TECHNISCHE UNIVERSITÄT MÜNCHEN

Fakultät Wissenschaftszentrum Weihenstephan

für Ernährung, Landnutzung und Umwelt

Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität

Systems for validation of brewing equipment hygiene on the basis of characteristic biofilm organisms

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktor der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

Vorsitzender: Prof. Dr. Horst-Christian Langowski

Prüfer der Dissertation:

1. Hon.-Prof. Dr.-Ing. Friedrich Jacob
2. Prof. Dr. Rudi F. Vogel

Die Dissertation wurde am 28.11.2019 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 06.02.2020 angenommen

Acknowledgement

First of all, I would like to thank Prof. Dr. Fritz Jacob for providing this outstanding topic, and for his support and trust throughout the last years.

Furthermore, I would like to thank Prof. Dr. Rudi F. Vogel for agreeing to review this dissertation and Prof. Dr. Horst-Christian Langowski for acting as chief examiner.

A great support throughout this time was my mentor and friend, Dr. Mathias Hutzler. Thank you for outstanding discussions, great talks, many ideas, reviewing my papers and helping whenever needed.

A big thank you goes out to my colleagues Prof. Dr. Mehmet Coelhan, Dr. Martin Zarnkow, Jennifer Schneiderbanger, Dr. Hubertus Schneiderbanger, Hubert Walter, Dario Cotterchio, Korbinian Haslbeck, Maximilian Michel, Tim Meier-Dörnberg, Florian Mallok, Dominique Stretz, and Friedrich Ampenberger for their collaboration, help and highly valuable discussions.

As this dissertation includes many analyses that would not have been possible without the support of many of the employees of the Research Center Weihenstephan I want to thank all employees especially Susan Illing, Tanja Spranger, Margit Grammer, Monika Mayerhofer, Anna Kleucker, Veronika Keindl, Karl-Heinz Bromig, Markus Schmidt and Joseph Pellmeier for their great support.

A special thank you needs to be expressed to all the students that supported this dissertation with their Diploma, Bachelor, and Master theses or as intern. Thank you to Alexandra Gonsior, Jörg Fütterer, Laura Ossenkopp, Lina Osorio, Nicole Dünzer, Paula Goderbauer, Simon Wengert and Thomas Schönbrunn.

My last thank you is dedicated to Sarah Silva who helped with quality editing of my English when needed.

I want to dedicate this work to my family, my parents Bernhard and Gudrun Riedl, my sisters and to my girlfriend Christina Wengbauer, who never stopped supporting me, showed great patience and were always by my side.

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EIDESSTATTLICHE ERKLÄHRUNG

Hiermit versichere ich eidesstattlich, dass ich die vorliegende Arbeit selbstständig und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus Veröffentlichungen entnommen sind, wurden als solche kenntlich gemacht.
Die Arbeit wurde in keiner gleichen oder ähnlichen Form einer anderen Prüfungsbehörde vorgelegt.

……..
Unterneukirchen,

Notations

% w/v	weight by volume
% w/w	weight by weight
% v/v	Volume percent
$(GTG)_5$	RAPD-PCR primer with the sequence GTGGTGGTGGTGGTG
°C	degree Celsius
µg/ml	microgram per millilitre
µl	microlitre
16S rDNA	area of the ribosomal DNA of bacteria
A	Adenosine
Å	Ångstrom (10^{-10} m)
A.	Acetobacter
approx.	approximate
ATPase	Adenosine triphosphatase
BLAST	Basic Logical Alignment Search Tool
bp	base pairs
C	Cytosine
C.	Candida
c.	circa
c-di-GMP	cyclic di-guanosine monophosphate
CLSM	confocal laser scanning microscopy
CO_2	Carbon dioxide
Cp	crossing point
ct	circuit threshold
Ct	cycle threshold
DNA	Deoxyribonucleic acid
DSMZ	German Collection of Microorganisms and Cell Cultures GmbH
E	optical extinction
e.g.	exempli gratia (for example)
EPS	Extracellular polymeric substances
et al.	et alia (and others)
FAM	fluorescent dye Carboxyfluorescein
Flo11	gene in *Saccharomyces* yeasts, connected with flocculation and adhesion of yeasts
Fo11p	Mannoprotein expressed from the gene Flo11, anchor protein
FRET	Förster resonance energy transfer probes
FZW-BLQ	Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität
G	Guanine
g	gravitational acceleration
g/L	gram per litre
H_2O	water
HEX	fluorescent dye Hexachloro-fluorescein
HitA	gene-coding proton pumps, connected with hop resistance in bacteria
HorA	gene-coding proton pumps, connected with hop resistance in bacteria
HorB	gene-coding proton pumps, connected with hop resistance in bacteria

Notations

HorC	gene-coding proton pumps, connected with hop resistance in bacteria
IAC	Internal amplification control
IBU	International bittering units
L.	*Lactobacillus/Lactococcus/Leuconostoc*
ml	millilitre
MRS	DeMan-Rogosa-Sharpe broth
Muc1	gene in *Saccharomyces* yeasts, connected with flocculation and adhesion of yeasts
MYPG	malt extract, yeast extract, peptone, glucose broth
NBB-B	Selective liquid medium for beer spoiling bacteria (Nachweis bierschädlicher Bakterien nach Back)
NBB-B-AM	Selective liquid medium for biofilm indicator species in beverage plants (Nachweis bierschädlicher Bakterien nach Back)
NBB-C	Selective concentrated liquid medium for beer-spoiling bacteria (Nachweis bierschädlicher Bakterien nach Back)
nM	10^{-9} mol/l
nmol	10^{-9} mol
No.	Number
P.	*Pseudomonas*
PCR	Polymerase chain reaction
pH	negative decadic logarithm of active oxonium ions
POF	Phenolic off-flavour
ppm	parts per million
PU	pasteurisation units
RAPD	Random amplified polymorphic DNA
rDNA	ribosomal DNA
rRNA	ribosomal RNA
S.	*Saccharomyces*
SD	Standard deviation
sp.	species
spp.	species pluralis
T	Thymine
T.	*Torulaspora*
TaqMan	real-time PCR fluorescence probe technique
var.	varians
VPNC	viable, but putative non-culturable
WBM-HR	Wheat beer media by Hutzler and Riedl
WBM-HR	Wheat beer media by Hutzler and Riedl

Zusammenfassung

Biofilme sind in der Umwelt allgegenwärtig. Ein Großteil des mikrobiologischen Lebens auf der Erde existiert in als Biofilm gebundenen Lebensgemeinschaften. Die Zusammensetzung von Biofilmen ist hoch variabel und abhängig vom umgebenden Medium. Austausch zwischen den besiedelten Spezies und die schützende Matrix des Biofilms machen die Lebensgemeinschaften hochgradig anpassungsfähig gegenüber wechselnden Umwelteinflüssen und Nähstoffangeboten und stellen damit einen echten Überlebensvorteil im Vergleich zu planktonisch vorkommenden Mikroorganismen dar.

Auch in Brauereien ist ein Großteil der vorkommenden Mikroorganismen in Biofilmen gebunden vor. Diese Biofilme sind Lebensraum und Brutstätte für Mikroorganismen, die für das Endprodukt als unbedenklich gelten, als auch bierverderbender Mikroorganismen. Der Aufbau und die Entwicklung von brauereigebundenen Biofilmen wurden insbesondere im Abfüllbereich in der Vergangenheit eingehend untersucht. Als Biofilm-startende Mikroorganismen gelten dabei zunächst ubiquitär vorkommende Bakterien, die Oberflächen besiedeln und durch ausgeschiedene Schleimstoffe sogenannte exopolymere Substanzen (EPS) einen stabilen Film bilden, der weiteren Mikroorganismen ein Habitat bieten. Insbesondere bierverderbende Laktobazillen und strikt anaerobe Bakterien wie Pectinatus sp und Megasphaera sp. benötigen ein sauerstoffreies Umfeld und im freien Medium nicht vorhandene Nährstoffe um zu wachsen. Im Mikrohabitat des Biofilms finden diese beide genannte Faktoren. Viele Resistenzen gegen Antibiotika finden sich im Genom der Bakterien auf Plasmiden codiert. So sind die für die biozid wirkenden Hopfenbittersäuren in Bier verantwortlichen Resistenzgene HorA, HorB, HorC und HitA in Laktobazillen ebenfalls plasmidcodiert. Da Bakterien in der Lage sind Plasmide über Ihre eigene Spezies hinaus auszutauschen, können auch die Resistenzen zwischen verschiedenen Spezies übertragen werden. Im räumlich engen Lebensraum des Biofilms ist ein solcher Austausch deutlich begünstigt. Durch diese Faktoren sind Biofilme in Brauereien ein großes Risiko für die mikrobiologische Stabilität des Bieres.

In der betrieblichen Laborpraxis werden Biofilme über Indikatororganismen über mikrobiologische Monitorings nachgewiesen. Dabei werden an kritischen Prozesspunkten mikrobiologische Proben genommen und auf diese Indikatororganismen untersucht. Der Nachweis erfolgt aber entweder quantitativ, wobei sich ein aufbauender Biofilm durch eine Erhöhung der nachgewiesenen Keimzahlen äußert, oder semiquantitativ durch Farbumschlag von Indikatorfarbstoffen und Veränderung des Testmediums in einer vorgegebenen Zeit. Beide Methoden haben den Nachteil durch die notwendige Bebrütungszeit zeitaufwendig zu sein. Eine Veränderung des Produktes in seiner Zusammensetzung kann zu einer Verschiebung in der Zusammensetzung des Biofilms führen und damit den Nachweis der Monitoring Systeme negativ beeinflussen. Ein in dieser

Arbeit behandeltes Beispiel für eine solche Verschiebung stellt der Trend zu hopfenarmen Bieren, sowie alkoholfreien Bieren und Biermischgetränken dar. PCR-basierte Methoden sind in der Lage durch verkürzte Anreicherungszeiten den Nachweisprozess zu verkürzen und können durch quantitative real-time PCR Methoden auch aussagen über die quantitative Verteilung einzelner Spezies liefern. Der quantitative Nachweis risikoorientiert ausgewählter Spezies hilft dabei den Reifegrad eines Biofilms und damit das Produktrisiko besser einzuschätzen.

Der erste Teil der vorliegenden Arbeit behandelt deshalb den Nachweis, Identifizierung von als potentiell bierschädlichen Milchsäurebakterien, namentlich *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Lactobacillus rossiae* und *Lactobacillus acetotolerans* als Biofilmindikatororganismen in Weizenbier. Letztere wurden bereits als Schadorganismen in schwach gehopften Biertypen wie Weizenbier beschrieben. Weiter wurde das initiale Biofilmbildungspotential verschiedener Stämme der genannten Spezies untersucht. Anhand des initialen Biofilmbildungspotentials und Bierschädlichkeit wurde eine risikoorientierte Einteilung für den Herstellungsprozess von Weizenbier und Biofilmreifung vorgenommen. Dabei wurde ein Nährmedium entwickelt und validiert, welches den spezifischen, schnellen Nachweis der genannten Spezies ermöglicht, sowie real-time PCR basierte Nachweissyteme der Einzelspezies entwickelt und validiert.

Ausgehend von der hohen Variabilität des Biofilmbildungspotentials von *Lactobacillus rossiae* und *Lactobacillus brevis* untersucht der zweite Teil die Varianz von *Lactobacillus brevis* in seinen Genotypen, als auch in seinen Phänotypen, repräsentiert in $(GTG)_5$ RAPD PCR Fingerprint, initialem Biofilmbildungspotential, als auch Wachstum in verschiedenen selektiven Medien. Die dabei auftretende Vielfalt an heterogenen Clustern innerhalb der Spezies konnte in einem Feldversuch in einer Brauerei über den kompletten Produktionsprozess beobachtet werten. Die verwendeten stammdifferenzierenden Methoden erwiesen sich dabei als wertvolles Werkzeug um Kontaminationen bis zu ihrem Ursprung zurückzuverfolgen.

Der dritte Teil behandelt Hefespezies, die als Bierschädlinge bekannt sind, als auch Hefen, die bereits als biofilmbildend beschrieben wurden oder ubiquitär im Brauprozess vorliegen. Es wurde ein hefespezifisches Nachweismedium entwickelt und anhand der Spezies *Saccharomyces cerevisiae* var. *diastaticus*, *Saccharomyces cerevisiae* TUM 68, *Saccharomyces pastorianus* var *carlsbergensis* TUM 34/70, *Dekkera anomala*, *Wickerhamomyces anomalus* und *Rhodotorula mucilaginosa* validiert. Das Nachweismedium wurde zur schnellen Detektion und Identifikation mit spezifischen real-time PCR basierten Nachweissystemen kombiniert, für *R. mucilaginosa* wurde diese de novo entwickelt und validiert. Anhand Wachstum in verschiedenen selektiven Medien, Produktschädlichkeit und

Zusammenfassung

Biofilmbildungspotential wurden die genannten Spezies in Produktschädlichkeit und Biofilmreifestadium kategorisiert.

Summary

Biofilms are ubiquitous in our environment. Most of the microbial life on earth exists in communities, bound as biofilm. The composition of biofilms is highly variable and depends on the surrounding medium. Exchange between settling species and the protecting matrix of the biofilm makes these communities highly adaptable to changing environments and nutrient supply, which gives them a survival advantage over planktonic living microorganisms.

In breweries, most of the occurring microorganisms are also bound in biofilms. These biofilms are a habitat and breeding ground for microorganisms, both those of no concern for bottled beer, and microorganisms known for beer spoilage. The structure and development of brewery-based biofilms, especially in the filling department has been reviewed in detail previously. Microorganisms that start biofilms include ubiquitous bacteria, which colonise surfaces and form a stable film by excreting slimy substances referred to as exopolymeric substances (EPS), and this film provides a habitat for other microorganisms. Beer-spoiling lactic acid bacteria and strict anaerobic bacteria such as *Pectinatus* sp. and *Megasphaera* sp. in particular need an oxygen-free environment and nutrients for growth that are unavailable in the free medium. The microhabitat of the biofilm provides both factors. Many resistances to antibiotics are coded within plasmids within the genome of bacteria. The resistance genes against the biocidal hop bitter acids HorA, HorB, HorC and HitA are also coded in plasmids. As bacteria are able to transfer plasmids between species, resistances can also be transferred between species. In the restricted space of the biofilm, such exchange is promoted. Through these factors, biofilms in breweries are a great risk to the microbiological stability of beer.

