.1016/B978-0-444-52149-1.00063-X.

[177] Lachenmeier, D. W. and Fügel, D. 2007. Reduction of nitrosamines in beer—review of a success story. *BrewingScience* 60, 84–89.

[178] Lakshmi Ragavan, M. and Das, N. 2017. Molecular identification of probiotic yeast strains and their characterization. *Asian J. Pharm. Clin. Res.* 10, 10, 339.

[179] Langstaff, S. A. and Lewis, M. J. 1993. The Mouthfeel of Beer - A Review. *J. Inst. Brew.* 99, 1, 31–37.

[180] Lara-Hidalgo, C., Hernández-Sánchez, H., Hernández-Rodríguez, C., and Dorantes-Álvarez, L. 2017. Yeasts in Fermented Foods and their Probiotic Potential. *Austin J. Nutr. Metab.* 4, 1, 1045.

[181] Lee, K. E., Lee, S. M., Choi, Y. H., Hurh, B. S., and Kim, Y.-S. 2013. Comparative volatile profiles in soy sauce according to inoculated microorganisms. *Biosci. Biotechnol. Biochem.* 77, 11, 2192–2200.

[182] Lee, P.-R., Ong, Y.-L., Yu, B., Curran, P., and Liu, S.-Q. 2010. Evolution of volatile compounds in papaya wine fermented with three *Williopsis saturnus* yeasts. *Int. J. Food Sci. Technol.* 45, 10, 2032–2041.

[183] Lee, S. M., Jung, J. H., Seo, J.-A., and Kim, Y.-S. 2018. Bioformation of Volatile and Nonvolatile Metabolites by *Saccharomycopsis fibuligera* KJJ81 Cultivated under Different Conditions-Carbon Sources and Cultivation Times. *Molecules* 23, 11, 2762.

[184] Lei, H., Zhao, H., Yu, Z., and Zhao, M. 2012. Effects of wort gravity and nitrogen level on fermentation performance of brewer's yeast and the formation of flavor volatiles. *Appl. Biochem. Biotechnol.* 166, 6, 1562–1574.

[185] Lentz, M. 2018. The Impact of Simple Phenolic Compounds on Beer Aroma and Flavor. *Fermentation* 4, 1, 20.

[186] Licker, J. L., Acree, T. E., and Henick-Kling, T. 1999. *What is "Brett" (Brettanomyces) Flavor?* Am. Chem. Soc. Symp., 213th Natl. Meet., San Francisco, USA.

[187] Limtong, S., Yongmanitchai, W., Tun, M. M., Kawasaki, H., and Seki, T. 2007. *Kazachstania siamensis* sp. nov., an ascomycetous yeast species from forest soil in Thailand. *Int. J. Syst. Evol. Microbiol.* 57, 2, 419–422.

[188] Lindner, P. 1893. *Schizosaccharomyces pombe* n. sp., ein neuer Gährungserreger. *Wochenschrift für Brauerei,* 10, 1298–1300.

[189] Liu, S.-Q. and Quek, A. Y. H. 2016. Evaluation of Beer Fermentation with a Novel Yeast *Williopsis saturnus*. *Food Technol. Biotechnol.* 54, 4, 403–412.

[190] Lloyd, N. D. R., Capone, D. L., Ugliano, M., Taylor, D. K., Skouroumounis, G. K., Sefton, M. A., and Elsey, G. M. 2011. Formation of Damascenone under both commercial and model fermentation conditions. *J. Agric. Food Chem.* 59, 4, 1338–1343.

[191] Lodolo, E. J., Kock, J. L. F., Axcell, B. C., and Brooks, M. 2008. The yeast *Saccharomyces cerevisiae* - the main character in beer brewing. *FEMS Yeast Res.* 8, 7, 1018–1036.

[192] Loret, S., Deloyer, P., and Dandrifosse, G. 2005. Levels of biogenic amines as a measure of the quality of the beer fermentation process: Data from Belgian samples. *Food Chem.* 89, 4, 519–525.

[193] Lüers, H. and Geys, K. 1922. Ueber die Flockung der Hefe. *Kolloid-Zeitschrift* 30, 6, 372–376.

[194] Magalhães, F., Vidgren, V., Ruohonen, L., and Gibson, B. 2016. Maltose and maltotriose utilisation by group I strains of the hybrid lager yeast *Saccharomyces pastorianus*. *FEMS Yeast Res.* 16, 5.

[195] Maier, A., Völker, B., Boles, E., and Fuhrmann, G. F. 2002. Characterisation of glucose transport in *Saccharomyces cerevisiae* with plasma membrane vesicles (countertransport) and intact cells (initial uptake) with single Hxt1, Hxt2, Hxt3, Hxt4, Hxt6, Hxt7 or Gal2 transporters. *FEMS Yeast Res.* 2, 4, 539–550.

[196] Malcorps, P., Cheval, J. M., Jamil, S., and Dufour, J. P. 1991. A new model for the regulation of ester synthesis by alcohol acetyltransferase in *Saccharomyces cerevisiae* during fermentation. *J. Am. Soc. Brew. Chem.* 49, 2, 47–53.

[197] Mangindaan, D., Khoiruddin, K., and Wenten, I. G. 2018. Beverage dealcoholization processes: Past, present, and future. *Trends Food Sci. Technol.* 71, 36–45.

[198] Maoura, N., Mbaiguinam, M., Nguyen, H. V., Gaillardin, C., and Pourquie, J. 2005. Identification and typing of the yeast strains isolated from bili bili, a traditional sorghum beer of Chad. *Afr. J. Biotechnol.* 4, 7, 646–656.

[199] Marquardt, H. 1962. Der Feinbau von Hefezellen im Elektronenmikroskop. *Zeitschrift für Naturforschung B* 17, 1, 42–48.

[200] Marten, R., Amul, G. G. H., and Casswell, S. 2020. Alcohol: global health's blind spot. *The Lancet Global Health* 8, 3, e329-e330.

[201] Mason, A. B. and Dufour, J.-P. 2000. Alcohol acetyltransferases and the significance of ester synthesis in yeast. *Yeast* 16, 14, 1287–1298.

[202] Masoud, W. and Jespersen, L. 2006. Pectin degrading enzymes in yeasts involved in fermentation of Coffea arabica in East Africa. *Int. J. Food Microbiol.* 110, 3, 291–296.

[203] Medeiros, A. B. P., Pandey, A., Christen, P., Fontoura, P. S. G., Freitas, R. J. S. de, and Soccol, C. R. 2001. Aroma compounds produced by *Kluyveromyces marxianus* in solid state fermentation on a packed bed column bioreactor. *World J. Microbiol. Biotechnol.* 17, 8, 767–771.

[204] Meier-Dörnberg, T. 2018. *Comparative characterization of selected* Saccharomyces *yeast strains as beer fermentation starter cultures*. Dissertation, Technische Universität München.

[205] Meier-Dörnberg, T., Hutzler, M., Jacob, F., and Schneiderbanger, H. 2015. Geschmacklich ansprechend. *Brauindustrie* 7, 12–15.

[206] Meier-Dörnberg, T., Hutzler, M., Michel, M., Methner, F.-J., and Jacob, F. 2017. The Importance of a Comparative Characterization of *Saccharomyces Cerevisiae* and *Saccharomyces Pastorianus* Strains for Brewing. *Fermentation* 3, 3, 41.