In brewing laboratory practice, biofilms are detected via the microbial monitoring of indicator germs. Microbiological samples are taken at critical process points and analysed for indicator organisms. Detection is quantitative with growing cell numbers representing the build-up of biofilm or semi quantitative, evidenced by a colour change in the indicator dye of the test medium over a defined time. Both methods have the disadvantage of being time consuming with a necessary incubation time. A change in product composition may result in a drift of species composition within the biofilm, leading to a potentially worse detection in monitoring systems. An example presented in this paper for such a drift is the trend to less hoped beer types, as well as alcohol-free beer types and beer mix beverages. PCR-based methods may accelerate the detection by shortening the incubation time and can provide hints about the quantitative species distribution, using quantitative real-time PCR methods. The quantitative detection of selected risk-orientated species, helps to categorise maturity and therefore the product risk of the biofilm.

The first part of this paper exams the detection and identification of lactic acid bacteria, classified as potential beer spoiling, in particular *Lactococcus lactis*, *Leuconostoc*

Summary

mesenteroides, *Lactobacillus rossiae* and *Lactobacillus acetotolerans* as indicator organisms for biofilms in Bavarian wheat beer. The latter two have already been described as spoilage organisms in low-hopped beer types and Bavarian wheat beer. The initial biofilm formation potential of various strain isolates of the named species was analysed. A risk-oriented categorisation of the wheat beer process and biofilm maturity was performed based on biofilm formation and beer spoilage. A detection medium was developed and validated for specific and fast detection of the species named above and combined with *de novo* developed and validated real-time PCR-based species-specific detection systems.

Starting from the observed high variance of the biofilm formation potential of *Lactobacillus rossiae* and *Lactobacillus brevis,* the variance of Lactobacillus brevis in genotype and phenotype was surveyed represented by its $(GTG)_5$ RAPD PCR fingerprint, biofilm formation and selective growth media. The observed diversity of heterogeneous clusters within the species was also observed in a field study across the entire production process within a brewery. The used strain differentiation methods proved to be valuable tools to track the source of a contamination.

In a comparable setting, yeast species that are known for beer spoilage were investigated, as well as species described as biofilm forming or ubiquitous in the brewing process. A yeast-specific medium for hygienic monitoring was developed and validated with strain isolates of the species *Saccharomyces cerevisiae* var. *diastaticus*, *Saccharomyces cerevisiae* TUM 68, *Saccharomyces pastorianus* var *carlsbergensis* TUM 34/70, *Dekkera anomala*, *Wickerhamomyces anomalus* und *Rhodotorula mucilaginosa*. The medium was combined with real-time PCR-based detection systems for the named species. The real-time PCR detection system for *R. mucilaginosa* was developed *de novo* and validated. The named species were categorised for product spoilage and biofilm maturation stage according to growth tests in selective media, product spoilage and biofilm formation potential.

1. Introduction and motivation

1.1. Biofilm definition and structure

Most microbial life on earth does not exist as planktonic, free cells, but is agglomerated in biofilms. Biofilm is a loose definition for microbial communities that normally settle at the border between aggregates of media, are surrounded by highly hydrated extracellular polymeric substances (EPS), and can be attached to surfaces or free-floating flocs [38]. Biofilms are anthropocentrically described as “cities of microbes” [40, 146] with the surrounding matrix being the “house of biofilm cells” [39, 40]. It has even been proposed that biofilms were the first living form, moving prebiotic gel to the position of first living biofilm [136]. The first reference of biofilm as a microbiological source of slime in paper production was in 1931, describing the structure of biofilm as an enmeshed mass, containing many bacterial organisms [9]. Later, the biofilm was reported to be part or product of the bacterial cell [160]. Biofilms come in various forms and consistencies, all with one goal: to immobilise the cell community and sustain a long-term diverse mixed species community with its interactions and gradients as a small-scale habitat with an external digestion system created by secreted enzymes, cooperation and competition between inhabitants [38, 40]. EPS make about 90 % dry substance of biofilms, giving them their 3-dimensional structure and trapping extracellular enzymes close to the cell [38]. EPS present a dominant part of bound carbon in soil, sediment and suspended matter in water, where they play an important role in the microbial ecology and nutrition among other functions [24-26, 37, 38, 40, 90]. The formation and structure of the microbial community that a biofilm represents, is strictly dependent on EPS production, composition and concentration [38, 40, 121]. The concentration, cohesion, charge, sorption capacity and composition of EPS, as well as their 3-dimensional structure determine the biofilm [38]. EPS, originally called ‘extrapolymer saccharides’ and later renamed when more information was gathered on their consistency, are a collection of various biopolymers, such as polysaccharides, proteins, lipids, humic substances and nucleic acids, and are mostly self-produced by the cells [38, 151]. The components of the biofilm matrix are not just a heap of macromolecules, they fulfil many important functions [35, 40].

Exopolysaccharides are still the major fraction of EPS [42, 153]. The complex network of polysaccharides attached to the cell surface was visualised using electron microscopy and confocal laser scanning microscopy (CLSM) combined with fluorescence dyes [76, 87, 151] or antibodies [87, 156]. Another approach is the combination of CLSM with Raman microscopy or Surface-Enhanced Raman Scattering (SERS) for a more in-depth analysis of EPS [62-65, 81, 82, 92, 143, 148]. One of the most-studied exopolysaccharide is alginate, produced, for example, by *Pseudomonas aeruginosa*, which is not essential for adhesion, but has an remarkable influence on biofilm formation of the originating species, as well as on

non-mucous species. The modification of alginate with acetyl groups, which are common substituents of exopolysaccharides, strongly increases the aggregation of bacteria and the structure of mature biofilms [38, 41, 120, 129]. The presence of alginates are also described as something that enhances *Saccharomyces* sp. brewing yeast strains [154]. Another common exopolysaccharide with a heavy influence on the biofilm formation of various species is cellulose [105, 137, 144, 153, 161]. The biofilm structure can be further secured under the influence of multivalent cations as Ca^{2+}, which can act as a bridge between polyanionic alginate molecules [73]. Most exopolysaccharides are polyanionic (e.g. alginate, xanthan, colanic acid) but there are some that are polycationic due to intercellular adhesion [48, 68].

Extracellular proteins may exceed the mass of exopolysaccharides in the biofilm matrix [18, 42, 67]. Diverse extracellular enzymes can be found in the EPS matrix, many able to degrade biopolymers. The degrading products may be absorbed as nutrients [38, 153, 159], while some enzymes seem to be involved in degrading structural EPS, enabling detachment of microorganisms from the biofilm [77, 150]. The detachment can be induced by starvation [47] or nutrient availability [109], resulting in enzymatic modification of the biofilm matrix [109]. Other enzymes are an integral part of microbial corrosion [15]. The extracellular enzymes are effectively retained within the biofilm by the interactions with exopolysaccharides [150, 151].

Extracellular DNA, long thought to be material from lysed cells proved to be an integral part of the biofilm matrix [152] and biofilm life cycle [84]. Due to its similarity to genomic DNA, the origin may be genomic DNA from lysed cells [114], but some species show distinct differences [11] so active excretion cannot be excluded [38]. The role of extracellular DNA can vary from being a major structural component to playing a minor role in the biofilm matrix, even between closely related species [66, 128]. The importance of extracellular DNA was observed in species from *Rhodovulum*, which produce EPS that consists of carbohydrates, proteins and nucleic acids [145]. The importance of structural integrity of the biofilm matrix was shown by treating *Pseudomonas aeruginosa* biofilms with nucleolytic enzymes, resulting in deflocculation. As neither degrading polysaccharides or proteins showed this effect, extracellular DNA functions as a connector between biofilm cells [158] and inhibits biofilm formation of the same species [149]. A comparable function of extracellular DNA was observed with *Bacillus cereus* [142]. Abiotic effects were observed as an additional function of extracellular DNA by chelating cations and denaturation of lipopolysaccharide and outer membrane, leading to cell lysis [85]. The exocellular DNA can vary in localisation and structural orientation in the biofilm matrix from grid-like structures [1], filamentous network [11], dense networks and thicker ‘ropes’ [70]. Further, eDNA may protect cells against antimicrobial effects, as shown with *Pseudomonas aeruginosa* against aminoglycosides [17].

Hydrophobic components of the EPS help to adhere to Teflon or waxy leaf surfaces [88]. The hydrophobic character is related to polysaccharide-linked methyl and acetyl groups [86]. Lipids can also be found within the matrix itself [18] and are essential for surface adherence [106, 108, 127].

Water is the biggest mass compound of EPS, providing a highly hydrated, slow-drying environment [38]. Bacteria are actively producing EPS in response to desiccation [104].

The EPS composition can vary greatly between biofilms, depending on species composition, temperature, shear forces and nutrients [139, 151]. Pili, flagella and other extracellular structures can also stabilise the biofilm [38, 161]. While the precise interactions of the integral polymers are not well described, some functions of EPS have been determined. Besides their influence on the three-dimensional structure of the biofilm, EPS have various functions and benefits for the microbial community. EPS are important for the adhesion to inert surfaces and therefore for the first step of biofilm formation. Related to this, EPS can also establish cell-cell connections [38]. Analyses with stained lectins and confocal laser imaging microscopy to differentiate various biofilm inhabitants and EPS, showed segregated microdomains, provided by the physical EPS structure [76]. These areas represent different biochemical environments, modified enzymatically to changing conditions. Chemical activity may be investigated spatially, using a combination of confocal laser scanning microscopy and Raman microscopy [38, 143]. This was used to monitor substances that were relevant for quorum-sensing activity within biofilms [94]. The EPS provide a highly porous matrix with a water phase, enabling an external digestive system using versatile extracellular enzymes to immobilise cells in close proximity. Cells of the biofilm community are embedded within EPS and EPS-forming capsules in particular, which are associated with the cell wall and influence the environment closest to the cell [121]. The matrix also keeps cell debris and lysed cells as nutrients within the community, while the EPS can be used as nutrients on their own [38]. Vesicles can carry various enzymes and biomolecules within the pore network, altering the matrix properties, sometimes with an abiotic effect on competing organisms [112]. The cell debris includes DNA, which can be ingested and partly included into the genome via horizontal gene transfer, providing a vast gene pool [38, 84]. The EPS matrix may work like a molecular sieve, binding ions, lipophilic substances and particles from the watery phase [36]. Once excreted, EPS may be altered by degradation, variation on composition, post-excretional addition of substituents, molecular structure or others as a reaction to external influences [34].

Many EPS form viscous gels that are linked by ion bonds and display varying gelling behaviour. Highly viscous EPS gels can even reform their 3D-structure after deformation by shear forces [120]. It may even react to increasing shear stress by forming ripples and rolling along a surface [95], which has been explained by the quorum-sensing controlled secretion

of biosurfactants [22]. The EPS highly influence the biofilm by their concentration, polarity, sorption and indirectly over viscosity, pores and channels [38]. The EPS work as a barrier against oxidation, charged biocides, some antibiotics and metallic cations [38, 40]. When drying out, bacteria strongly produce EPS [104] and the surface EPS layer hardens, protecting the deeper layers of desiccation [38, 120]. The selective pressure caused by competition and cooperation within the biofilm appears to be an evolutionary benefit for polymer producers over non-producers and favours biodiversity [38, 157]. Desiccation seems to be the condition in which all members of the biofilm community, those that produce and don't produce EPS, benefit from the EPS matrix [96]. EPS are not restricted to bacterial biofilms, but can also be found in microalgae [19], yeasts [7, 16, 141] and moulds that are involved in flocculation, adhesion and biofilm formation. The growth of heterotrophic bacteria that can use EPS as a substrate is supported [38]. EPS often influence biofilms far longer than they actually exist in their original form, as they merge into a complex three-dimensional matrix structure, inhabited by various species [26, 38].

1.2. Biofilm development

There are many different models that show how the detailed biofilm development takes place, but they all have 4 phases in common: 1. Surface conditioning, 2. Initial adherence, 3. Physical irreversible adherence, involving the production of macromolecules, 4. Cell growth and formation of microcolonies and coaggregations, which leads to an established biofilm [3, 14]. An important step in the initial biofilm formation is the depolarisation of the surface by positively charged polymers, enabling cells that cannot normally attach, to adhere to surfaces as steel or plastic, which can be enhanced by EPS [115, 154]. Yeast biofilms and bacterial biofilms develop similarly, but dimorphic yeasts such as *Candida albicans* and *S. cerevisiae* can form monolayers of spherical cells, as well as pseudohyphae during biofilm maturation [44]. Yeast are able to grow initially on inert surfaces as plastic [101] or stainless steel [14] or can colonise existing biofilms of fungal [147] or bacterial [69] origin.

A very interesting part of the EPS are adhesins as Flo11/Muc1 flocculin in *Saccharomyces cerevisiae* and *Saccharomyces cerevisiae* var. *diastaticus*, which can be very variable in its phenotype and is seen as being responsible for the formation of pseudohyphae, surface adhesion, agar invasion and biofilm development [33]. Depending on the expression of Fo11p, the mannoprotein is able to anchor cells expressing *FLO11* to other cells or surfaces [33]. Flo11p increases the hydrophobicity of the cell wall, making it easier to adhere to hydrophobic surfaces such as stainless steel or plastic [101]. The mechanism is also present in *Saccharomyces cerevisiae* ssp. used in industry. The expression of FLO11 and the controlled cell adhesion was studied in baker's yeast and was the most expressed in reduced-glucose medium, while N-starvation of the culture triggered the formation of pseudohyphae, the expression of FLO11 was suppressed by glucose [101]. Depending on

the FLO11 expression, a similar mechanism for the biofilm formation of *Saccharomyces* strains used for brewing seems possible with a low fermentable sugar concentration.
Epifluorescence microscopy was used on biofilms located on stainless steel using staining dye methods to visualise and quantify biofilm formation. For example, Concanavalin A can be used to stain EPS while DNA-staining dyes such as SytoBC or acridine orange that stain the nucleus mark the cell positions within the biofilm [44, 52, 116]. The quantitative biofilm formation was also observed using spectral photometric cell-culture-staining methods [133]. Within a biofilm, complex communities with multiple species can develop physiochemical gradients and produce microhabitats. Intense cell-cell communication, as well as horizontal gene transfer can happen between cells, making biofilms highly competitive and complex environments [38, 84]. A critical biofilm stage is the distribution of sessile cells from the biofilm, which enables new biofilm formation. The degradation of the binding EPS, which stabilise the biofilm, is induced by extracellular enzymes, segregated by the biofilm-inhabiting species as a reaction to environmental changes such as nutrient starvation or sudden nutrient availability, which requires a rearrangement of the biofilm matrix. The complex biochemical inte-cellular communication system that causes biofilm matrix deformation is called quorum sensing [38, 99]. An example of this complex adhesion and detachment regulation is the role of cyclic di-guanosine monophosphate (c-di-GMP), which works as a secondary messenger, stimulating the synthesis of adhesins and EPS substances across many species. It also inhibits forms of motility and therefore controls the transition between planktonic and biofilm life. The synthesis and degrading of c-di-GMP can be triggered by environmental signals [35, 49, 51]. Under starvation, the intracellular concentration of c-di-GMP, for example, in *Pseudomonas putida* changes the activity of protease LapG, resulting in lysis of the amyloid-like proteins, anchoring the cells to the surface. Thus the cells become motile and can change position [35, 46, 155]. In comparison with this reaction, some bacteria produce eDNA or polysaccharide-degrading enzymes, resulting in the polymeric breakdown of the biofilm matrix [35, 79, 80].

1.3. Biofilms in breweries

In breweries, especially in the filling department, areas that are difficult to access for cleaning and disinfection and dead ends are a perfect environment for biofilm formation. Areas in direct product contact in particular enable beer-spoiling bacteria to adapt to the hostile environment of beer as a medium [5]. In the brewery environment, the spectrum of species to be found in biofilms can be quite variable, dependent on the process step and therefore the substrate available in the microbial habitat. Indirect weak spots are often richly populated with common slime-forming species such as *Pseudomonas* sp., *Enterobacteriaceae*, yeasts especially *Rhodotorula* sp. and moulds [5]. Bacteria of the genera *Pseudomonas*, *Enterobacter*, *Klebsiella*, *Alcaligenes*, *Flavobacterium*, *Lactobacillus*, *Bacillus* and *Arthrobacter* can be found, for example, on conveyor belts. Yeasts of the genera *Saccharomyces*, *Candida*, *Rhodotorula, Cryptococcus* and *Trichosporum* and moulds of the genera *Cladosporium*, *Penicillium*, *Geotrichum*, *Trichiderma*, *Mucor*, *Hormonconis*, *Aureobasidium*, *Moniliella* sp. and *Paecilomyces* are also reported to form slimy biofilms [5, 8, 78, 131-133, 135]. In places in direct beer contact, acetic acid bacteria such as *Acetobacter* sp. and *Gluconobacter* sp. are often described as being the dominant slime-forming group [5]. Within biofilms related to brewery environment, beer-spoiling bacteria such as *Lactobacillus brevis*, *Lactobacillus lindneri*, *Pediococcus pentosaceus*, can be found as well as the potentially beer-spoiling lactic acid bacteria *Lactococcus lactis* and *Lactobacillus paracasei* [78, 116, 131]. The latter being able to strongly bond to surfaces [116]. Isolates of *Lactobacillus brevis*, *Lactobacillus lindneri*, *Pediococcus pentosaceus* and *Lactococcus lactis* were observed to form weak biofilms, while non-beer-spoiling genera as *Acetobacter*, *Enterobacteriaceae* and *Pseudomonas* could form strong biofilms that were highly resistant to peracetic acid [78]. Some later studies were not able to detect acetic acid bacteria as initial biofilm starters from brewery isolates at all. Isolates thought to be acetic acid bacteria according to morphology, acid formation and aerobic growth, turned out to be *Enterobacteriaceae* [116, 131, 135]. While acetic acid bacteria tend not to have spoilage potential in beer, they and some *Enterobacteriaceae* such as *Hafnia* sp., *Obesumbacterium* sp., *Klebsiella* sp. and *Citrobacter* sp. were reported to be associated with the spoilage of unfermented and fermenting wort [91, 98, 138]. *Wickerhamomyces anomalus* could be found as one of the first biofilm colonisers with quite strong biofilm-forming potential [116, 133]. This is interesting because the weak fermenting yeast is quite common in the brewing and beverage environment and is categorised as a potential beer-spoiling organism [2, 6, 75]. Additionally, *Wickerhamomyces anomalus* is able to produce toxins, killing other yeasts [75]. *Yarrowia lipolitica* can also be found in biofilms from breweries [116]. Some *Saccharomyces cerevisiae strains* used in rice wine are known to form mixed biofilms with lactic acid bacteria, specifically *Leuconostoc mesenteroides* and *Lactobacillus casei*, which are also known to

appear in a brewery environment [2, 4, 6, 43, 72]. The biofilm-forming potential is highly strain and substrate dependent and [115] fermentable sugar and sweeteners generally enhance biofilm growth [116]. It was also observed that some species such as *Saccharomyces cerevisiae* appear to form biofilms not as a single culture, but need metabolic products from other species (e.g. lactic acid bacteria) [43, 72]. The biofilm-formation potential in biofilm-forming *Saccharomyces cerevisiae* var. *diastaticus* was shown to be connected with the yeast flocculation gene Flo11/Muc1, which seems to have a special phenotype in biofilm-forming strains and is more strongly expressed in glucose-deprived media [33]. Comparable gene expression reactions to starvation situations, leading to the formation of pseudohyphae and adhesins by expression of Flo11 and Flo8 were observed in bakery *Saccharomyces cerevisiae* strains [101]. Oxygen distribution within biofilms is not homogeneous and may result in anaerobic pockets, with aerobic microorganisms consuming oxygen faster than is resupplied by diffusion [21].

The anaerobic environment within these biofilms enables growth that is protected from oxidative stress of strictly anaerobic species, such as *Pectinatus* sp. and *Megasphaera* sp., *Selenomonas* sp. and *Propionispira* sp. [5, 71]. The presence of *Pectinatus* sp. in brewery grown biofilms was confirmed [71, 132]. *Pectinatus* sp. and *Megasphaera* sp. were predominantly found in the filling area of breweries, especially at difficult-to-clean places such as the underside of conveyor belts and various pipe and monoblock constructions below the filler, as well as in cracks in the floor and drainage system [71]. Lactic acid bacteria tend to form biofilms *de novo* under the chemical stress of ethanol and acids [74]. Contaminations with lactic acid bacteria may promote the growth of *Pectinatus* sp. and *Megasphaera* sp., as those are able to utilise lactic acid as a carbon source [71].

The formation and maturation of brewery-based biofilms is often described as a multiphase development. Phase 1 is the carryover of single cells into the brewery via an empty bottle, airborne or via personnel. Phase 2 describes the start of growth in difficult-to-clean areas. Phase 3 is the persistent growth and coexistence of a wide variety of species of yeasts, lactic acid bacteria, non-beer-spoiling aerobic bacteria and other organisms. Within these biofilms the anaerobic microhabitat and enrichment of fermentation products such as lactic acid as carbon sources, and the rising pH due to autolysis enables the growth of strict anaerobic beer-spoiling bacteria such as *Pectinatus* sp. and *Megasphaera* sp.. With constant biofilm growth, parts of the stationary biofilms loosen in phase 4 and can be transferred as aerosols via rotating equipment into single containers while filling. The last phase relates to the phase of constant consumer complaints and health inspections, caused by the uncontrollable spread of beer-spoiling organisms [5].

1.4. Beer spoilage

From a microbiological perspective, beer is a relatively stable product. Due to its content of hop bitter acids (approx. 17-55 ppm iso-α-acids) and ethanol (0.5-10 % w/w), the anaerobic atmosphere (less than 0.3 ppm oxygen) and high carbon dioxide content (approx. 0.5 % w/v, low pH (3.8-4.7) and lack of nutritive substances, pathogenic microorganisms cannot survive in beer. Diminishing one or more of these 'microbial hurdles' may enable the growth of a wider spectrum of microorganisms [83, 107, 122, 124]. Apart from modified product parameters, there are only a few bacteria that can grow in beer. The most prominent are gram-positive lactic acid bacteria of the genera *Lactobacillus* and *Pediococcus*, as well as gram-negative bacteria *Pectinatus* and *Megasphaera*, and some super-attenuating yeasts [55, 57, 71, 107, 122, 124]. Persistent biofilms in breweries are potential habitats for beer-spoiling organisms or even pathogens. An anaerobic environment and fermentable metabolism product (e.g. lactate) enable the growth of strict anaerobic bacteria such as *Megasphaera* sp. or *Pectinatus* sp., *Selenomonas* sp. and *Propionispira* sp. [5, 71, 107]. Lactic acid bacteria, normally sensitive to hop bitter acids are able to adapt to beer as a substrate in the protected environment of biofilms with beer contact [5]. This adaption may happen by expression of genes connected with hop resistance (e.g. HorA, HorB, HorC or HitA) or transfer of these plasmid-coded genes between resistant and non-resistant species and strains in the case of *Lactobacillus* sp. [97, 122, 123]. The reduction of cell membrane fluidity by incorporating more unsaturated fatty acids into the cytoplasmic membrane and modification of the cell wall with lipoteichoic acids, was reported to be a passive protective strategy to prevent hop bitter acids from entering the cell and reducing the intracellular loss of Mn^{2+} [10, 122]. Aside from these hop resistance mechanisms, species that can form slime capsules are more resistant to disinfectants and heat treatment (up to 25 PU) as observed with some strains of *Lactobacillus brevis* (formerly *Lactobacillus frigidus*) [2, 122]. In contrast to other beer-spoiling organisms, strictly anaerobic bacteria as *Pectinatus* sp. and *Megasphaera* sp. require a virtually oxygen-free environment to grow in beer and first appeared in the late 1970s when progress was made to produce beer with low oxygen levels. Parallel contaminations with lactic acid bacteria may promote the growth of *Pectinatus* sp. and *Megasphaera* sp. due to lactic acid utilisation of this species as a carbon source. The hop resistance of strictly anaerobic beer-spoiling bacteria is higher than that of lactic acid bacteria and they are able to spoil all beer types, causing turbidity and crass off-flavours [71]. Mature biofilms, rich in beer-spoiling bacteria can be the cause of irregular contaminations in bottled beer [5]. Additionally, biofilm-bound contaminants are more resistant to chemical cleaning and disinfection measurements [38]. Depending on the environment, this process may take months or it may only be short term. Persistent biofilms should therefore be eliminated from the production environment [5].

1.5. Hygiene monitoring methods in breweries

The ATPase (Adenosine Triphosphatase) test is widely used for hygienic monitoring, but other tests such as protein detection and the oxidoreductase test are also used for this purpose [113]. The ATPase tests are based on bioluminescence with ATP and luciferase, therefore indicating the presence of living cell material and cell debris and insufficient cleaning [102, 113]. The alternative test, the oxidoreductase test, is based on the presence of NAD(P) (nicotinamide adenine dinucleotides(phosphate)) and/or NAD(P)H and indicates living cells by transforming tetrazolium salt into coloured formazan salt [113]. The most common industrial microbiological method for biofilm monitoring is trace indicator organisms that are connected with the early stages of biofilms or common contamination paths [5]. Biofilm indicator germs are unpretentious organisms, easily cultivated and can be easily detected with swab samples or contact dishes from critical sampling points [5]. If biofilms can be detected that may present a host to beer-spoiling organisms, more detailed analysis can be performed [5]. For the brewing process, NBB-B-AM is the most common medium used for monitoring biofilms. The medium is optimised for the growth of lactic acid bacteria, but is less selective for beer-spoiling lactic acid bacteria than NBB-B, enabling the aerobic growth of major beverage biofilm indicator germs [5]. Incubated at 28 °C, samples from relevant biofilms (potential host to spoilage organisms) show indicator colour change from red to yellow, due to acid formation [5]. As the indicator germ composition is comparable to other beverage industry sectors, this medium may also be used in lemonade and fruit juice factories, as well in wineries and mineral water factories [5]. To identify species from mixed cultures, it may be possible to use in situ hybridisation detection systems, based on specific marking fluorescence probes [113]. There is a wide range of probes, oligonucleotides, composed of up to 20 nucleotides, some with fluorescence markers, that target mirror sequences in the 16S rRNA, 23S rRNA or other specific sequences [113]. Using rRNA to detect microorganisms is interesting, as RNA is only available in living cells in sufficient numbers and no PCR is required [113]. Another widely used cluster method is based on PCR (Polymerase Chain Reaction) and many different applications for detection and identification have been developed [13, 53, 54, 56, 113]. The simplest method based on PCR is endpoint PCR, which detect the PCR product via visualisation with fluorescence dye and electrophoresis in agarose or acrylamide gel. This fluorescence dye can be, for example, ethidium bromide or SYBR Green I. Real-time PCR, based on labelling and measuring the PCR product in the PCR process is the next step in the development of this technique. The disadvantage of not being able to distinguish between PCR products in the simple method using fluorescent dyes can be countered by using fluorescently labelled oligonucleotide probes [113]. Förster resonance energy transfer probes (FRET) are based on the effect of a donor and an acceptor molecule, represented as two additional oligonucleotides.

Fluorescence of the acceptor fluorophore is only emitted when the donator fluorophore is near (1-10 Å). Therefore the specific binding areas for the probes in the PCR product need to be near to each other [113]. A similar principle is used in the TaqMan® probes, dual-labelled probes with 5'-quencher and 3'-flourescences molecule. The probes bind to their target area and are destroyed by the polymerase, separating the quencher and fluorescence molecule and resulting in a rising fluorescence signal [113]. All real-time PCR methods are capable of relative quantification of the initial DNA, using the Ct value (cycle threshold), or Cp (crossing point). This point is the PCR cycle with significantly increasing fluorescence. The earlier the Ct can be observed, the more initial DNA was in the sample [113]. The aim of these methods in the beverage industry is primarily the fast detection and identification of spoiling microorganisms and they are used with high throughput with various automated systems [113].

1.6. Problems encountered in biofilm detection and motivation

The available media for biofilm monitoring in the brewing industry (e.g. NBB-B-AM) are more selective for bacteria and yeasts encountered in the filling environment of breweries and beverage plants. The used detection media to cultivate these indicator organisms may therefore only detect an incomplete spectrum of species encountered in biofilms bound on surfaces of brewery equipment. Even strict spoilage organisms in beer, such as *Megasphaera* sp. cannot be detected for sure, as they are not cultivable in most used media. With a change in the product range to beer-based products that are more microbiologically sensitive (e.g. low hopped beer, alcohol-free beer and beer mix beverages), the spectrum of spoilage organisms, as well as of biofilm inhabitant is also changing. The indicator organisms for relevant biofilms therefore also change.

Conventional microbiological methods involve time-consuming analysis. The most common swab sample medium in breweries takes up to three days for cultivation. A more detailed specific media test may take even longer, depending on the species and medium. This leads to a high discrepancy between the analysis result and the present microbiological status.

Most theories on biofilm formation in breweries are based on ubiquitous microorganisms that build biofilms, which are inhabited by spoilage organisms in later biofilm stages. The detection of these indicator organisms shows the appearance of biofilms. A key process step for biofilm monitoring within breweries is in the filling department, as most beer-spoilage organisms (e.g. lactic acid bacteria) need higher temperatures to prevail against the used yeast culture strains and the contamination paths are more common due to the unsterile environment within the bottling cellar. As most incidents with spoiled beer can be traced back to secondary contamination within the filling process, this may apply to most instances. For scattered contaminations from the primary production process (e.g. fermentation and storage), this monitoring is not applicable, as the direct product contact inhibits the growth of

most indicator organisms and the biofilm causing the issue is mostly formed by beer-spoiling organisms in recesses and dead ends within the process. There is not much data about the initial biofilm formation potential of most spoilage organisms such as *Lactobacillus brevis* or spoilage yeasts such as *Saccharomyces cerevisiae* var. *diastaticus*. Other species such as the non-spoiling yeasts *Wickerhamomyces anomalus* or *Rhodotorula mucilaginosa* are known for biofilm formation, but ignored in existing biofilm-formation models in breweries.