[207] Meier-Dörnberg, T., Schneiderbanger, H., Jacob, F., and Hutzler, M. 2014. Alcohol-Free Wheat Beer with Maltose Negative Yeast Strain *Saccharomycodes ludwigii*. Young Scientists Symposium On Malting, Brewing And Distilling. Ghent, Belgium.

[208] Meilgaard, M. 2001. Effects on Flavour of Innovations in Brewery Equipment and Processing: A Review. *J. Inst. Brew.* 107, 5, 271–286.

[209] Meilgaard, M., Aymá, M., and Ruano, J. I. 1971. A study of carbonyl compounds in beer. IV. Carbonyl compounds identified in heat-treated wort and beer. *J. Am. Soc. Brew. Chem.* 1, 219–229.

[210] Meilgaard, M. C. 1975. Flavor chemistry of beer. II. Flavor and threshold of 239 aroma volatiles. *MBAA TQ* 12, 151–168.

[211] Meilgaard, M. C. 1976. Wort composition: with special reference to the use of ajuncts. *MBAA TQ* 13, 78–90.

[212] Meilgaard, M. C., Reid, D. S., and Wyborski, K. A. 1982. Reference standards for beer flavor terminology system. *J. Am. Soc. Brew. Chem.* 40, 4, 119–128.

[213] Menz, G., Vriesekoop, F., Zarei, M., Zhu, B., and Aldred, P. 2010. The growth and survival of foodborne pathogens in sweet and fermenting brewers' wort. *Int. J. Food Microbiol.* 140, 1, 19–25.

[214] Mertens, S., Steensels, J., Saels, V., Rouck, G. de, Aerts, G., and Verstrepen, K. J. 2015. A large set of newly created interspecific *Saccharomyces* hybrids increases aromatic diversity in lager beers. *Appl. Environ. Microbiol.* 81, 23, 8202–8214.

[215] Methner, F.-J. 1987. *Über die Aromabildung beim Berliner Weissbier unter besonderer Berücksichtigung von Säuren und Estern*. Dissertation, Technische Universität Berlin.

[216] Meussdoerffer, F. G. 2009. A Comprehensive History of Beer Brewing. In *Handbook of Brewing*, H. M. Esslinger, Ed. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 1–42. https://doi.org/10.1002/9783527623488.ch1.

[217] Michel, M. 2018. *Use of non*-Saccharomyces *yeast for beer fermentation as illustrated by* Torulaspora delbrueckii. Dissertation, Technische Universität München.

[218] Michel, M., Cocuzza, S., Biendl, M., Peifer, F., Hans, S., Methner, Y., Pehl, F., Back, W., Jacob, F., and Hutzler, M. 2020. The impact of different hop compounds on the growth of selected beer spoilage bacteria in beer. *J. Inst. Brew.* 126, 4, 354–361.

[219] Michel, M., Kopecká, J., Meier-Dörnberg, T., Zarnkow, M., Jacob, F., and Hutzler, M. 2016. Screening for new brewing yeasts in the non-*Saccharomyces* sector with *Torulaspora delbrueckii* as model. *Yeast* 33, 4, 129–144.

[220] Michel, M., Meier-Dörnberg, T., Jacob, F., Methner, F.-J., Wagner, R. S., and Hutzler, M. 2016. Pure non-*Saccharomyces* starter cultures for beer fermentation with a focus on secondary metabolites and practical applications. *J. Inst. Brew.* 122, 4, 569–587.

[221] Michel, M., Meier-Dörnberg, T., Jacob, F., Schneiderbanger, H., Haselbeck, K., Zarnkow, M., and Hutzler, M. 2017. Optimization of Beer Fermentation with a Novel Brewing Strain *Torulaspora delbrueckii* Using Response Surface Methodology. *MBAA TQ* 54, 23–33.

[222] Miguel, G. A., Carlsen, S., Arneborg, N., Saerens, S. M. G., Laulund, S., and Knudsen, G. M. 2022. Non-*Saccharomyces* yeasts for beer production: Insights into safety aspects and considerations. *Int. J. Food Microbiol.* 383, 109951.

[223] Mikyška, A., Hrabák, M., Hašková, D., and Šrogl, J. 2002. The Role of Malt and Hop Polyphenols in Beer Quality, Flavour and Haze Stability. *J. Inst. Brew.* 108, 1, 78–85.

[224] Minetoki, T., Bogaki, T., Iwamatsu, A., Fujii, T., and Hamachi, M. 1993. The purification, properties and internal peptide sequences of alcohol acetyltransferase isolated from *Saccharomyces cerevisiae* Kyokai No. 7. *Biosci. Biotechnol. Biochem.* 57, 12, 2094–2098.

[225] Misihairabgwi, J. M., Kock, L., Pretorious, E., Pohl, C., and Zvauya, R. 2015. Characterisation of yeasts isolated from traditional opaque beer beverages brewed in Zimbabwean households. *Afr. J. Microbiol. Res.* 9, 8, 549–556.

[226] Moir, M. 2000. Hops—A Millennium Review. *J. Am. Soc. Brew. Chem.* 58, 4, 131–146.

[227] Montanari, L., Marconi, O., Mayer, H., and Fantozzi, P. 2009. Production of Alcohol-Free Beer. In *Beer in health and disease prevention*, V. R. Preedy, Ed. Elsevier/Academic Press, Amsterdam, London, 61–75. https://doi.org/10.1016/B978-0-12-373891-2.00006-7.

[228] Montandon, G., Borelli, B., Causmaecker, B. de, Praet, T., Jaskula-Goiris, B., Rosa, A., and Aerts, G., Eds. 2016. *Selection of indigenous* Saccharomyces cerevisiae *strains for top fermentation beer production*. Trends in Brewing. Ghent, Belgium.

[229] Moreira, N., Mendes, F., Guedes de Pinho, P., Hogg, T., and Vasconcelos, I. 2008. Heavy sulphur compounds, higher alcohols and esters production profile of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* grown as pure and mixed cultures in grape must. *Int. J. Food Microbiol.* 124, 3, 231–238.

[230] Mortazavian, A. M., Razavi, S. H., Mousavi, S. M., Malganji, S., and Sohrabvandi, S. 2014. The effect of *Saccharomyces* strain and fermentation conditions on quality prameters of non-alcoholic beer. *J. paramed. sci.* 5, 2, 21–26.

[231] Mukai, N., Masaki, K., Fujii, T., Kawamukai, M., and Iefuji, H. 2010. PAD1 and FDC1 are essential for the decarboxylation of phenylacrylic acids in *Saccharomyces cerevisiae*. *J. Biosci. Bioeng.* 109, 6, 564–569.

[232] Muller, R. 1990. The production of low-alcohol and alcohol-free beers by limited fermentations. *Ferment* 3, 4, 224–230.

[233] Müller, M., Bellut, K., Tippmann, J., and Becker, T. 2017. Physical Methods for Dealcoholization of Beverage Matrices and their Impact on Quality Attributes. *ChemBioEng Reviews* 4, 5, 310–326.

[234] Muller, R. E., J. Barrett, und E. D. Baxter, Ed. 1991. *Enhancement of flavor in beers with reduced alcohol contents*. Proceedings of the European Brewing Convention Congress, Lisbon, Portugal.

[235] Müller-Auffermann, K., Caldera, A., Jacob, F., and Hutzler, M. 2015. Characterization of different bottom fermenting *Saccharomyces pastorianus* brewing yeast strains. *BrewingScience* 68, 46–57.