The aim of this study is to adapt media to detect a wider spectrum of biofilm inhabitants and to adapt these media to more sensitive products such as lactic acid bacteria biofilms in Bavarian wheat beer (2.2) or yeast-bound biofilms (2.4). The combination with species-selective real-life PCR methods with adapted media for different biofilm-formation species was developed and used as a tool to gather more information about the biofilm formation and maturation. Selected species were tested for their initial biofilm potential, for classification as an initial biofilm former or biofilm coloniser. The distribution of selected species indicates the maturation stage of the biofilm. Molecular detection techniques such as real-time PCR enable the detection of far lower cell numbers than classical microbiological methods, resulting in a shorter incubation time. Both systems are designed to be modular and it is possible to extend the detected species selection accordingly to adapt it to other beverages or investigations.

Beer-spoiling lactic acid bacteria such as *Lactobacillus brevis* are generally handled as biofilm inhabitants, not as biofilm constructors, which often mean that the source of contamination in bottled beer remains unidentified. Classical hygienic monitoring is reaching its limits, especially with regard to primary contamination incidents, and identification purely at the species level is often not enough to isolate the contamination source. *Lactobacillus brevis* as the most common beer spoiler is detected only at the species level, giving very little information about the possible contamination source and biofilm association. A more differentiating identification at the strain level was established and linked to detailed physiological profiles, including initial biofilm-formation potential and beer-spoiling potential will help to fight this brewing enemy No. 1 in the future (2.3).

2. Results (Thesis Publications)

2.1. Summary of results

The publication papers are summarised individually in paragraphs 2.2 to 2.4. with a description of authorship contributions and a full copy of each attached. Table 1 shows the overall overview of the publications and their content. Permissions of publishers for imprint of publications are listed in the Appendix.

Table 1: Short overview of the three publications with title of the publication, major objective, applied method and main findings

Publication Title		
Publication 1 Bavarian Wheat Beer, an example of a special microbe habitat – Cultivation, detection, biofilm formation, characterisation of selected lactic acid bacteria hygiene indicators and spoilers	**Publication 2** Brewing Enemy Number One: Genetic diversity, physiology and biofilm formation of *Lactobacillus brevis*	**Publication 3** Combined yeast biofilm screening – Characterisation and validation of yeast related biofilms in the brewing environment with combined cultivation and specific real-time PCR screening of selected indicator species
Major objective		
Fast detection and biofilm formation of biofilm-related lactic acid bacteria, categorised as potential beer spoiling by combination of a wheat beer specific medium and specific real-time PCR detection.	Genetic strain differentiation and physiological characterisation (growth potential and biofilm formation in various media) of a strain-set of brewery isolates of *Lactobacillus brevis* spp., source tracking of various strain types of *Lactobacillus brevis* within one brewery was done.	Fast detection of yeast-related biofilms with a combination of cultural and real-time PCR-based detection, Biofilm formation of characteristic yeast species
Applied methods		
TaqMan® real-time PCR detection system design using Primer Express©, a specific wheat beer medium was developed and combined with real-time PCR detection of lactic acid bacteria species, fluorescence and colorimetric microtiter culture	Rep-PCR fingerprinting $(GTG)_5$ primer, capillary gel-electrophoresis (Bioanalyzer 2100 expert, Agilent), Bionumerics 7.6 fingerprint data analysis, real-time PCR species identification, colorimetric microtiter culture	TaqMan® real-time PCR detection system design using Primer Express©, a specific swab sample medium with growth indicator dye resazurin was developed and combined with real-time PCR detection of characteristic yeast species, fluorescence and colorimetric microtiter culture
Main findings/ conclusion		
De novo real-time PCR detection system for *Lactobacillus acetotolerans*, *Lactobacillus rossiae*, *Lactococcus lactis* and *Leuconostoc mesenteroides* and specific culture medium for wheat beer-spoiling lactic acid bacteria. Biofilm formation in MRS of *Lactobacillus brevis*, *Lactobacillus rossiae*, *Lactococcus lactis* and *Leuconostoc mesenteroides* was proven.	A high genetic diversity of Lactobacillus brevis strain types, isolated from various beer types and breweries could be proven. No direct correlation could be found between the biofilm formation and growth and the genetic fingerprint profile. The genetic fingerprint profiling proved to be a highly usable method for tracking contamination sources throughout a brewery.	De novo real-time PCR detection system for *Rhodotorula mucilaginosa* and specific culture medium with indicator dye for the fast detection of yeast-related biofilms. Medium is applicable in combination with real-time PCR detection for hygienic monitoring and microbiological trouble shooting. Biofilm formation in modified MYPG for *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae*, *Saccharomyces cerevisiae* var. *diastaticus, Saccharomyces pastorianus* var. *carlsbergensis* and *Wickerhamomyces anomalus* was proven.

2.2. Bavarian Wheat Beer, an Example of a Special Microbe Habitat – Cultivation, Detection, Biofilm Formation, Characterization of Selected Lactic Acid Bacteria Hygiene Indicators and Spoilers

Abstract

For the food industry, hygiene conditions of production plants are of high relevance to product quality. Most microbiological quality issues can be traced back to inadequate plant hygiene. In particular, the formation of mature biofilms is highly connected with product spoilage. The formation of biofilms depends on the provision of nutrients and therefore of the product. With a wider range of beer types and beer-like products, new spoilage organisms are becoming relevant. For Bavarian Wheat Beer types, other low-hopped beer types and beer mix beverages, the potential beer-spoiling bacteria *Lactobacillus acetotolerans*, *Lactobacillus rossiae*, *Lactococcus lactis* and *Leuconostoc mesenteroides* can be critical. Either because of the spoilage potential or because of the biofilm-formation potential. The majority of strains of the above-mentioned species proved that they could develop biofilms *de novo* in MRS, which makes them important hygienic indicator germs. An adapted media to detect Bavarian Wheat beer-spoiling bacteria (Wheat Beer media by Hutzler and Riedl (WBM-HR) was developed. For rapid detection and identification, real-time PCR systems with compatible standard protocols were developed for the specified species. The detection limit and the detection time of obligate slow-growing Bavarian Wheat Beer-spoiling species *Lactobacillus acetotolerans* were significantly reduced. The developed methods can be applied to specific contamination tracking and to evaluating the hygiene status of breweries that produce Bavarian Wheat Beer.

Authors/Authorship contribution:

Riedl R.: Literature search, writing, data creation, study conception and design; **Goderbauer P.:** Data analysis and interpretation (Biofilm formation), critical review of draft; **Brandl A.:** consultation of real-time PCR design, critical content review; **Jacob F.:** Supervised the project; **Hutzler M.:** Creation of the research plan, media design, critical content review

39 January / February 2017 (Vol. 70) BrewingScience

Riedl, R., Goderbauer, P., Brandl, A., Jacob, F. and Hutzler, M.

Bavarian Wheat Beer, an Example of a Special Microbe Habitat – Cultivation, Detection, Biofilm Formation, Characterization of Selected Lactic Acid Bacteria Hygiene Indicators and Spoilers

For the food industry, hygiene conditions within production plants are of high relevance to product quality. Most microbiological quality issues can be traced back to inadequate plant hygiene. In particular, the formation of mature biofilms is highly connected with product spoilage. The formation of biofilms depends on the provision of nutrients and therefore on the product. With a wider range of beer types and beer-like products, new spoilage organisms are becoming relevant. For Bavarian Wheat Beer types, other low-hopped beer types and beer mix beverages, the potential beer-spoiling bacteria *Lactobacillus acetotolerans*, *Lactobacillus rossiae*, *Lactococcus lactis* and *Leuconostoc mesenteroides* can be critical, either because of the spoilage potential or because of the biofilm formation potential. The majority of strains of the above-mentioned species proved that they could develop biofilms de novo in MRS, which makes them important hygienic indicator germs. An adapted media to detect Bavarian Wheat Beer spoiling bacteria (Wheat Beer media by Hutzler and Riedl (WBM-HR)) was developed. For rapid detection and identification, real-time PCR systems with compatible standard protocols were developed for the specified species. The detection of obligate slow-growing Bavarian Wheat Beer-spoiling species *Lactobacillus acetotolerans* was modified. The developed methods can be applied to specific contamination tracking and to evaluating the hygiene status of breweries that produce Bavarian Wheat Beer.

Descriptors: Bavarian Wheat Beer spoilage, *Lactobacillus rossiae*, *Lactobacillus acetotolerans*, *Lactococcus lactis*, *Leuconostoc mesenteroides*

1 Introduction

The overall beer consumption in Germany has decreased by 18 % in recent years [20]. While global beer sales are stagnating, even decreasing by about 0.6 % in 2014 [25], consumer preferences are changing. While the export lager beer sector in Germany is diminishing, other sectors are flourishing. The Bavarian Wheat Beer sector has increased its share of the German beer market to 8.8 % since 1970 when 98 % of the beer market was dominated by bottom fermented beer types. Beer mix beverages increased to 6.8 % and alcohol-free beer to 3.6 % of the market in 2010 [21]. In 2015, the Bavarian Wheat Beer sector alone increased to 7.2 % [23]. While beer mix beverages decreased by 2.9 % in 2015, alcohol-free beer-based beverages are still popular in Germany [22].

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The changing product portfolios in breweries present new challenges for microbiological quality control. In comparison with other beverages and foods, beer is a very stable product in terms of its microbiology. The spectrum of microorganisms able to grow in beer is reduced by the presence of ethanol (0.5–10 % w/w), hop bitter compounds (approx. 17–55 ppm of iso-α-acids), high levels of carbon dioxide (approx. 0.5 % w/w), low pH (3.8–4.7) and (at least for industrially produced and filled beer) very low amounts of oxygen (<0.1 ppm), as well as traces of fermentable carbohydrates and amino acids [32]. Due to the high microbial selectivity of beer, non-culture organisms inhabiting breweries are categorized according to their spoilage potential as absolute harmful organisms, potentially harmful organisms, indirectly harmful organisms and indicator germs [4]. There are only a few bacteria that can grow in beer. The most prominent are gram-positive lactic acid bacteria of the genus *Lactobacillus* and *Pediococcus*, as well as gram-negative bacteria *Pectinatus* and *Megasphaera*, and some super-attenuating yeasts [2, 17, 18].

Producing Bavarian Wheat Beer or beer mix beverages involves varying the parameters that protect the product against microbio-

logical spoilage as mentioned above. In Bavarian Wheat Beer the content of hop bitter acids is reduced compared to most bottom fermented beer types (10–14 IBU in Bavarian Wheat Beer to 15–55 IBU in bottom fermented beer types [13, 27]). This beer type also contains a higher level of nutrients, – amino acids and fatty acids in particular in the case of unfiltered Bavarian Wheat Beer – and can therefore be spoiled by a higher number of species, which are normally in the category of potentially harmful organisms (*Lactobacillus rossiae*, *Lactobacillus acetotolerans*) [18]. Beer mix beverages, alcohol reduced and alcohol-free beer have even greater variety in these parameters.

In industrial settings the Bavarian Wheat Beer spoiling bacteria *L. rossiae* and *L. acetotolerans* are being found more often in Bavarian Wheat Beer samples than in previous years. The former was first isolated in 2005 from sourdough [6] and can often be found as persistent contamination of bottling plants. It is considered biofilm relevant due to its slime-forming potential [18]. The latter was originally found as a spoilage organism in rice vinegar broth [12]. It was later described as a spoilage organism in low-hopped Chinese beer and alcohol-free and regular Bavarian Wheat Beer [8; 16; 31]. In 2016 this species even attracted the attention of non-scientific media in reports of a massive beer spoilage in the US caused by *L. acetotolerans* [28]. This species is mostly found as a spoiler in fermentation vessels and pitching yeast and is hard to detect due to its ability to enter a viable, but putatively non-culturable (VPNC) state in beer. In addition, under cold stress *L. acetolerans* grows slowly and requires an incubation time of more than two weeks using classic media until detection [8, 9]. *Leuconostoc mesenteroides* and *Lactococcus lactis* are common in the filling area. They are considered to be potential beer-spoilage bacteria [4, 17, 34] and are known to be able to spoil non-alcoholic beverages [4]. There are no recently documented cases of these species spoiling beer, but they can be widespread in the filling area [3, 4]. They are considered to be biofilm relevant due to the production of exopolysaccharides (EPS), which also makes them potential biofilm initiators [4, 10, 29, 34]. The production of EPS is not necessarily required for initial cell-surface attachment, but is essential for the growth of mature biofilms [1, 19]. *L. lactis* strains, isolated from dairy plants were described as having biofilm-producing potential [10]. Besides the spoilage risk for beer mix and non-alcoholic beer types, this makes them important as organisms that indicate hygiene problems, since biofilms containing non-product-spoiling lactic acid bacteria can provide habitats for strictly anaerobic, obligate beer spoiling *Acidaminococcaceae* such as *Pectinatus* sp. and *Megasphaera* sp. [34]. Real-time PCR-based detection systems have already been designed for most known beer-spoiling bacteria and yeasts by *Brandl* and *Hutzler* [5, 14, 15], but no rapid detection and identification methods have been published for *L. acetotolerans*, *L. rossiae*, *L. lactis* or *L. mesenteroides*.