[236] N'guessan, K. F., Brou, K., Jacques, N., Casaregola, S., and Dje, K. M. 2011. Identification of yeasts during alcoholic fermentation of *tchapalo*, a traditional sorghum beer from Côte d'Ivoire. *Antonie van Leeuwenhoek* 99, 4, 855–864.

[237] Nagami, K., Takahashi, T., Nakatani, K., and Kumada, J. 1980. Hydrogen sulfide in brewing. *MBAA TQ* 17, 64–68.

[238] Nagasawa, N., Bogaki, T., Iwamatsu, A., Hamachi, M., and Kumagai, C. 1998. Cloning and nucleotide sequence of the alcohol acetyltransferase II gene (ATF2) from *Saccharomyces cerevisiae* Kyokai No. 7. *Biosci. Biotechnol. Biochem*. 62, 1852–1857.

[239] Narziß, L. 1986. Technological factors of flavour stability. *J. Inst. Brew.* 92, 4, 346–353.

[240] Narziß, L., Back, W., Gastl, M., and Zarnkow, M. 2017. *Abriss der Bierbrauerei*. John Wiley & Sons Incorporated, Newark.

[241] Narziß, L., Miedaner, H., Kern, E., and Leibhard, M. 1992. Technology and composition of non-alcoholic beers. Processes using arrested fermentation. *Brauwelt Int.* 4, 396–410.

[242] Nelson, G. and Young, T. W. 1986. Yeast extracellular proteolytic enzymes for chill-proofing beer. *J. Inst. Brew.* 92, 6, 599–603.

[243] Nikulin, J., Aisala, H., and Gibson, B. 2022. Production of non-alcoholic beer via cold contact fermentation with *Torulaspora delbrueckii*. *J. Inst. Brew.* 128, 1, 28–35.

[244] Nikulin, J., Eerikäinen, R., Hutzler, M., and Gibson, B. 2020. Brewing Characteristics of the Maltotriose-Positive Yeast *Zygotorulaspora florentina* Isolated from Oak. *Beverages* 6, 4, 58.

[245] Nyanga, L. K., Nout, M. J. R., Smid, E. J., Boekhout, T., and Zwietering, M. H. 2013. Fermentation characteristics of yeasts isolated from traditionally fermented masau (*Ziziphus mauritiana*) fruits. *Int. J. Food Microbiol.* 166, 3, 426–432.

[246] Nykänen, L. 1986. Formation and Occurrence of Flavor Compounds in Wine and Distilled Alcoholic Beverages. *Am. J. Enol. Vitic.* 37, 1, 84–96.

[247] O'Connor, B. 2020. Winning with wheat beer. Brewing non-alcoholic wheat beer with sour wort produces better quality – and maximises yield through targeted adjustment of pH value. *Brewer and distiller international*, 44–47.

[248] Ogane, O., Imai, T., Ogawa, Y., and Ohkochi, M. 2006. Influence of wort boiling and wort clarification conditions on aging-relevant carbonyl compounds in beer. *MBAA TQ* 43, 2, 121–126.

[249] Oka, K., Hayashi, T., Matsumoto, N., and Yanase, H. 2008. Decrease in hydrogen sulfide content during the final stage of beer fermentation due to involvement of yeast and not carbon dioxide gas purging. *J. Biosci. Bioeng.* 106, 3, 253–257.

[250] Olaniran, A. O., Hiralal, L., Mokoena, M. P., and Pillay, B. 2017. Flavour-active volatile compounds in beer: production, regulation and control. *J. Inst. Brew.* 123, 1, 13–23.

[251] Oliveira, D. R., Lopes, A. C. A., Pereira, R. A., Cardoso, P. G., and Duarte, W. F. 2019. Selection of potentially probiotic *Kluyveromyces lactis* for the fermentation of cheese whey–based beverage. *Ann. Microbiol.* 69, 13, 1361–1372.

[252] Oliver, G. und T. Colicchio. 2011. *The Oxford companion to beer*. Oxford University Press.

[253] Olšovská, J., Vrzal, T., Štěrba, K., Slabý, M., Kubizniaková, P., and Čejka, P. 2019. The chemical profiling of fatty acids during the brewing process. *J. Sci. Food Agric.* 99, 4, 1772–1779.

[254] Osburn, K., Amaral, J., Metcalf, S. R., Nickens, D. M., Rogers, C. M., Sausen, C., Caputo, R., Miller, J., Li, H., Tennessen, J. M., and Bochman, M. L. 2018. Primary souring: A novel bacteria-free method for sour beer production. *Food Microbiol.* 70, 76–84.

[255] Osorio-Paz, I., Brunauer, R., and Alavez, S. 2020. Beer and its non-alcoholic compounds in health and disease. *Crit. Rev. Food Sci. Nutr.* 60, 20, 3492–3505.

[256] Parise, A., Kovaleva, E. G., Glukhareva, T. V., and Shatunova, S. A. 2017. Biotechnological production of non-traditional beer. *AIP Conf. Proc.* 1886, 1, 20098.

[257] Pfeiffer, T. and Morley, A. 2014. An evolutionary perspective on the Crabtree effect. *Front. Mol. Biosci.* 1, 17.

[258] Piornos, J. A., Balagiannis, D. P., Methven, L., Koussissi, E., Brouwer, E., and Parker, J. K. 2020. Elucidating the odor-active aroma compounds in alcohol-free beer and their contribution to the worty flavor. *J. Agric. Food Chem.* 68, 37, 10088–10096.

[259] Piper, P. W. 1995. The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. *FEMS Microbiol. Lett.* 134, 2-3, 121–127.

[260] Pires, E. J., Teixeira, J. A., Brányik, T., and Vicente, A. A. 2014. Yeast: the soul of beer's aroma—a review of flavour-active esters and higher alcohols produced by the brewing yeast. *Appl. Microbiol. Biotechnol.* 98, 5, 1937–1949.

[261] Piškur, J. and Langkjaer, R. B. 2004. Yeast genome sequencing: the power of comparative genomics. *Mol. Microbiol.* 53, 2, 381–389.

[262] Piškur, J., Rozpedowska, E., Polakova, S., Merico, A., and Compagno, C. 2006. How did *Saccharomyces* evolve to become a good brewer? *Trends Genet.* 22, 4, 183–186.

[263] Puerari, C., Strejc, J., Souza, A. C., Karabín, M., Schwan, R. F., and Brányik, T. 2016. Optimization of alcohol-free beer production by lager and cachaça yeast strains using response surface methodology. *J. Inst. Brew.* 122, 1, 69–75.

[264] Querol, A., Fernández-Espinar, M. T., and Belloch, C. 2008. Genetics of Yeasts. In *Meat biotechnology*, F. Toldrá, Ed. Springer, New York, London, 167–179. https://doi.org/10.1007/978-0-387-79382-5_7.

[265] Rautio, J. and Londesborough, J. 2003. Maltose Transport by Brewer's Yeasts in Brewer's Wort. *J. Inst. Brew.* 109, 3, 251–261.

[266] Ravasio, D., Carlin, S., Boekhout, T., Groenewald, M., Vrhovsek, U., Walther, A., and Wendland, J. 2018. Adding Flavor to Beverages with Non-Conventional Yeasts. *Fermentation* 4, 1, 15.