The aim of this study was to improve the detection of bacteria that are relevant to Bavarian Wheat Beer as an example of beer types with reduced selectivity by developing an adapted enrichment medium. To date, the modular and multiplex compatible, TaqMan based real-time PCR systems developed by Brandl [5] and Hutzler

Table 1 **Strain set of selected obligate and potential beer spoilage organisms and brewery related non-target microorganisms, used to validate WBM-HR Medium against NBB**

Species	Strain	Origin	Properties
Acetobacter pasteurianus	DSM 3509 T	Type strain, DSMZ culture collection, originally isolated from beer	aerobic background flora, non-target germ, most common slime-forming species at critical points with direct product contact [3]
Lactobacillus acetotolerans	TUM BP 111012019-2001	Brewery, Bavarian Wheat Beer, culture collection of the Research Center Weihenstephan BLQ	target germ, potential beer spoiler/ Bavarian Wheat Beer spoiler, slow growth [18]
Lactobacillus brevis	TUM BP 120816044-2790	Brewery, rinsing water, dealcoholizing plant, culture collection of the Research Center Weihenstephan BLQ	target germ, obligate beer spoiler
Lactobacillus rossiae	TUM BP 130607017-2573	Brewery, Bavarian Wheat Beer light, culture collection of the Research Center Weihenstephan BLQ	target germ, potential beer spoiler/ Bavarian Wheat Beer spoiler [18]
Lactococcus lactis	TUM BP 120611046-8448	Brewery, beer, culture collection of the Research Center Weihenstephan BLQ	target germ, potential Bavarian Wheat Beer spoiler (only when severe production errors occur), biofilm potential, common background flora
Leuconostoc mesenteroides	TUM BP 130927040-0820	Brewery, swab sample filler, culture collection of the Research Center Weihenstephan BLQ	target germ, potential Bavarian Wheat Beer spoiler (only when severe production errors occur), biofilm potential, common background flora
Pseudomonas fluorescens	DSM 50090 T	Type strain, DSMZ culture collection	aerobic background flora, water- and soil bacterium, non-target germ, non-pathogenic representor of *Pseudomonas* sp., *Pseudomonas* sp. common in biofilms at secondary critical points in filling area [3, 34, 35]
Saccharomyces cerevisiae	TUM 68 (FZW-BLQ)	most common top fermenting Bavarian Wheat Beer yeast, culture collection of the Research Center Weihenstephan BLQ	top fermenting culture yeast, non-target germ
Saccharomyces pastorianus var. carlsbergensis	TUM 34/70 (FZW-BLQ)	most common bottom-fermenting lager yeast, culture collection of the Research Center Weihenstephan BLQ	bottom-fermenting culture yeast, non-target germ

[14, 15] were not able to detect *L. acetotolerans*, *L. rossiae*, *L. lactis* and *L. mesenteroides*. As a result, new, compatible specific systems for those species were developed. The biofilm formation potential of strains of the four target species was also evaluated.

2 Materials and methods

2.1 Biofilm formation potential in stationary phase

For testing the biofilm formation potential of the used microorganisms (Table 6) an adapted test design according to *Kolari* et al. [24] and modified by *Timke* [36] in a 96-well microtiter format was used. This test was selected as it is described as straightforward and usable for quantifying a broad range of microbial biofilms with the exception of *P. aeruginosa* biofilms [30]. The test was carried out in sterile, black, flat-bottomed polystyrene microtiter 96 well plates with polymeric optical bottoms for fluorescence applications (Thermofisher Scientific, Rochester, USA). Each well was filled with 250 µl MRS-broth (DeMan-Rogosa-Sharpe broth) [7] with 0.02 µg/ml resazurin as a redox indicator for growth and cysteine hydrochloride 0.006 µg/ml as an oxidative quencher. Overnight cultures of the test strains were washed by suspending and centrifuging in sterile distilled water and adjusted to 10*E6 cells/ml. The filled wells were incubated with 2.5 µl of the washed and adjusted cultures. Afterwards, the microtiter plates were sealed with gas permeable foil and anaerobically incubated at 28 °C without disturbance for 24 hours. The fluorescence at 530 nm extinction and 590 nm emission (Multi-Detection-Reader Synergy 2, BioTek, Bad Friedrichshall, Germany) was measured directly after sealing the plates and after incubation as indirect growth control. The plates were emptied and rinsed afterwards, using sterile distilled water. 300 µl of crystal violet solution (4 g/l in 20 % vol ethanol) was added to stain residing cells, forming a biofilm in the wells for 5 minutes at room temperature. All wells are emptied and rinsed 3 times using 400 µl sterile distilled water. The remaining crystal violet that was still bound to the cells was dissolved in 300 µl 96 % vol ethanol at 10 °C overnight and A590 was measured using the Synergy 2 Multi-detection reader. Means were calculated using four independent measurements of four biological replicates per strain and normalized against the mean of four independent measurements of four blank samples. All wells were counter-checked and documented microscopically for adherent cells and trub particles using a Nikon inverted research microscope Ti-E, using a CFI S P-Fluor ELWD ADM 60x C air objective for phase contrast microscopy.

2.2 Test strains used for media validation

For media validation, 9 representative species were selected to cover the possible spectrum from obligate beer spoiling bacteria

Table 2 Formulation of developed Wheat Beer media

	WBM-HR-Broth (WBM-HR-B)	WBM-HR-Agar (WBM-HR-A)
	concentration [g/L]	concentration [g/L]
Chlorophenol red	0.04	0.04
D(–)fructose	2.00	2.00
D(–)ribose	0.54	0.54
D(+)glucose	15.99	15.99
Diammonium hydrogen citrate	1.20	1.20
Dipotassium hydrogen phosphate	1.20	1.20
Dipotassium hydrogen phosphate trihydrate	0.80	0.80
Meat peptone	1.99	1.99
Meat extract	4.80	4.80
Yeast extract	10.38	10.38
L-arginine mono hydrochloride	0.08	0.08
L-cysteine mono hydrochloride	0.20	0.20
Magnesium sulfate hexahydrate	0.12	0.12
Maltose monohydrate	1.50	1.50
Manganese sulfate1hydrate	0.03	0.03
Natamax®	0.05	0.05
Sodium acetatetrihydrate	3.00	3.00
Casein peptone	6.00	6.00
Sucrose	1.00	1.00
Trisodium citratedihydrate	0.16	0.16
Tween 80	0.24	0.24
Agar-Agar		18.00
Decarbonized Bavarian Wheat Beer (alcohol free)	600 ml	600 ml
H_2O	400 ml	400 ml

(*Lactobacillus. brevis*), potential beer spoiling bacteria (*L. rossiae*, *L. acetotolerans*, *L. lactis* and *L. mesenteroides*) to non-spoilage bacteria (*Acetobacter pasteurianus* and *Pseudomonas fluorescens*), as well as the omnipresent brewing yeasts (*Saccharomyces cerevisiae* and *Saccharomyces pastorianus* var. *carlsbergensis*). The exact test strains used are shown in table 1, which also provides origin and property information.

2.3 Media properties for enrichment cultures

To improve the enrichment of Bavarian Wheat Beer spoiling *Lactobacillus* sp. a new media, Wheat Beer media by Hutzler and *Riedl* (referred to as WBM-HR in the following) was developed by Hutzler and Riedl primarily to provide a medium with properties closer to those of Bavarian Wheat Beer with a known composition for the detection of *L. acetotolerans* at the Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität. Deng described catalase as an appropriate additive to overcome the VPNC state of *L. acetotolerans* by reducing oxidative damage of the non-media-adapted cells [8; 9]. Using an alternative method, the medium was created using L-cysteine monohydrochloride as an antioxidative agent. The medium was designed as a broth medium and as an agar plate medium. For validation, NBB (Doehler, Darmstadt Germany) was used as a comparable reference medium according to the instructions for use. The detailed formulations of the developed media are listed in table 2.

2.4 Media validation

The media were validated by using five target germ species, which are supposed to grow on the developed media. Those target germs are namely *L. brevis* as obligate beer spoilage organism the four potential beer spoiling organisms *L. rossiae*, *L. acetotolerans*, *L. lactis* and *L. mesenteroides*. For the non-target species, which are not supposed to grow under the intended conditions, *A. pasteurianus*, *P. fluorescens*, *S. cerevisiae* and *S. pastorianus* var. *carlsbergensis* were used. The detailed strain numbers and properties are listed in table 1.

All strains were cultivated in liquid culture and standardized to 1000 cells/ml. All media were inoculated with about 100 cells, the liquid media (WBM-HR-B and NBB-B) with 100 µl of the standardized cell suspension, the agar media (WBM-HR-A and NBB-A) as a pour plate culture with 1 ml of a 1:10 dilution with sterile ringer solution of the standardized cell suspension.

The broth media and pour plate agar cultures were inoculated and incubated at 28 °C for 7 days.

To validate the growth rate in WBM-HR-B, 25 µl of an MRS culture, which was inoculated from a cryogenic storage culture and incubated for 48 h at 28 °C, was inoculated in tubes with 10 ml WBM-HR-B and NBB-B in parallel and incubated anaerobically for 14 days at

Table 3 TaqMan® based real-time PCR detection systems for potential Bavarian Wheat Beer spoilage bacteria

Target organism	System Name	Probe	Primer	Target Area	Nucleotide sequences (5' → 3')	Reference
internal amplification control	IAC135	IAC135-S	IAC135-f	IAC135	TGGATAGATTCGATGACCCTAGAAC	This study
			IAC135-r		TGAGTCCATTTTCGCAGATAACTT	This study
Lactobacillus acetotolerans	Lac	Lac-S	Lac-f	16S rDNA	CGAGCCGAACCAATTGATTAC	This study
			Lac-r		TGTGATCTCTCCTTTTATCCGGTAT	This study
Lactobacillus rossiae	Lro	Lro-S	Lro-f	16S rDNA	GGCGTGCCTAATACATGCAAR	This study
			Lro-r		TGTCTCGTCAATCTGGTGCAA	This study
Lactococcus lactis	iLacLa	i200	Llac-f	16S rDNA	GAAAGATGCAATTGCATCACTCAA	This study
			LP-r		ATTCCCTACTGCTGCCTCCC	[5]
Leuconostoc mesenteroides	iLeu	i200	Leu-f	16S rDNA	GCGCTTCGGCGTCACC	This study
			LP-r		ATTCCCTACTGCTGCCTCCC	[5]

Table 4 Probes used for TaqMan® real-time PCR detection systems for potential Bavarian Wheat Beer spoiler bacteria

Probe	Reporter	Quencher	Nucleotide sequence (5' → 3')	Reference
i200	FAM	BHQ-1	CCACATTGGGACTGAGACACGGCC	[5]
Lro_S	FAM	BHQ-1	TCGAACGCACTTCGGKTTTTGATTGA	This study
Lac_S	FAM	BHQ-1	CCTACCCTATAGTCTGGGATACCACTTGGAAACAG	This study
IAC135-S	HEX	BHQ-1	TGGGAGGATGCATTAGGAGCATTGTAAGAGAG	This study

Table 5 Artificial target sequence nucleotide IAC135 for internal amplification control

Nucleotide	Nucleotide sequence (5' → 3')	Reference
IAC135	TGCTAGAGAATGGATAGATTCGATGACCCTAGAACTAGTGG GAGGATGCATTAGGAGCATTGTAAGAGAGTCGGAAGTTA	This study
IAC135-rev	TGCGACACCTTGGGCGACCGTCAATAGGCCACTCGAAT GAGTCCATTTTCGCAGATAACTTCCGACTCTCTTACAATGCT	This study

Table 6 Biofilm formation potential of selected obligate and potential Bavarian Wheat Beer spoiling lactic acid bacteria

Species	Test strain	$\bar{E}_{590} - \overline{SD}_{590}$	Biofilm formation potential[1]	observable microscopical cell adhension to surface
Lactobacillus acetotolerans	TUM BP 111012019-2001	0,188	–	yes
Lactobacillus acetotolerans	TUM BP 120706025-2505	0,290	–	yes
Lactobacillus brevis	TUM BP 120816044-2790	0,745	++	yes
Lactobacillus brevis	TUM BP 120816004-2781	0,385	–	yes
Lactobacillus brevis	TUM BP 120827005-2823	5,00	+++	yes
Lactobacillus rossiae	TUM BP 131022000-2858	0,302	–	no
Lactobacillus rossiae	TUM BP 131022011-2866	0,357	–	yes
Lactobacillus rossiae	TUM BP 131011001-2846	0,345	–	yes
Lactobacillus rossiae	TUM BP 130607017-2573	1,00	++	yes
Lactococcus lactis	DSM20481T	0,826	+	yes
Lactococcus lactis	TUM BP 120611046-8446	0,619	+	yes
Leucocostoc mesenteroides	TUM BP 130927040-0820	1,13	+	yes
Leuconostoc mesenteroides	TUM BP 130920005-0816	1,81	++	yes

[1] Biofilm formation according to Diaz [10]

- : no biofilm formation $\bar{E}_{590} - \overline{SD}_{590} < E_{CTRL\ cutoff}$

+ : weak biofilm formation $E_{590\ cutoff} < \bar{E}_{590} - \overline{SD}_{CTRL} < 2 * E_{590\ cutoff}$

++ : moderate biofilm formation $2 * E_{590\ cutoff} < \bar{E}_{590} - \overline{SD}_{590} < 4 * E_{590\ cutoff}$

+++ : strong biofilm formation $\bar{E}_{590} - \overline{SD}_{590} > 4 * E_{CTRL\ cutoff}$

28 °C in duplets. The time of the pH-indicator change was documented visually against blind samples of a non-inoculated medium.

2.5 DNA-extraction

For the DNA extraction, a modified protocol using the InstaGene Matrix™ (Bio-Rad, Hercules, CA, USA) was used [26]. Hence 200 µl of a dense liquid enrichment culture was transferred into a 1.5 ml Eppendorf reaction tube and centrifuged for 1 minute at 15,114 x g (Hettich Mikro 200). The supernatant was discarded and 200 µl InstaGene™ DNA isolation buffer was added to the residing cell pellet and incubated at 56 °C for 30 minutes (Eppendorf Thermomix comfort). After a 10 second vortex step, the tube was incubated for another 8 minutes at 95 °C then centrifuged for 1 min at 15,114 x g. The DNA concentration was measured using a Nanodrop ND 2000 (Thermofisher Scientific, Rochester, USA). For the validation, the DNA concentration of the test strains was adjusted with PCR-clean ddH2O to 5 ng/µl. The DNA sample material and sample strain set were stored at –20 °C.

2.6 Real-time-PCR

For the rapid detection of the potential beer-spoiling target species *L. lactis*, *L. mesenteroides*, *L. rossiae* and *L. acetotolerans*, TaqMan® real-time PCR assays were designed. The target sequence for all four species is located in the 16S rDNA region. To identify usable, specific primer binding areas, the 16S rDNA-sequences of 99 strains of common brewery microorganisms collected from the NCBI-Database (http://www.ncbi.nlm.nih.gov) were aligned (DNASTAR, MegAlign, Lasergene, Version 11). To achieve the greatest specificity possible, primer target areas were selected that had specific polymorphisms. Primers and probes were designed using Primer Express (Primer Express 1.5, Applied Biosystems, Thermofisher Scientific). For internal amplification control, a synthetic, random DNA sequence was generated and a specific TaqMan real-time PCR system with this sequence as target area was designed (Table 3, Table 4 and Table 5). All primers and probes were tested in silico using BLAST (Basic Local Alignment Search Tool) for homologies with other sites or species. All real-time PCR systems were designed to be compatible with other systems for spoilage organism detection, designed by Brandl [5] and Hutzler [14, 15]. The real-time PCR parameters used for the design were therefore:

- Annealing temperature primers: 60 °C.
- Annealing temperature probes: 10 °C above annealing temperature primers.
- Maximum amplicon length: 200 bp.