[267] Ricci, A., Allende, A., Bolton, D., Chemaly, M., Davies, R., Fernández Escámez, P. S., Girones, R., Koutsoumanis, K., Lindqvist, R., Nørrung, B., Robertson, L., Ru, G., Sanaa, M., Simmons, M., Skandamis, P., Snary, E., Speybroeck, N., Ter Kuile, B., Threlfall, J., Wahlström, H., Cocconcelli, P. S., Peixe, L., Maradona, M. P., Querol, A., Suarez, J. E., Sundh, I., Vlak, J., Barizzone, F., Correia, S., and Herman, L. 2018. Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 8: suitability of taxonomic units notified to EFSA until March 2018. *EFSA Journal* 16, 7, e05315.

[268] Rodrigues, F., Ludovico, P., and Leão, C. 2006. Sugar Metabolism in Yeasts: an Overview of Aerobic and Anaerobic Glucose Catabolism. In *Biodiversity and ecophysiology of yeasts*, C. A. Rosa

and G. Péter, Eds. The yeast handbook. Springer, Berlin, London, 101–121. https://doi.org/10.1007/3-540-30985-3_6.

[269] Rodríguez, M. E., Lopes, C. A., Barbagelata, R. J., Barda, N. B., and Caballero, A. C. 2010. Influence of *Candida pulcherrima* Patagonian strain on alcoholic fermentation behaviour and wine aroma. *Int. J. Food Microbiol.* 138, 1-2, 19–25.

[270] Rodríguez Madrera, R., Pando Bedriñana, R., and Suárez Valles, B. 2021. Evaluation of indigenous non-*Saccharomyces* cider yeasts for use in brewing. *Eur. Food Res. Technol.* 247, 4, 819–828.

[271] Romero, R., Bagur, M. G., Sánchez-Viñas, M., and Gázquez, D. 2003. The influence of the brewing process on the formation of biogenic amines in beers. *Anal. Bioanal. Chem.* 376, 2, 162–167.

[272] Rückle, L. 2005. Hop acids as natural antibacterials in ethanol fermentation. *Int. Sugar J.* 107, 162–165.

[273] Sadoudi, M., Tourdot-Maréchal, R., Rousseaux, S., Steyer, D., Gallardo-Chacón, J.-J., Ballester, J., Vichi, S., Guérin-Schneider, R., Caixach, J., and Alexandre, H. 2012. Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. *Food Microbiol.* 32, 2, 243–253.

[274] Saerens, S. and Swiegers, J. H. 2014. *Production of low-alcohol or alcohol-free beer with* Pichia kluyveri *yeast strains*. Google Patents, WO2014/135673A2.

[275] Saerens, S. M. G., Verbelen, P. J., Vanbeneden, N., Thevelein, J. M., and Delvaux, F. R. 2008. Monitoring the influence of high-gravity brewing and fermentation temperature on flavour formation by analysis of gene expression levels in brewing yeast. *Appl. Microbiol. Biotechnol.* 80, 6, 1039–1051.

[276] Saerens, S. M.G., Delvaux, F., Verstrepen, K. J., van Dijck, P., Thevelein, J. M., and Delvaux, F. R. 2008. Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation. *Appl. Environ. Microbiol.* 74, 2, 454–461.

[277] Saison, D., Schutter, D. P. de, Uyttenhove, B., Delvaux, F., and Delvaux, F. R. 2009. Contribution of staling compounds to the aged flavour of lager beer by studying their flavour thresholds. *Food Chem.* 114, 4, 1206–1215.

[278] Saison, D., Schutter, D. P. de, Vanbeneden, N., Daenen, L., Delvaux, F., and Delvaux, F. R. 2010. Decrease of aged beer aroma by the reducing activity of brewing yeast. *J. Agric. Food Chem.* 58, 5, 3107–3115.

[279] Sakamoto, K. and Konings, W. N. 2003. Beer spoilage bacteria and hop resistance. *Int. J. Food Microbiol.* 89, 2-3, 105–124.

[280] Sampaio J. P., Libkind D., and Hutzler M. submitted 2016. *Brewing Microbiology: Current Research, Omics and Microbial Ecology*. *Yeast taxonomy and typing (Chapter 2)*. Horizon Press, Norfolk.

[281] Sanders, M. E. 2006. Summary of probiotic activities of *Bifidobacterium lactis* HN019. *J. Clin. Gastroenterol.* 40, 9, 776–783.

[282] Sanders, M. E. 2008. Probiotics: definition, sources, selection, and uses. *Clin. Infect. Dis.* 46 Supplement 2, S58-61; discussion S144-51.

[283] Sannino, C., Mezzasoma, A., Buzzini, P., and Turchetti, B. 2019. Non-conventional Yeasts for Producing Alternative Beers. In *Non-conventional Yeasts: from Basic Research to Application*, A. Sibirny, Ed. Springer International Publishing, Cham, 361–388. https://doi.org/10.1007/978-3-030-21110-3_11.

[284] Satora, P., Skotniczny, M., Strnad, S., and Ženišová, K. 2020. Yeast Microbiota during Sauerkraut Fermentation and Its Characteristics. *Int. J. Mol. Sci.* 21, 24, 9699.

[285] Sawadogo-Lingani, H., Lei, V., Diawara, B., Nielsen, D. S., Møller, P. L., Traoré, A. S., and Jakobsen, M. 2007. The biodiversity of predominant lactic acid bacteria in dolo and pito wort for the production of sorghum beer. *J. Appl. Microbiol.* 103, 4, 765–777.

[286] Schieberle, P. 1991. Primary odorants of pale lager beer. *Eur. Food Res. Technol.* 193, 6, 558–565.

[287] Schmalreck, A. F. and Teuber, M. 1975. Structural features determining the antibiotic potencies of natural and synthetic hop bitter resins, their precursors and derivatives. *Can. J. Microbiol.* 21, 2, 205–212.

[288] Schneiderbanger, H. J. 2016. *Entstehung, Abbau und potentielle Verlustquellen wertgebender Aromen während der Produktion von Weizenbieren*. Dissertation, Technische Universität München.

[289] Schneiderbanger, J. 2019. *Occurrence, Detection, Characterization and Description of Selected Beer-Spoilage Lactic Acid Bacteria*. Dissertation, Technische Universität München.

[290] Schönberger, C. and Kostelecky, T. 2011. 125th Anniversary Review: The Role of Hops in Brewing. *J. Inst. Brew.* 117, 3, 259–267.

[291] Schutter, D. P. de, Meester, M.-R. de, Saison, D., Delvaux, F., Derdelinckx, G., Rock, J.-M., Neven, H., and Delvaux, F. R. 2008. Characterization and quantification of thermal load during wort boiling. *BrewingScience* 52, 121–134.

[292] Schutter, D. P. de, Saison, D., Delvaux, F., Derdelinckx, G., Rock, J.-M., Neven, H., and Delvaux, F. R. 2008. Characterization of volatiles in unhopped wort. *J. Agric. Food Chem.* 56, 1, 246–254.

[293] Schutter, D. P. de, Saison, D., Delvaux, F., Derdelinckx, G., Rock, J.-M., Neven, H., and Delvaux, F. R. 2008. Release and evaporation of volatiles during boiling of unhopped wort. *J. Agric. Food Chem.* 56, 13, 5172–5180.

[294] Schutter, D. de. 2008. *The influence of thermal load during wort boiling on the flavour stability of beer*. Dissertation, K.U. Leuven.

[295] Sefa-Dedeh, S., Sanni, A. I., Tetteh, G., and Sakyi-Dawson, E. 1999. Yeasts in the traditional brewing of pito in Ghana. *World J. Microbiol. Biotechnol.* 15, 5, 593–597.