All nucleotides were synthesized by biomers.net GmbH, Ulm, Germany. All PCR-runs were carried out on a LightCycler® 480 Instrument II (Roche Diagnostics Deutschland GmbH, Mannheim, Germany), using 10 µl LightCycler® 480 Probes Master in a 20 µl volume assay with a 5 µl sample. The real-time PCR was performed using 400 nmol l^{-1} of forward and reverse primer and 200 nmol l^{-1} TaqMan probe of the main PCR system. The Primers were added in aliquots of 0.8 µl and 10^{-5} mol l^{-1}, the probes were added in 0.4 µl aliquots of 10^{-5} mol l-1.

The internal amplification control IAC135 was added using 250 nM of IAC135-f and IAC135r, 200 nM and IAC135-S. The target DNA of the internal amplification control IAC135 and IAC135-rev was used at a concentration of $5*10^{-20}$ mol l^{-1}. The primers were added in aliquots of 0.5 µl and 10^{-5} mol l^{-1}, the probes were added

Table 7 Quantitative validation WBM-HR-A according to DIN EN ISO 11133:2015-01 [11] after 7 days incubation at 28 °C with selected obligate and potential Bavarian Wheat Beer spoiling bacteria and brewery related non-target microorganisms

	WM-A				NBB-A					
	#1 [CFU]	#2 [CFU]	#3 [CFU]	Ø	#1 [CFU]	#2 [CFU]	#3 [CFU]	Ø	Productivity P_R=WM-A/NBB-A (WBMHR-A versus NBB-A) $P_R \geq 0.5$	Selectivity (growth level)
Acetobacter pasteurianus	0	0	0	0	0	0	0	0	complete inhibition	0
Lactobacillus acetotolerans	235	230	245	237	257	277	284	273	0.87	2
Lactobacillus brevis	49	42	44	45	46	50	53	50	0.90	2
Lactobacillus rossiae	79	72	78	76	82	92	76	83	0.92	2
Lactococcus lactis	88	76	52	72	57	81	96	78	0.92	2
Leuconostoc mesenteroides	79	82	84	82	102	91	75	89	0.92	2
Pseudomonas fluorescens	0	0	0	0	0	0	0	0	complete inhibition	0
Saccharomyces cerevisiae TUM 68	5	7	7	6	0	0	0	0	partial inhibition	1
Saccharomyces pastorianus var. carlsbergensis TUM 34/70	16	13	11	13	0	0	0	0	partial inhibition	1

Selectivity (growth levels): 0 = no growth, 1 = weak growth, 2 = good growth
CFU = Colony Forming Units

Table 8 Qualitative validation with selected obligate and potential Bavarian Wheat Beer spoiling bacteria and brewery related non-target microorganisms of WBM-HR-B, incubated at 28 °C for 7 days (4-field test) [33]

Medium	WBM-HR-B (7 d)			NBB-B (7 d)				
	#1	#2	#3	#1	#2	#3		
Acetobacter pasteurianus	–	–	–	–	–	–		
Lactobacillus acetotolerans	+	+	+	+	+	+		
Lactobacillus brevis	+	+	+	+	+	+		
Lactobacillus rossiae	+	+	+	+	+	+	**a:** number of positive analysis results in both methods:	30
Lactococcus lactis	+	+	+	+	+	+	**b:** number of wrong negative analysis results versus the reference method (NBB-B):	0
Leuconostoc mesenteroides	+	+	+	+	+	+	**c:** number of wrong positive analysis results versus the reference method (NBB-B):	0
Pseudomonas fluorescens	–	–	–	–	–	–	**d:** number of overall negative results in both methods:	24
Saccharomyces cerevisiae TUM 68	–	–	–	–	–	–	**n:** overall analysis results:	54
Saccharomyces pasteurianus var. carlsbergensis TUM 34/70	–	–	–	–	–	–	**relative specificity WBM-HR-C (d/(c+d))*100%**	100 %
							relative accuracy WBM-HR-C ((a+d)/n)*100 %	100 %
							relative sensitivity (a/(a+b))*100%	100 %

- : no visible indicator color change + : visible color change/growth

Table 9 Detection speed validation of WBM-HR-B with selected obligate and potential Bavarian Wheat Beer spoiling bacteria, incubated anaerobically at 28 °C for 14 days

Species	Medium	Day 1	Day 2	Day 3	Day 4	Day 7	Day 10	Day 14
Lactobacillus acetotolerans TUM BP 120706025-2967	WBM-HR-B	–	–	–	+/–	+	+	+
	NBB-B	–	–	–	–	+	+	+
Lactobacillus backi TUM BP 140407001-2242	WBM-HR-B	–	+	+	+	+	+	+
	NBB-B	–	+	+	+	+	+	+
Lactobacillus brevis TUM BP 120711011-2578	WBM-HR-B	–	+	+	+	+	+	+
	NBB-B	–	+	+	+	+	+	+
Lactobacillus casei TUM BP 120509129-2360	WBM-HR-B	–	+	+	+	+	+	+
	NBB-B	+	+	+	+	+	+	+
Lactobacillus collinoides TUM BP 000-2061	WBM-HR-B	–	–	+	+	+	+	+
	NBB-B	–	–	+	+	+	+	+
Lactobacillus lindneri TUM BP 121213056-2397	WBM-HR-B	–	–	–	+/-	+	+	+
	NBB-B	–	–	–	–	+	+	+
Lactobacillus parabuchneri TUM BP 121008043-2282	WBM-HR-B	–	+	+	+	+	+	+
	NBB-B	–	+	+	+	+	+	+
Lactobacillus perolens TUM BP 130000240-2596	WBM-HR-B	–	+	+	+	+	+	+
	NBB-B	–	+	+	+	+	+	+
Lactobacillus plantarum TUM BP 121121170-2217	WBM-HR-B	–	+	+	+	+	+	+
	NBB-B	+	+	+	+	+	+	+
Lactobacillus rossiae TUM BP 130906019-2754	WBM-HR-B	–	+	+	+	+	+	+
	NBB-B	–	+	+	+	+	+	+
Leuconostoc mesenteroides TUM BP 000-0983	WBM-HR-B	–	–	–	–	–	+/–	+/–
	NBB-B	–	+	+	+	+	+	+
Megasphaera cerevisiae TUM BP 121011015-5986	WBM-HR-B	–	–	+/–	+	+	+	+
	NBB-B	–	–	–	–	+	+	+
Pectinatus frisingensis TUM BP 000-4327	WBM-HR-B	–	–	+	+	+	+	+
	NBB-B	–	–	–	–	–	+	+
Pediococcus damnosus TUM BP 140313142-2243	WBM-HR-B	–	–	+	+	+	+	+
	NBB-B	–	–	+	+	+	+	+

- : = no color indicator change, + : = color indicator change from red to yellow, +/- : = incomplete, partial color indicator change from red to yellow

in 0.4 µl aliquots of 10^{-5} mol l^{-1}, the internal amplification control target DNA was added in 0.1 µl aliquots of 10^{-17} mol l^{-1}. The initial heating at 95 °C was held for 10 minutes, then 40 cycles were performed at 95 °C for 10 seconds and 60 °C for 30 seconds. The fluorescence was measured at the end of the 60 °C step of each cycle (modified [5, 14, 15]).

2.7 Validation real-time-PCR

As a strain set to validate the PCR specificity, 99 representative species, known to be common in the brewing process were selected. All strains listed in table 10 (see page 46) were grown as dense three-day cultures in WBM-HR-B, the DNA isolated according to section 2.5 and adjusted to 5 ng/µl.

3 Results and discussion

3.1 Biofilm formation

To validate the biofilm formation potential, cut-off values were used to differentiate between biofilm-forming strains and non-biofilm-forming strains. The biofilm-forming cut-off for non-biofilm forming organisms ($E_{590\ cutoff}$) was defined as the mean of the measured optical density of four independent negative samples ($E_{590\ blank}$) plus three times the mean standard deviation ($SD_{590\ blank}$) and the mean measurement of the blank value ($E_{590\ blank}$) plus the mean standard deviation ($SD_{590\ blank}$). The mean of four independent measurements of a strain minus the standard deviation of these measurement $\overline{SD}_{590}$ equal or below this threshold was defined as no biofilm formation, values between $E_{590\ cutoff}$ and $2 * E_{590\ cutoff}$ were defined as weak biofilm formation, between $2 * E_{590\ cutoff}$ and $4 * E_{590\ cutoff}$ as moderate biofilm formation and above $4 * E_{590\ cutoff}$ as strong biofilm formation according to *Diaz* [10]. All wells in the microtiter plate were checked microscopically for cell adhesion.

As shown in table 6, two of the three tested *L. brevis* showed biofilm formation in MRS, of which TUM BP 120827005-2823 showed very strong biofilm formation. Of the four tested strains of *L. rossiae*, only the strain TUM BP 130607017-2573 showed biofilm formation. All test strains of the species *L. lactis* and *L. mesenteroides* were able to build biofilm in MRS.

Table 10 Results of real-time PCR specificity validation with DNA isolates adjusted to 5 ng/µl of target species and brewery related non-target microorganisms

Species	Strain	Real-time PCR-identification *L. rossiae*	Real-time PCR-Screening *L. acetotolerans*	Real-time PCR-Screening *L. lactis*	Real-time PCR-Screening *L. mesenteroides*
Acetobacter aceti	TUM BP 000-1991	negative	negative	negative	negative
Acetobacter pasteurianus	TUM BP 000-1990	negative	negative	negative	negative
Asaia lannensis	TUM BP 000-0994	negative	negative	negative	negative
Bacillus subtilis	TUM BP 000-0980	negative	negative	negative	negative
Candida boindinii	TUM YP 000-6007	negative	negative	negative	negative
Cryptococcus laurentii	TUM YP 000-0011	negative	negative	negative	negative
Debaryomyces hansenii	TUM YP 000-0006	negative	negative	negative	negative
Dekkera anomala	TUM YP 000-3040	negative	negative	negative	negative
Dekkera bruxellensis	TUM YP 000-3096	negative	negative	negative	negative
Enterobacter sp.	TUM BP 000-6088	negative	negative	negative	negative
Enterococcus sp.	TUM BP 111206005-0075	negative	negative	negative	negative
Escherichia coli	TUM BP 000-0981	negative	negative	negative	negative
Gluconacetobacter liquefaciens	TUM BP 000-0105 (DSM 5603 (BS 279))	negative	negative	negative	negative
Gluconobacter oxydans	TUM BP 000-0078	negative	negative	negative	negative
Hafnia alvei	TUM BP 000-0993	negative	negative	negative	negative
Hanseniaspora uvarum	TUM YP 000-0054 (CBS 5074)	negative	negative	negative	negative
Kazachstania exigua	TUM YP 000-337	negative	negative	negative	negative
Kluyvera ascorbata	TUM BP 131213038-0099	negative	negative	negative	negative
Kluyveromyces marxianus	TUM YP 000-0005	negative	negative	negative	negative
Kocuria kristinae	TUM BP 000-0083 (DSMZ 22032)	negative	negative	negative	negative
Lactobacillus acetotolerans	TUM BP 120706025-2967	negative	positive (ct 24.35)	negative	negative
Lactobacillus acidophilus	TUM BP 000-2081 (DSMZ 20079)	negative	negative	negative	negative
Lactobacillus alimentarius	TUM BP 000-2979	negative	negative	positive (ct 28.89)	negative
Lactobacillus amylolyticus	TUM BP 000-2969	negative	negative	negativ	negative
Lactobacillus amylophilus	TUM BP 000-2068	negative	negative	negativ	negative
Lactobacillus amylovorus	TUM BP 000-2080 (DSMZ 20531)	negative	negative	negativ	negative
Lactobacillus backi	TUM BP 140407001-2242	negative	negative	negativ	negative
Lactobacillus bifermentans	TUM BP 000-2014 (DSMZ 20003)	negative	negative	negativ	negative
Lactobacillus brevis	TUM BP 120711011-2578	negative	negative	negativ	negative
Lactobacillus brevis (formerly Lactobacillus brevisimilis)	TUM BP 000-2976	negative	negative	negativ	negative
Lactobacillus buchneri	TUM BP 000-2060	negative	negative	negativ	negative
Lactobacillus casei	TUM BP 120509129-2360	negative	negative	negativ	negative
Lactobacillus collinoides	TUM BP 000-2061	negative	negative	negativ	negative
Lactobacillus coryniformis	TUM BP 000-2978	negative	negative	negativ	negative
Lactobacillus curvatus	TUM BP 000-2977 (BS 218)	negative	negative	positive (ct 28.83)	negative
Lactobacillus delbrueckii ssp. delbrueckii	TUM BP 000-2968	negative	negative	negative	negative
Lactobacillus dextrinicus	TUM BP 000-2987	negative	negative	positive (ct 28.96)	negative
Lactobacillus fermentum	TUM BP 000-2069	negative	negative	negative	negative
Lactobacillus frisingensis	TUM BP 130919043-2789	negative	negative	negative	negative
Lactobacillus fructivorans	TUM BP 000-2038	negative	negative	negative	negative
Lactobacillus gasseri	TUM BP 000-2970	negative	negative	negative	negative
Lactobacillus ghanensis	TUM BP 000-2931	negative	negative	negative	negative
Lactobacillus harbinensis	TUM BP 120906016-2993	negative	negative	negative	negative
Lactobacillus helveticus	TUM BP 000-2971	negative	negative	negative	negative
Lactobacillus hilgardii	TUM BP 000-2975	negative	negative	negative	negative
Lactobacillus johnsonii	TUM BP 000-2972 (BS 224)	negative	negative	negative	negative
Lactobacillus kefiri	TUM BP 000-2037	negative	negative	negative	negative