[296] Sefton, M. A., Skouroumounis, G. K., Elsey, G. M., and Taylor, D. K. 2011. Occurrence, sensory impact, formation, and fate of damascenone in grapes, wines, and other foods and beverages. *J. Agric. Food Chem.* 59, 18, 9717–9746.

[297] Senkarcinova, B., Graça Dias, I. A., Nespor, J., and Branyik, T. 2019. Probiotic alcohol-free beer made with *Saccharomyces cerevisiae* var. *boulardii*. *LWT* 100, 362–367.

[298] Sgouros, G., Mallouchos, A., Filippousi, M.-E., Banilas, G., and Nisiotou, A. 2020. Molecular Characterization and Enological Potential of A High Lactic Acid-Producing *Lachancea thermotolerans* Vineyard Strain. *Foods* 9, 5, 595.

[299] Shimwell, J. L. 1945. The Gram stain and its significance in brewing bacteriology. *Wallerstein Lab. Commun.* 8, 23-33.

[300] Shinohara, T., Kubodera, S., and Yanagida, F. 2000. Distribution of phenolic yeasts and production of phenolic off-flavors in wine fermentation. *J. Biosci. Bioeng.* 90, 1, 90–97.

[301] Siefker, J. A. and Pollock, G. E. 1956. Melanoidins in the Brewing Processes. I. Formation of Aldehydes during Wort Boiling. *Proceedings. Annual meeting - American Society of Brewing Chemists* 14, 1, 5–12.

[302] Silva, D. P., Brányik, T., Teixeira, J. A., and Almeida E Silva, J. B. 2010. Alcohol-free beer (written in Portuguese). In *Bebidas Alcoólicas: Ciência e Tecnologia*, W. G. Venturini Filho, Ed. 1. Edgard Blucher Ltda, São Paulo, 69–83.

[303] Sohrabvandi, S., Mousavi, S. M., Razavi, S. H., Mortazavian, A. M., and Rezaei, K. 2010. Alcohol-free Beer: Methods of Production, Sensorial Defects, and Healthful Effects. *Food Rev. Int.* 26, 4, 335–352.

[304] Sohrabvandi, S., Razavi, S. H., Mousavi, S. M., Mortazavian, A., and Rezaei, K. 2009. Application of *Saccharomyces rouxii* for the production of non-alcoholic beer. *Food Sci. Biotechnol.* 18, 5, 1132–1137.

[305] Solieri, L., Landi, S., Vero, L. de, and Giudici, P. 2006. Molecular assessment of indigenous yeast population from traditional balsamic vinegar. *J. Appl. Microbiol.* 101, 1, 63–71.

[306] Song, Y.-R., Jeong, D.-Y., and Baik, S.-H. 2015. Monitoring of Yeast Communities and Volatile Flavor Changes During Traditional Korean Soy Sauce Fermentation. *J. Food Sci.* 80, 9, M2005-14.

[307] Spano, G., Russo, P., Lonvaud-Funel, A., Lucas, P., Alexandre, H., Grandvalet, C., Coton, E., Coton, M., Barnavon, L., Bach, B., Rattray, F., Bunte, A., Magni, C., Ladero, V., Alvarez, M., Fernández, M., Lopez, P., Palencia, P. F. de, Corbi, A., Trip, H., and Lolkema, J. S. 2010. Biogenic amines in fermented foods. *Eur. J.Clin. Nutr.* 64 Supplement 3, S95-100.

[308] Spitaels, F., Wieme, A. D., Janssens, M., Aerts, M., Daniel, H.-M., van Landschoot, A., Vuyst, L. de, and Vandamme, P. 2014. The microbial diversity of traditional spontaneously fermented lambic beer. *PloS one* 9, 4, e95384.

[309] Srinivasan, V., Goldberg, D., and Haas, G. J. 2004. Contributions to the Antimicrobial Spectrum of Hop Constituents. *Econ. Bot.* 58, 1, S230-S238.

[310] Stambuk, B. U., da Silva, M. A., Panek, A. D., and Araujo, P. S. de. 1999. Active alpha-glucoside transport in *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* 170, 1, 105–110.

[311] Staniszewski, A. and Kordowska-Wiater, M. 2021. Probiotic and Potentially Probiotic Yeasts-Characteristics and Food Application. *Foods* 10, 6, 1306.

[312] Statista. 2022. *Alkoholfreies Bier - Weltweit*. https://de.statista.com/outlook/cmo/alkoholische-getraenke/bier/alkoholfreies-bier/weltweit. Accessed October 3, 2022.

[313] Statistisches Bundesamt. 2022. *Produktion von alkoholfreiem Bier in Deutschland in den Jahren 2005 bis 2021 (in 1.000 Hektoliter)*. https://de.statista.com/statistik/daten/studie/425881/umfrage/produktion-von-alkoholfreiem-bier-in-deutschland/. Accessed August 16, 2022.

[314] Steensels, J. and Verstrepen, K. J. 2014. Taming wild yeast: potential of conventional and non-conventional yeasts in industrial fermentations. *Annu. Rev. Microbiol.* 68, 61–80.

[315] Stewart, G. G. 2006. Studies on the Uptake and Metabolism of Wort Sugars During Brewing Fermentations. *MBAA TQ* 43, 4, 265-269.

[316] Tataridis, P., Drosou, F., Kanellis, A., Kechagia, D., Stylianos, L., and Chatzilazarou, A., Eds. 2016. *Differentiating beer aroma, flavor and alcohol content through the use of* Torulaspora delbrueckii. Young Scientists Symposium. Chico, California, USA.

[317] Tataridis, P., Kanellis, A., Logothetis, S., and Nerantzis, E. 2013. Use of non-*Saccharomyces Torulaspora delbrueckii* yeast strains in winemaking and brewing. *Matica Srpska Journal for Natural Sciences,* 124, 415–426.

[318] Teoh, A. L., Heard, G., and Cox, J. 2004. Yeast ecology of Kombucha fermentation. *Int. J. Food Microbiol.* 95, 2, 119–126.

[319] Thurston, P. A. and Tubb, R. S. 1981. Screening Yeast Strains for their Ability to Produce Phenolic Off-Flavors: A Simple Method for Determining Phenols in Wort and Beer. *J. Inst. Brew.* 87, 3, 177–179.

[320] Titze, J., Beermann, M., Blieninger, S., and Kaltenbrunner, A., Eds. 2014. *Sour wort concentrate as an efficient alternative to traditional biological acidification or the use of acidified malt*. Trends in Brewing. Ghent, Belgium.

[321] Toh, D. W. K., Chua, J. Y., and Liu, S. Q. 2018. Impact of simultaneous fermentation with *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* on volatile and non-volatile constituents in beer. *LWT* 91, 26–33.

[322] Toh, D. W. K., Chua, J. Y., Lu, Y., and Liu, S. Q. 2019. Evaluation of the potential of commercial non-*Saccharomyces* yeast strains of *Torulaspora delbrueckii* and *Lachancea thermotolerans* in beer fermentation. *Int. J. Food Sci. Technol.* 235, 303.

[323] Tokpohozin, S. E., Lauterbach, A., Fischer, S., Behr, J., Sacher, B., and Becker, T. 2016. Phenotypical and molecular characterization of yeast content in the starter of "*Tchoukoutou*," a Beninese African sorghum beer. *Eur. Food Res. Technol.* 242, 12, 2147–2160.

[324] Tominaga, T., Okuzawa, Y., Kato, S., and Suzuki, M. 2003. The first isolation of two types of trifluoroleucine resistant mutants of *Saccharomyces servazzii*. *Biotechnol. Lett.* 25, 20, 1735–1738.