Lactobacillus lindneri	TUM BP 121213056-2397	negative	negative	negative	negative
Lactobacillus malefermentans	TUM BP 000-2974	negative	negative	positive (ct 28.83)	negative
Lactobacillus parabrevis T	TUM BP 000-2080 (DSMZ 20531)	negative	negative	negative	negative
Lactobacillus parabuchneri	TUM BP 121008043-2282	negative	negative	negative	negative
Lactobacillus paracollinoides	TUM BP 150113003-2371	negative	negative	negative	negative
Lactobacillus perolens	TUM BP 130000240-2596	negative	negative	negative	negative
Lactobacillus plantarum	TUM BP 121121170-2217	negative	negative	negative	negative
Lactobacillus reuteri	TUM BP 000-2055 (BS 227)	negative	negative	negative	negative
Lactobacillus rhamnosus	TUM BP 000-2996	negative	negative	negative	negative
Lactobacillus rossiae	TUM BP 130806019-2754	positive (ct 22.82)	negative	negative	negative
Lactobacillus salivarius	TUM BP 000-2997	negative	negative	negative	negative
Lactobacillus sanfranciscensis	TUM BP 000-2982	negative	negative	negative	negative
Lactococcus lactis	TUM BP 000-8973	negative	negative	positive (ct 16.43)	negative
Leuconostoc mesenteroides	TUM BP 000-0983	negative	negative	negative	positive (ct 22.43)
Megasphaera cerevisiae	TUM BP 121011015-5986	negative	negative	negative	negative
Meyerozyma guilliermondii	TUM YP 000-0041	negative	negative	negative	negative
Micrococcus luteus	TUM BP 000-0995	negative	negative	negative	negative
Oenococcus oeni	TUM BP 000-0013	negative	negative	negative	negative
Pantoea dispersa	TUM BP 000-0992	negative	negative	negative	negative
Pectinatus cerevisiiphilus	TUM BP 120919033-4402	negative	negative	negative	negative
Pectinatus frisingensis	TUM BP 000-4327	negative	negative	negative	negative
Pectinatus haikarae	TUM BP 120919239-4404	negative	negative	negative	negative
Pediococcus clausenii	TUM BP 000-3986	negative	negative	negative	negative
Pediococcus damnosus	TUM BP 140313142-2243	negative	negative	negative	negative
Pediococcus inopinatus	TUM BP 000-3984	negative	negative	negative	negative
Pediococcus pentosaceus	TUM BP 000-3985	negative	negative	negative	negative
Pichia membranifaciens	TUM YP 000-2009	negative	negative	negative	negative
Pseudomonas poae	TUM BP 000-7057	negative	negative	negative	negative
Pseudomons fluorescens	DSM 50090 (BS236)	negative	negative	negative	negative
Rhodosporidium toruloides	TUM YP 000-0110	negative	negative	negative	negative
Rhodotorula mucilaginosa	TUM YP 120306011-7159	negative	negative	negative	negative
Saccharomyces bayanus	TUM YP 000-1999	negative	negative	negative	negative
Saccharomyces cer. var. diastaticus	TUM YP 000-1042 (DSM 70487)	negative	negative	negative	negative
Saccharomyces cerevisiae TUM 184	TUM YP 000-1001	negative	negative	negative	negative
Saccharomyces cerevisiae TUM 66/70	TUM YP 000-1044	negative	negative	negative	negative
Saccharomyces cerevisiae TUM 68	TUM YP 000-1045	negative	negative	negative	negative
Saccharomyces paradoxus	TUM YP 000-1043 (BS 11 (WYSC 63))	negative	negative	negative	negative
Saccharomyces pastorianus	TUM YP 000-1010	negative	negative	negative	negative
Saccharomyces pastorianus TUM 34/70	TUM YP 000-1008	negative	negative	negative	negative
Saccharomyces pastorianus TUM 34/78	TUM YP 000-1010	negative	negative	negative	negative
Saccharomyces uvarum	TUM YP 000-1090	negative	negative	negative	negative
Saccharomycodes ludwigii	TUM YP 000-0046 (SL17)	negative	negative	negative	negative
Schizosaccharomyces pombe	TUM YP 000-0039	negative	negative	negative	negative
Selenomonas lacticifex	TUM BP 000-0998	negative	negative	negative	negative
Torulaspora delbrueckii	TUM YP 000-0003	negative	negative	negative	negative
Weissella paramesenteroides	TUM BP 000-0988	negative	negative	negative	negative
Weissella viridescens	TUM BP 000-0989 (BS 198)	negative	negative	negative	negative
Wickerhamomyces anomalus	TUM YP 000-2004	negative	negative	negative	negative
Zygosaccharomyces bailii	TUM YP 000-5094	negative	negative	negative	negative
Zygosaccharomyces rouxii	TUM YP 000-5092	negative	negative	negative	negative
Zymomonas mobilis	TUM BP 000-0036 (DSMZ 424)	negative	negative	negative	negative

Biofilm formation could not be measured in either of the tested strains of *L. acetotolerans* under the described standardized experiment parameters, but cell adhesion could be observed microscopically The appearance of this species in fermentation tanks and vessels [8, 9] may be linked to its adhesion to surfaces in substrates with good growth factor distribution such as MRS. Further tests with standardized Bavarian Wheat Beer wort and finished Bavarian Wheat Beer should be conducted and the incubation time should be increased for this species, to evaluate the potential risk of this species permanently inhabiting this production area.

The *L. rossiae* species includes very heterogenic strain types that differ physiologically and in their genetic profile [6]. It is therefore only logical that these have different biofilm building potentials. The experimental procedure was designed to analyze the biofilm formation potential of lactic acid bacteria in general, using MRS as the universal medium for this group. As *L. rossiae* is a bigger problem in the filling and packaging area of breweries [17], the biofilm-forming potential in media that are closer in composition to fermented beer/ diluted beer will be tested in addition to conducting the standard test with MRS. As EPS development is a reaction of microorganisms to their environment and correlated to the composition of their substrates, lower distribution of growth factors and higher concentrations of growth inhibitors can lead to different biofilm-building potentials.

The tested *L. lactis* and *L. mesenteroides* strains could all develop biofilms in MRS. The ability to form initial biofilms correlates with the appearance of both species in brewery-related biofilms has already been described [4, 34].

3.2 Media validation

The agar medium (WBM-HR-A) was validated using the modified method according to the DIN ISO 11133 [11] as shown in table 7. The target organisms *L. acetotolerans*, *L. rossiae*, *L. brevis*, *L. mesenteroides* and *L. lactis* showed good growth on WBM-HR-A. The productivity of all 5 species was above 0.7 compared with NBB-A. *A. pasteurianus* and *P. aeruginosa* were both completely inhibited by WBM-HR-A and NBB-B. The two culture yeast strains *S. cerevisiae* TUM 68 and *S. pastorianus* TUM 34/70 showed partial inhibition in WBM-HR-A.

The minimum productivity proportion PR is set as 0.7 according to the DIN EN ISO 11133 for the validation of alternative media in comparison with a similar reference medium for target germs. This criterion was fulfilled by WBM-HR-A. The partial inhibition of *S. cerevisiae* TUM 68 and *S. pastorianus* TUM 34/70 can relate to a process step in the preparation of the agar medium. The sole yeast-inhibiting agent in all three new developed media is Natamax® (Natamycin) which is not heat stable in solution. For pouring the agar plates, the medium has to be heated for melting the agarose, which can lead to reduced antimicrobial effectiveness. Adding further antimycotics such as actidione (cycloheximide) can easily mitigate this disadvantage.

As shown in table 8 and table 9, the new broth medium was evaluated using the 4-field test [33]. All target organisms (*L. acetotolerans*, *L. brevis*, *L. rossiae*, *L. lactis* and *L. mesenteroides*) were able to grow in WBM-HR-B. The non-target organisms (*A. pasteurianus*, *P. fluorescens*, *S. cerevisiae* TUM 68 and *S. pastorianus* TUM 34/70) did not grow. The relative specificity, the relative accuracy and the relative sensitivity according to the 4-field test [33] are therefore 100 %.

To inhibit the dilution effects, it is possible to use the medium with higher amounts of sample volume, for example with 50 % medium using a double concentrated medium, or 10 % medium with a 10-fold concentrated medium. In that case, the final pH and bitter units in the mixture of sample and concentrated medium have to be checked.

The detection speed of WBM-HR-B was checked against NBB-B with a selection of target organisms as shown in table 9 in duplets. The detection speed of both media is comparable. *L. acetotolerans* TUM BP 120706025-2967, *Lactobacillus lindneri* TUM BP 121213056-2397, *Megasphaera cerevisiae* TUM BP 121011015-5986 and *Pectinatus frisingensis* TUM BP 000-4327 show an earlier detectable change of the indicator color in WBM-HR-B than in NBB-B. The test strains *L. casei* TUM BP 120509129-2360, *L. plantarum* TUM BP 121121170-2217 and *L. mesenteroides* TUM BP 000-0983 can be detected earlier in NBB-B.

3.3 Real-time-PCR

The real-time PCR systems for *L. acetotolerans*, *L. rossiae*, *L. lactis* and *L. mesenteroides* were validated against a strain set of 99 species that belong to the group of lactic acid bacteria or that are known to be present in breweries and beverage bottling plants (Table 10).

The identification systems for *L. acetotolerans*, *L. rossiae* and *L. mesenteroides* proved to be highly specific. Only the target species showed a significant signal within 40 PCR cycles. The relative specificity, the relative accuracy and relative sensitivity are therefore at 100 % [33].The identification system for *L. lactis* showed positive reactions after 28 cycles with the DNA of the following strains: *Lactobacillus malefermentans* TUM BP 000-2974, *Lactobacillus curvatus* TUM BP 000-2977 (BS 218), *Lactobacillus alimentarius* TUM BP 000-2979 and *Lactobacillus dextrinicus* TUM BP 000-2987. This means 4 cross reactions within the specificity evaluation and therefore a relative specificity of the *L. lactis* identification system of 96 % [33].

4 Conclusion/Summary

With the exception of some *L. rossiae* strains, all the tested bacteria could build biofilms in MRS, a nutrient rich medium, as used in the standard method to evaluate biofilm formation potential. While this medium is comparable in nutrient composition with wort and most alcohol-free beer types, most intermediate stages in the beer production offer quite different nutrient profiles. The missing biofilm formation of *L. acetotolerans* sustains the thesis that this germ mostly originates from the brewery sections that contain yeast such as fermentation tanks and vessels in the case of contamination cases as described by *Deng* [8].

L. rossiae is mostly described as a germ that originates in filling devices. Heterogenic phenotypes concerning biofilm formation potential in different strains of this species could be observed. *L. rossiae* has already been described as having a wide intra-species variation of biochemical properties and RAPD genotypes [6]. This high variance also seems to affect the biofilm-forming potential. The risk to breweries that are confronted with this species of building persistent biofilms on filling equipment can therefore vary. It is still possible for non-biofilm forming strains of *L. rossiae* to occur in symbiosis with other biofilm forming microorganisms.

All tested strains of the *L. lactis* and *L. mesenteroides* species showed biofilm-forming potential in MRS. There are no cases reported of these species having a product spoiling potential in Bavarian Wheat Beer under normal production conditions, but the de novo biofilm production by these species can generate a habitat for spoilage organisms.

The developed and validated WBM-HR media proved to be sufficient. Compared with the reference medium NBB, the broth variant showed comparable results. The advantage is that the formula/recipe is known and that it can be modified in any direction (concentrate, double-concentration, beer content, brewery specific beer, etc.). It also detects slow-growing germs such as *L. acetotolerans* very rapidly compared with comparable enrichment media. The WBM-HR agar can be used after modifying the yeast inhibition substance. The addition of cycloheximide is a possible option that should be tested in the future.

The real-time PCR identification systems developed for *L. acetotolerans*, *L. rossiae* and *L. mesenteroides* proved to be highly specific with the target species. No cross reactions could be observed in vitro. The identification system for *L. lactis* showed a positive PCR reaction after 28 cycles with the strains of following species *Lactobacillus malefermentans*, *Lactobacillus curvatus*, *Lactobacillus alimentarius* and *Lactobacillus dextrinicus*. The species that showed cross reactions are not potential or obligate beer spoilers and are lactic acid bacteria that seldom occur or do not occur in a brewery environment. *L. lactis* pure culture DNA isolate was detected much earlier at a PCR ct value of 16 (16 PCR cycles). Cross reactions of the *L. lactis* could therefore additionally be excluded by checking the ct value of a pure culture or enriched culture analysis. No influence of the developed medium on the PCR reaction could be observed in any of the tests. A combined use of the WBM-HR with the PCR screening systems is of great value for quality management in breweries, especially Bavarian Wheat Beer breweries, confronted with the discussed species.

5 Funding

Parts of this study were funded by the Wissenschaftliche Station für Brauerei in München e.V.. The funding source did not have any influence on the study design, the data collection, the analysis and interpretation, the report writing or article submission.

Acknowledgement

The authors are very grateful to Mrs. Jennifer Koob, Mrs Margit Grammer and Mrs Siglinde Huber for supporting this project in the Laboratories of the Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität.

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Received 8 December 2016, accepted 10 February 2017

2.3. Brewing Enemy Number One: Genetic diversity, physiology and biofilm formation of *Lactobacillus brevis*

Abstract

Lactobacillus brevis is the most significant beer-spoilage bacteria worldwide. It is found as a contaminant at all stages of brewing, including during primary and secondary fermentation, storage, filtration and the packaging process. In production with flash pasteurisation and subsequent hygienic filling, avoiding and tracing secondary contaminations is the key to a microbiologically stable product. However, *L. brevis* strains vary in their spoilage potential and can grow in many different beer types. This study presents a physiological test scheme for growth potential and biofilm formation in various media. It was determined that a large number of *L. brevis* strains can form biofilms as a first coloniser. The identification of the species alone is therefore not enough to be sure of the spoilage risk, which shows the need for a more in-depth differentiation. DNA fingerprint techniques are crucial to differentiate isolates of this species at strain level. The rep-PCR fingerprint system (GTG)5 was used to differentiate a selected collection of 20 isolates, which were characterised in growth and biofilm formation in various media. The data showed a high variation within the selected isolates. As a second step, generated fingerprint clusters of *L. brevis* were traced back to contamination sources in a German brewery, revealing a high number of isolates with potentially varying growth, spoilage and biofilm potential. Using *L. brevis* as the demonstrator species, the PCR system used is a powerful and compatible tracing and troubleshooting tool for all kinds of spoilage bacteria in the brewing industry.