[325] Trius-Soler, M., Vilas-Franquesa, A., Tresserra-Rimbau, A., Sasot, G., Storniolo, C. E., Estruch, R., and Lamuela-Raventós, R. M. 2020. Effects of the Non-Alcoholic Fraction of Beer on Abdominal Fat, Osteoporosis, and Body Hydration in Women. *Molecules* 25, 17, 3910.

[326] Turck, D., Bresson, J.-L., Burlingame, B., Dean, T., Fairweather-Tait, S., Heinonen, M., Hirsch-Ernst, K. I., Mangelsdorf, I., McArdle, H., Naska, A., Neuhäuser-Berthold, M., Nowicka, G., Pentieva, K., Sanz, Y., Siani, A., Sjödin, A., Stern, M., Tomé, D., Vinceti, M., Willatts, P., Engel, K.-H., Marchelli, R., Pöting, A., Poulsen, M., Salminen, S., Schlatter, J., Arcella, D., Gelbmann, W., Sesmaisons-Lecarré, A. de, Verhagen, H., and van Loveren, H. 2016. Guidance on the preparation and presentation of an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283. *EFS2* 14, 11.

[327] U.S. Food and Drug Administration. 2018. *Microorganisms & Microbial-Derived Ingredients Used in Food (Partial List)*. https://www.fda.gov/food/generally-recognized-safe-gras/microorganisms-microbial-derived-ingredients-used-food-partial-list. Accessed July 15, 2022.

[328] van Haecht, J. L. and Dufour, J. P. 1995. The production of sulfur compounds by brewing yeast: A review. *Cerevisia and biotechnology* 20, 1, 51–64.

[329] van Laere, S. D. M., Saerens, S. M. G., Verstrepen, K. J., van Dijck, P., Thevelein, J. M., and Delvaux, F. R. 2008. Flavour formation in fungi: characterisation of KlAtf, the *Kluyveromyces lactis* orthologue of the *Saccharomyces cerevisiae* alcohol acetyltransferases Atf1 and Atf2. *Appl. Microbiol. Biotechnol.* 78, 5, 783–792.

[330] van Rijswijck, I. M. H., van Mastrigt, O., Pijffers, G., Wolkers-Rooijackers, J. C. M., Abee, T., Zwietering, M. H., and Smid, E. J. 2019. Dynamic modelling of brewers' yeast and *Cyberlindnera fabianii* co-culture behaviour for steering fermentation performance. *Food Microbiol.* 83, 113–121.

[331] van Rijswijck, I. M. H., Wolkers-Rooijackers, J. C. M., Abee, T., and Smid, E. J. 2017. Performance of non-conventional yeasts in co-culture with brewers' yeast for steering ethanol and aroma production. *Microb. Biotechnol.* 10, 6, 1591–1602.

[332] Vanbeneden, N., Gils, F., Delvaux, F., and Delvaux, F. R. 2008. Formation of 4-vinyl and 4-ethyl derivatives from hydroxycinnamic acids: Occurrence of volatile phenolic flavour compounds in beer and distribution of Pad1-activity among brewing yeasts. *Food Chem.* 107, 1, 221–230.

[333] Vanderhaegen, B., Neven, H., Coghe, S., Verstrepen, K. J., Derdelinckx, G., and Verachtert, H. 2003. Bioflavoring and beer refermentation. *Appl. Microbiol. Biotechnol.* 62, 2-3, 140–150.

[334] Vanderhaegen, B., Neven, H., Verachtert, H., and Derdelinckx, G. 2006. The chemistry of beer aging – a critical review. *Food Chem.* 95, 3, 357–381.

[335] Vaštík, P., Šmogrovičová, D., Kafková, V., Sulo, P., Furdíková, K., and Špánik, I. 2020. Production and characterisation of non-alcoholic beer using special yeast. *Kvasný Průmysl/Fermentation Industry* 66, 5, 336–344.

[336] Verachtert, H., Kumara, H. M. C., and Dawoud, E. 1989. Yeasts in mixed cultures with emphasis on lambic beer brewing. In *Yeast—biotechnology and biocatalysis*, H. Verachtert and R. de Mot, Eds. Dekker, New York, 429–478.

[337] Verstrepen, K. J., Derdelinckx, G., and Delvaux, F. R. 2003. Esters in beer-part 1: The fermentation process: more than ethanol formation. *Cerevisia* 28, 3, 41–49.

[338] Verstrepen, K. J., Derdelinckx, G., and Delvaux, F. R. 2003. Esters in beer-part 2: Controlling ester levels during beer fermentation: a biochemical approach. *Cerevisia* 28, 4, 22-33.

[339] Verstrepen, K. J., Derdelinckx, G., Dufour, J.-P., Winderickx, J., Thevelein, J. M., Pretorius, I. S., and Delvaux, F. R. 2003. Flavor-active esters: adding fruitiness to beer. *J. Biosci. Bioeng.* 96, 2, 110–118.

[340] Verstrepen, K. J., Derdelinckx, G., Verachtert, H., and Delvaux, F. R. 2003. Yeast flocculation: what brewers should know. *Appl. Microbiol. Biotechnol.* 61, 3, 197–205.

[341] Verstrepen, K. J., Iserentant, D., Malcorps, P., Derdelinckx, G., van Dijck, P., Winderickx, J., Pretorius, I. S., Thevelein, J. M., and Delvaux, F. R. 2004. Glucose and sucrose: hazardous fast-food for industrial yeast? *Trends Biotechnol.* 22, 10, 531–537.

[342] Verstrepen, K. J., Jansen, A., Lewitter, F., and Fink, G. R. 2005. Intragenic tandem repeats generate functional variability. *Nat. Genet.* 37, 9, 986–990.

[343] Vriesekoop, F., Krahl, M., Hucker, B., and Menz, G. 2012. 125th Anniversary Review: Bacteria in brewing: The good, the bad and the ugly. *J. Inst. Brew.* 118, 4, 335–345.

[344] Vuyst, L. de, Harth, H., van Kerrebroeck, S., and Leroy, F. 2016. Yeast diversity of sourdoughs and associated metabolic properties and functionalities. *Int. J. Food Microbiol.* 239, 26–34.

[345] Weigert, C. 2010. *Erhöhung der Zelldichte während der Propagation von Bierhefe*. Dissertation, Technische Universität Berlin.

[346] Weinrich, R., Nitzko, S., Spiller, A., and Zühlsdorf, A. 2015. Verbraucherverständnis von Verkehrsbezeichnungen. *J. Verbr. Lebensm.* 10, 1, 13–22.

[347] Wickerham, L. J. 1965. New Heterothallic species of *Hansenula*. *Mycopathol. Mycol. Appl.* 26, 1, 79–86.

[348] Willaert, R. and Nedovic, V. A. 2006. Primary beer fermentation by immobilised yeast—a review on flavour formation and control strategies. *J. Chem. Technol. Biotechnol.* 81, 8, 1353–1367.

[349] Wu, H., Ito, K., and Shimoi, H. 2005. Identification and characterization of a novel biotin biosynthesis gene in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 71, 11, 6845–6855.

[350] Xie, Z.-B., Zhang, K.-Z., Kang, Z.-H., and Yang, J.-G. 2021. *Saccharomycopsis fibuligera* in liquor production: A review. *Eur. Food Res. Technol.* 247, 7, 1569–1577.

[351] Xu, D., Chen, T., and Liu, Y. 2021. The physical properties, antioxidant and antimicrobial activity of chitosan–gelatin edible films incorporated with the extract from hop plant. *Polym. Bull.* 78, 7, 3607–3624.

[352] Yang, Y., Zhong, H., Yang, T., Lan, C., and Zhu, H. 2021. Characterization of the key aroma compounds of a sweet rice alcoholic beverage fermented with *Saccharomycopsis fibuligera*. *J. Food Sci. Technol.* 58, 10, 3752–3764.

[353] Yeo, H. Q. and Liu, S.-Q. 2014. An overview of selected specialty beers: developments, challenges and prospects. *Int. J. Food Sci. Technol.* 49, 7, 1607–1618.

[354] Yilmaztekin, M., Erten, H., and Cabaroglu, T. 2008. Production of Isoamyl Acetate from Sugar Beet Molasses by *Williopsis saturnus* var. *saturnus*. *J. Inst. Brew.* 114, 1, 34–38.

[355] Yoshimoto, H., Fujiwara, D., Momma, T., Ito, C., Sone, H., Kaneko, Y., and Tamai, Y. 1998. Characterization of the ATF1 and Lg-ATF1 genes encoding alcohol acetyltransferases in the bottom fermenting yeast *Saccharomyces pastorianus*. *Journal of Fermentation and Bioengineering* 86, 1, 15–20.

[356] Yoshimoto, H., Fujiwara, D., Momma, T., Tanaka, K., Sone, H., Nagasawa, N., and Tamai, Y. 1999. Isolation and characterization of the ATF2 gene encoding alcohol acetyltransferase II in the bottom fermenting yeast *Saccharomyces pastorianus*. *Yeast* 15, 5, 409–417.

[357] Yoshioka, K. and Hashimoto, N. 1981. Ester Formation by Alcohol Acetyltransferase from Brewers' Yeast. *Agricultural and Biological Chemistry* 45, 10, 2183–2190.

[358] Yoshioka, K. and Hashimoto, N. 1983. Cellular fatty acid and ester formation by brewers' yeast. *Agricultural and Biological Chemistry* 47, 10, 2287–2294.

[359] Yoshioka, K. and Hashimoto, N. 1984. Acetyl-CoA of brewers' yeast and formation of acetate esters. *Agricultural and Biological Chemistry* 48, 1, 207–209.

[360] Yun, J. W., Park, K. S., Ki, C.-S., and Lee, N. Y. 2013. Catheter-related bloodstream infection by *Lindnera fabianii* in a neutropenic patient. *J. Med. Microbiol.* 62, 6, 922–925.

[361] Zdaniewicz, M., Satora, P., Pater, A., and Bogacz, S. 2020. Low Lactic Acid-Producing Strain of *Lachancea thermotolerans* as a New Starter for Beer Production. *Biomolecules* 10, 2,256.

[362] Zghair, F. S. 2020. First record of *Cyberlindnera fabianii* that causes Vulvovaginitis in Iraq. *EurAsian J. BioSci.* 14, 1.

5 Permission of publishers for imprints of publications

Publication 1

Methner, Y.; Hutzler, M.; Matoulková, D.; Jacob, F.; Michel, M. (2019). **Screening for the Brewing Ability of Different Non-*Saccharomyces* Yeasts.** Fermentation 5 (4): 101. DOI: 10.3390/fermentation5040101.

Methner, Yvonne

Von: Mayora Li <mayora.li@mdpi.com>
Gesendet: 20 June 2022 04:28
An: Methner, Yvonne
Cc: fermentation@mdpi.com
Betreff: Re: Informal permission DOI: 10.3390/fermentation5040101

Dear Yvonne,

Thank you for your e-mail. As all our papers are fully open access and distributed under the terms and conditions of the Creative Commons Attribution License (CC BY), i.e., authors retain the copyright of their own paper. You can re-use the materials under the conditions stated
here: https://creativecommons.org/licenses/by/4.0/. Therefore, please feel free to reuse, but cite the paper where you will reference.

By the way, /Fermentation/ will receive a new IF (about 5) this month.
You are very welcome to contribute to /Fermentation/ again in the future. We look forward to cooperating with you.

Please feel free to contact us if you have any questions.

Kind regards,

Ms. Mayora Li
Managing Editor

- Submit to Fermentation:
https://susy.mdpi.com/user/manuscripts/upload?journal=fermentation

- Recruiting Editorial Board Members for Fermentation:
https://www.mdpi.com/journal/fermentation/announcements/2726

- Topical Advisory Panel Application
https://www.mdpi.com/journal/fermentation/topical_advisory_panel_application

Fermentation (http://www.mdpi.com/journal/fermentation)
Fermentation received its first Impact Factor 3.975 (2021 Journal Citation Reports®)
LinkedIn: https://www.linkedin.com/company/76270919/admin/
Fermentation channel on Twitter (@Ferment_MDPI)

--

On 2022/6/19 23:23, Methner, Yvonne wrote:
> CAUTION - EXTERNAL: This email originated from outside of MDPI
> organisation. BE CAUTIOUS especially to click links or open attachments.
>
> Dear Ms. Mayora Li,
>
> As I am preparing my doctoral thesis (PhD), which is intended to be a

> compilation of an overall summary (overview) and research papers bound
> together, I would greatly appreciate if you would allow me to include
> the following publication in my dissertation:
>
> Methner, Y.; Hutzler, M.; Matoulková, D.; Jacob, F.; Michel, M. (2019).
> *Screening for the Brewing Ability of Different Non-/Saccharomyces
> Yeasts/.*Fermentation 5 (4): 101. DOI: 10.3390/fermentation5040101.
>
> An informal permission would be fine, which I could include in the
> appendix of my dissertation.
>
> Yours sincerely,
>
> Yvonne Methner
>
> M. Eng. Yvonne Methner
>
> Forschung & Entwicklung / Research & Development
>
> Forschungszentrum Weihenstephan
>
> für Brau- und Lebensmittelqualität
>
> Technische Universität München
>
> Alte Akademie 3
>
> D-85354 Freising-Weihenstephan
>
> Tel.: +49 8161 71-4531
>
> Fax: +49 8161 71-4181
>
> Email: yvonne.methner@tum.de <mailto:yvonne.methner@tum.de>
>
> Internet: http://www.blq-weihenstephan.de
> <http://www.blq-weihenstephan.de/>

Publication 2

Methner, Y.; Hutzler, M.; Zarnkow, M.; Prowald, A.; Endres, F.; Jacob, F. (2022). **Investigation of Non-*Saccharomyces* Yeast Strains for Their Suitability for the Production of Non-Alcoholic Beers with Novel Flavor Profiles.** Journal of the American Society of Brewing Chemists 80 (4): 341-355. DOI: 10.1080/03610470.2021.2012747.

Methner, Yvonne

Von: Inge Russell <jasbceditor@gmail.com>
Gesendet: 20 June 2022 19:12
An: Methner, Yvonne; Inge Russell
Betreff: Permission DOI: 10.1080/03610470.2021.2012747 granted to include in thesis

Dear Yvonne,

Please accept this email as formal permission to reproduce the article DOI **10.1080/03610470.2021.2012747** in your thesis.

For a PhD student publishing a thesis, in your author agreement that you previously signed for your article, you were already covered in terms of permission. See the section below from the Taylor and Francis Agreement.

"Section 4 Viii You have the right to include your paper in a thesis or dissertation that is not to be published commercially, provided that acknowledgement of prior publication in the Journal is given. "

Best regards
Inge

Dr. Inge Russell
Editor - JASBC

From: "Methner, Yvonne" <yvonne.methner@tum.de>
Date: June 19, 2022 at 11:18:55 AM EDT
To: Inge Russell <irussell@alltech.com>
Subject: Formal **permission DOI: 10.1080/03610470.2021.2012747**

I would greatly appreciate if you would allow me to include the following publication in my dissertation:

Methner, Y.; Hutzler, M.; Zarnkow, M.; Prowald, A.; Endres, F.; Jacob, F. (2022). Investigation of Non-*Saccharomyces* Yeast Strains for Their Suitability for the Production of Non-Alcoholic Beers with Novel Flavor Profiles. Journal of the American Society of Brewing Chemists: 1–15, DOI: 10.1080/03610470.2021.2012747.

Publication 3

Methner, Y.; Magalhães, F.; Raihofer, L.; Zarnkow, M.; Jacob, F.; Hutzler, M. (2022). **Beer fermentation performance and sugar uptake of *Saccharomycopsis fibuligera*–A novel option for low-alcohol beer.** Frontiers in Microbiology 13: 1011155. DOI: 10.3389/fmicb.2022.1011155.

Methner, Yvonne

Von: Frontiers in Microbiology - Production <microbiology.production.office@frontiersin.org>
Gesendet: 13 October 2022 13:11
An: Methner, Yvonne
Betreff: Copyright (permissions request)

Dear Yvonne Methner,

All Frontiers content is published under a CC-BY Creative Commons attribution license that enables anyone to use the publication freely, given appropriate attribution to the author(s) and citing Frontiers as the original publisher. Therefore, when reusing a figure, or the article contents, the original publication should be cited. For example:

Figure 1, Chen L, Li J, Zhu W, Kuang Y, Liu T, Zhang W, Chen X and Peng C (2020) Skin and Gut Microbiome in Psoriasis: Gaining Insight Into the Pathophysiology of It and Finding Novel Therapeutic Strategies. Front. Microbiol. 11:589726. doi: 10.3389/fmicb.2020.589726

I hope this answers your query, please let me know if you have any other questions.

Best regards,

Sidra

Production Team
Production Manager: Catriona

Frontiers | Production Office

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne
Switzerland
Office T +41 21 510 17 06
www.frontiersin.org

---------- Original Message ----------
From: Methner, Yvonne [yvonne.methner@tum.de]
Sent: 08.10.2022 11:32
To: microbiology.production.office@frontiersin.org
Subject: AW: Frontiers: Congratulations! Your article is published

Dear Frontiers Microbiology Production Office,

Thanks a lot for the information that our article has been published with you. We are very pleased and appreciate that we were able to publish our article with you.

As I am preparing my doctoral thesis (PhD), which is intended to be a compilation of an overall summary (overview) and research papers bound together, I would greatly appreciate if you would allow me to include the publication in my dissertation:

Methner, Y.; Magalhães, F.; Raihofer, L.; Zarnkow, M.; Jacob, F.; Hutzler, M. (2022). Beer fermentation performance and sugar uptake of *Saccharomycopsis fibuligera*–A novel option for low-alcohol beer. Frontiers in Microbiology 13: 1011155. DOI: 10.3389/fmicb.2022.1011155.

An informal permission would be fine, which I could include in the appendix of my dissertation.

Thank you in advance and kind regards

Yvonne Methner

Publication 4

Methner, Y.; Dancker, P.; Maier, R.; Latorre, M.; Hutzler, M.; Zarnkow, M.; Steinhaus, M.; Libkind, D.; Frank, S.; Jacob, F. (2022). **Influence of Varying Fermentation Parameters of the Yeast Strain *Cyberlindnera saturnus* on the Concentrations of Selected Flavor Components in Non-Alcoholic Beer Focusing on (*E*)-*β*-Damascenone.** Foods 11 (7): 1038. DOI: 10.3390/foods11071038.

Methner, Yvonne

Von: Natalija Knezevic <natalija.knezevic@mdpi.com>
Gesendet: 20 June 2022 09:52
An: Methner, Yvonne
Cc: foods@mdpi.com
Betreff: Re: Informal permission DOI: 10.3390/foods11071038

Dear Yvonne,

Thank you for your mail.

You do not need any permission, the author owns the copyright of the article published by our journal. Please, note that the citation of the paper is needed so please add the citation to your doctoral thesis.

In case of any question, feel free to contact us.

Kind regards,

Ms. Natalija Knežević
Assistant Editor, MDPI Novi Sad
E-Mail: natalija.knezevic@mdpi.com

MDPI Branch Office, Novi Sad
Bulevar oslobođenja 83, 21000 Novi Sad, Serbia Tel. +381 21 300 14 49 Foods (http://www.mdpi.com/journal/foods)
Twitter: @Foods_MDPI

Reviewer Article Selector:
New Tool for Reviewers to Pick Papers in their Area of Expertise:
https://www.mdpi.com/about/announcements/3943

Disclaimer: The information contained in this message is confidential and intended solely for the use of the individual or entity to whom they are addressed. If you have received this message in error, please inform us by an email reply and then delete the message. You may not copy this message in its entirety or in part, or disclose its contents to anyone.

On 2022-06-19 17:27, Methner, Yvonne wrote:
> Dear Natalija,
>
>
>
> As I am preparing my doctoral thesis (PhD), which is intended to be a
> compilation of an overall summary (overview) and research papers bound
> together, I would greatly appreciate if you would allow me to include
> the following publication in my dissertation:
>
>
>
> Methner, Y.; Dancker, P.; Maier, R.; Latorre, M.; Hutzler, M.;
> Zarnkow, M.; Steinhaus, M.; Libkind, D.; Frank, S.; Jacob, F. (2022).
> *Influence of Varying Fermentation Parameters of the Yeast Strain
> /Cyberlindnera saturnus/ on the Concentrations of Selected Flavor
> Components in Non-Alcoholic Beer Focusing on (/E/)-/β/-Damascenone.*Foods 11 (7):
> 1038. DOI: 10.3390/foods11071038.

Publication 5

Methner, Y.; Weber, N.; Kunz, O.; Zarnkow, M.; Rychlik, M.; Hutzler, M.; Jacob, F. (2022). **Investigations into metabolic properties and selected nutritional metabolic byproducts of different non-*Saccharomyces* yeast strains when producing nonalcoholic beer.** FEMS Yeast Research: foac042. DOI: 10.1093/femsyr/foac042.

Methner, Yvonne

Von: jnls.author.support@oup.com
Gesendet: 04 January 2023 08:47
An: Methner, Yvonne
Cc: femsyr@aptaracorp.com
Betreff: RE: Request informal permission DOI: 10.1093/femsyr/foac042 ~4069090

Dear Mrs Methner,

Thank you for your query regarding use of OUP journal FEMS Yeast Research article DOI 10.1093/femsyr/foac042.

As the author of the paper, you retain the right to include the paper in full or in part in a thesis or dissertation, as long as a full acknowledgment is made to the original source of the material including the journal name, volume, issue, page numbers, year of publication, title of article and to Oxford University Press and/or the learned society.

If re-use were to be commercial, status regarding permissions can be seen via the Permissions link for this article (Rightslink ® service).

If you should have any further queries or concerns, please do let us know.

With best regards,
Lorna

Lorna Stevenson Walker
Author Specialist | Oxford Journals
Oxford University Press