Authors/Authorship contribution:

Riedl R.: Literature search, writing, data creation, study conception and design; **Dünzer N.:** Data analysis and interpretation (rep-PCR fingerprinting), critical review of draft; **Michel M.:** critical content review; **Jacob F.:** Supervised the project; **Hutzler M.:** Creation of the research plan, critical content review

Research article

Received: 7 February 2018 Revised: 2 November 2018 Accepted: 11 November 2018 Published online in Wiley Online Library: 28 January 2019

(wileyonlinelibrary.com) DOI 10.1002/jib.553

Beer enemy number one: genetic diversity, physiology and biofilm formation of *Lactobacillus brevis*

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Lactobacillus brevis* is the most significant beer spoilage bacteria worldwide. It is found as a contaminant at all stages of brewing, including during primary and secondary fermentation, storage, filtration and the packaging process. In production with flash pasteurisation and subsequent hygienic filling, avoiding and tracing secondary contaminations is the key to a microbiologically stable product. However, *L. brevis* strains vary in their spoilage potential and can grow in many different beer types. This study presents a physiological test scheme for growth potential and biofilm formation in various media. It was determined that a large number of *L. brevis* strains can form biofilms as a first coloniser. The identification of the species alone is therefore not enough to be sure of the spoilage risk, which shows the need for a more in depth differentiation. DNA fingerprint techniques are crucial to differentiate isolates of this species at strain level. The rep-PCR fingerprint system $(GTG)_5$ was used to differentiate a selected collection of 20 isolates, which were characterised in growth and biofilm formation in various media. The data showed a high variation within the selected isolates. As second step, generated fingerprint clusters of *L. brevis* were traced back to contamination sources in a German brewery, revealing a high number of isolates with potentially varying growth, spoilage and biofilm potential. *L. brevis* being the demonstrator species, the PCR system used is a powerful and compatible tracing and troubleshooting tool for all kinds of spoilage bacteria in the brewing industry. © 2019 The Institute of Brewing & Distilling

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Keywords: *Lactobacillus brevis*; biofilm formation; brewing; contamination tracking; strain differentiation; rep-PCR

Introduction

In microbiological terms, beer is a relatively stable beverage. Only a small group of species is able to grow in the hostile environment that beer represents, being rich in ethanol, carbon dioxide, hop bitter acids, having a low pH and being poor in nutrients *(1)*. Within this group *Lactobacilli* are the most common with *Lactobacillus brevis* being the most significant beer spoilage bacteria worldwide *(1,2)*. From 2010 to 2016, between 41 and 53% of the samples sent to the Research Center Weihenstephan for Brewing and Food Quality that scored positive for beer spoiling bacteria were identified as containing *Lactobacillus brevis (3–5)*. This insight suggests that *L. brevis* is one of the most common species responsible for beer spoilage in breweries *(6)*. It is found as a contaminant at all stages of brewing, including primary and secondary fermentation, storage and filtration and in the packaging process. In addition to *L. lindneri*, *L. backi*, *L. paracollinoides* and *Pediococcus damnosus*, *L. brevis* is recognised as one of the most potent beer spoiling Gram positive bacteria *(1,7)*. All beer spoiling bacteria, especially *L. brevis*, have the ability to grow in beer and change the product negatively in appearance, organoleptic propertie, taste and flavour *(2,6)*. *L. brevis* can been historically classified into two types according to its morphological appearance (colony form/colour, cell form) *(2,6)*. However the beer spoiling potential caused by hop tolerance depends on the strain and origin, and cannot be predicted by cell or colony morphology *(8,9)*. In most cases beer spoiling bacteria, including *L. brevis*, are detected using traditional selective culture media or observing metabolic products *(7,10)*. Detection using standard media is time consuming (3–14 days, depending on the matrix and medium) and has limited value in predicting the beer spoiling potential and origin of the contamination *(2,6,7)*. Specific, rapid detection and identification of beer spoiling bacteria can be achieved with immunoassays, DNA–DNA hybridisation and polymerase chain reaction *(7,11)*.

L. brevis, as well as other beer spoiling bacteria, has been described as a late biofilm coloniser in brewery biofilms, often benefiting from prior colonisation of different species *(12)*. What makes biofilms so dangerous for any hygienic production is that they provide protection against various environmental stress factors such as oxidation, desiccation, biocides, antibiotics, metal ions and UV *(13)*. For example, biofilms consisting of single strains of *L. plantarum* subsp. *plantarum* had a significantly higher resistance to acetic acid and ethanol than their planktonic equivalents, making biofilm formation an important indicator for hygienic countermeasures *(14)*. Lactic acid bacteria (LAB) influence biofilm consistency in mixed biofilms and can enhance biofilm formation in mixed species biofilms with yeasts *(15,16)*. It was further possible to grow weak biofilms in MRS using *L. brevis* strains that were

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isolated from a brewery site (17). The media composition has a major influence on biofilm formation, as demonstrated with strains of *L. rhamnosus* (18). As the biofilm formation is described as being strongly dependent on the substrate and strain (19), no precise data is available for beer spoiling strains in a brewery environment. While media richer in nutrients are generally described as enabling strong biofilm formation owing to high cell growth, chemical and physical stress factors can enhance cell adherence and the production of extracellular substances resulting in stronger biofilms (13). In addition, the examination of other species (*L. rossiae* and *Lactococcus lactis*) with regard to their biofilm formation in earlier studies showed great strain dependent differences in the biofilm formation (20,21). Accordingly, strain and media specific tests for *L. brevis* biofilm formation and growth potential appear to be necessary.

For strain differentiation, RAPD–PCR (randomly amplified polymorphic DNA–polymerase chain reaction) fingerprinting methods are a powerful and easy-to-use tool to look for genetic variations (22,23). Differentiation at strain level, which is not possible when using 16S rRNA-based PCR, can be achieved using RAPD–PCR fingerprinting and has already been applied to identify genetic markers correlating with hop tolerance and beer spoiling ability (22,24,25). To differentiate LAB, rep-PCR (repetitive sequence-based-polymerase chain reaction) is described as having a higher discriminatory power compared with RAPD-PCR, the most used system for differentiation (26). Additionally, the reproducibility of RAPD–PCR patterns is rather poor (27–29). For species identification, the total genomic DNA–DNA homology is considered the 'gold standard' and a value of ≥70% similarity represents an internationally accepted criterion to define the bacterial species (30). The correlation between DNA–DNA homologies and rep-PCR fingerprinting methods is very high, which makes rep-PCR methods useful for rapid and discriminatory screening to determine the taxonomic and phylogenetic structures of bacterial populations (31). The bacterial genome contains many highly dispersed repetitive sequences (32). Many of those are conserved regions at various positions throughout the genome in many different eubacteria and can be used as a primer target sequence (33). The rep-PCR, based on short repetitive sequences dispersed widely over the genome as primer target areas, is described as a method to classify and type Gram positive and negative bacteria, as well as eukaryotic species (28,29,34,35). One example of such a repetitive unit is the poly-trinucleotide $(GTG)_5$, which appears to be highly repetitive within the genome of *Escherichia coli* and *Salmonella typhimurium* (36). This region does not code on its own, but is widely dispersed throughout the bacterial genome (37). The $(GTG)_5$ rep-PCR primer has been described as being highly applicable to the differentiation of LAB at a subspecies and strain level (26), but also for acetic acid bacteria (38), enterococci (39) and yeasts (28).

The aim of this study was to investigate if DNA $(GTG)_5$ fingerprinting methods can be used to discriminate different strains of *L. brevis* in a brewery and track the source of contamination. In addition, this study investigated the ability of different strains to form biofilms and whether strains that readily form biofilms, based on their physiology, can be tracked back to a source in a brewery. A set of *L. brevis* isolates was therefore selected from the culture collection of the Research Center Weihenstephan for Brewing and Food Quality. All isolates are known to have different origins, as well as variation in their product spoiling effect. A genotype database, using $(GTG)_5$ fingerprinting, was used as a tool for investigating the variety of beer spoiling bacteria in breweries at a genetic level. For physiological differentiation, the growth as well as the biofilm formation in various media was tested and compared for correlation within the genotype database. As a second step, a field study was conducted within one brewery with a persistent presence of *L. brevis* to examine the variance within a single brewery. The field study was performed in order to track the contamination of single fingerprint type from the bottled end product back to the first process step.

Materials and methods

To investigate the genetic and physiological diversity of *L. brevis*, an isolate set ($N = 20$) was selected from the culture collection of the Research Center Weihenstephan for Brewing and Food Quality. The selected isolates were known to originate from various sources and showed different product spoilage. The isolates were examined for growth and biofilm potential in different media and their genotype was classified using the $(GTG)_5$ fingerprint. Finally, DNA was extracted from pure cultures and the species was confirmed as *L. brevis*, using the foodproof® Beer Screening Kit (Biotecon Diagnostics, Potsdam, Germany). $(GTG)_5$ fingerprints were generated by DNA fingerprint amplification, followed by capillary gel electrophoresis and data processing of the generated fingerprints. The physiology was investigated as growth and biofilm forming potential in the stationary phase for the same isolates. The field study, to track the contamination of *L. brevis* fingerprint clusters in a German brewery, was performed by means of close mesh hygienic sampling of the entire brewing process. All isolates of *L. brevis* were processed along with the reference isolate set for gaining genetic fingerprints and processing the genotype data. The genotypes were tracked throughout the process.

Cell cultures

All reference isolates were stored as cryo cultures at −80°C in Roti®-Store Cryo Tubes (Carl Roth GmbH+Co. KG, Karlsruhe, Germany) at the Research Center Weihenstephan before reviving and incubation in NBB-B (Döhler GmbH, Darmstadt, Germany) at 28°C, anaerobically for 4–5 days until growth was visible. Reference isolates were selected from different brewery samples when cultures scored positive with a species identity of *L. brevis*, using the foodproof® Beer Screening Kit as shown below.

DNA isolation

For DNA isolation, a modified protocol using the InstaGene Matrix™ (Bio-rad, Hercules, CA, USA) was used (20,40). A 1 mL aliquot of a homogenous cell suspension (between 10^7 and 10^8 cells/mL) was transferred to a 1.5 mL Eppendorf reaction tube and centrifuged for 2 min at 15,114*g* (Hettich MIKRO 200 (Hettich, Tuttlingen)). The cell pellet (about 2 mm in diameter) was suspended in 200 µL of InstaGene™ Matrix. A lysis buffer was added and the sample was incubated at 56°C for 30 min (Eppendorf Thermomix comfort, Eppendorf, Wesseling-Berzdorf). After vortexing for 10 s, the tube was incubated for 8 min at 95°C then centrifuged for 1 min at 15,114*g*. The DNA concentration was measured using a Nanodrop ND 2000 (ThermoFisher Scientific, Rochester, USA).

For the fingerprint PCR amplification, the DNA concentration of the test isolates was adjusted using PCR-clean ddH_2O to

175 ng/µL. The DNA sample and sample isolate were stored at −20°C.

Confirmation of species identity

The species identity of each isolate was confirmed using a Lightcycler 480 II real-time PCR System (Roche Diagnostics Ltd, Rotkreuz, Schweiz) with the PCR FRET hybridisation probe foodproof® Beer Screening Kit, according to manufacturing instructions.

DNA fingerprint amplification

$(GTG)_5$ fingerprints were generated by DNA fingerprint amplification, followed by capillary gel electrophoresis and the data processing of the generated fingerprints. The DNA amplification was carried out in 25 µL batches, containing 12.5 µL RedTaq Mastermix (2×) 2-fold (Genaxxon bioscience, Ulm), 10 µL Primer Solution (50 pmol L^{-1}), 5 µL PCR-clean bidistilled water (ddH_2O) and 2.5 µL sample DNA. The primer used for the PCR reaction was the $(GTG)_5$ primer: 5′-GTGGTGGTGGTGGTG-3′ according to Versalovic *et al. (34)*. The PCR temperature protocol included an activation phase for the Taq Polymerase of 5 min at 95°C. Thirty cycles were performed, each cycle lasting 0.5 min at 95°C for denaturation, 1 min for annealing and 8 min for amplification. The final extension with a duration of 16 min was performed at 72°C before cooling to 4°C. The annealing and time of amplification were conducted according to Versalovic *et al. (34)*, and the other parameters were set to match the requirements of the RedTaq Mastermix (2×) 2-fold polymerase (Genaxxon bioscience, Ulm).

Capillary gel electrophoresis

The capillary gel electrophoresis was performed with an Agilent Bioanalyzer 2100 expert system, using the Agilent 7500 DNA Kit (Agilent Technologies, Santa Clara, CA, USA) according to the operation manual for generating electrographic PCR fingerprints, ranging from 100 to 7500 base pairs.

Data processing

Electropherograms were analysed using BioNumerics version 7.6.2 (Applied Maths NV, Keistraat BL). The raw data was processed using an 11% disk size background subtraction and a filter of the arithmetic average with a least square filtering cutoff <0.35%. For normalisation, the ladder run performed for each microcapillary chip was set as the reference lane. The bands of the ladder run were set as reference bands with sizing according to the Agilent DNA 7500 kit manual, as well as the upper and lower marker band of each run. The similarity coefficient for fingerprint comparison was calculated using a Pearson correlation with optimisation 0%, curve smoothing 0.5% and negative similarities clip to zero. The cluster calculation was calculated using the UPGMA with secondary criterion clustering method. The highest overall similarity and degeneracy calculation was clustering + secondary criterion.

Growth and biofilm forming potential in stationary phase

The biofilm formation potential and growth control of the microorganisms used (Table 1) were tested using an adapted test design according to Kolari *et al. (42)* and modified by Timke *et al. (41)* in a 96 well microtitre format that was used in a recent study *(20)*. This test was selected as being straightforward for quantifying a broad range of microbial biofilms with the exception of *P. aeruginosa* biofilms *(43)*. The test was carried out in sterile, black, flat bottomed polystyrene microtitre 96 well plates with polymeric optical bottoms for fluorescence applications (Thermofisher Scientific, Rochester, USA). Each well was filled with 250 µL MRS DeMan–Rogosa–Sharpe) broth *(44)*, filtered wheat beer, lager beer and pilsener-type lager beer. To simulate the conditions in filler surroundings (e.g. over-foaming beer and rinsing water), a second set of tests was carried out with each medium diluted with 50% ddH_2O. Overnight cultures of the test isolates were washed by suspending and centrifuging in sterile distilled water and adjusted to 10×10^6 cells/mL. The filled wells were incubated with 2.5 µL of the washed cultures in four replicates. A blank value was measured by preparing four replicates of each medium with 2.5 µL sterile distilled water instead of the cell suspension for each isolate and medium. Afterwards, the microtitre plates were sealed with gas permeable foil and incubated anaerobically at 28°C for 24 h. The optical density (OD_{590}) (Multi-Detection-Reader Synergy 2, BioTek, Bad Friedrichshall, Germany) was measured directly after sealing the plates and after 24 h incubation as a growth control. The plates were emptied and rinsed using sterile distilled water. A 300 µL aliquot of crystal violet solution (4 g/L in 20% v/v ethanol) was added to cells forming a biofilm in the wells for 5 min at room temperature. All wells were emptied and rinsed three times using 400 µL sterile distilled water. The remaining crystal violet that was still bound to the cells was dissolved in 300 µL 96% v/v ethanol at 10°C overnight and OD_{590} was measured using the Synergy 2 Multi-detection reader. To calculate the growth and biofilm formation, means were calculated using four independent measurements of four biological replicates per isolate, normalised against the mean of four independent measurements of four blank samples and categorised as shown in Table 1. All wells were cochecked and documented microscopically for adherent cells and trub particles using a Nikon inverted research microscope Ti-E, using a CFI S P-Fluor ELWD ADM 60× C air objective for phase contrast microscopy.

To validate growth, cutoff values were used to differentiate the growth potential in different media. The growth cutoff for no growth ($E_{590\ cutoff}$) was defined as the mean difference of the measured optical density of four independent negative samples directly after inoculation ($\overline{E}_{590\ blank\ t_0}$) and 24 h incubation ($\overline{E}_{590\ blank\ t_{24h}}$) plus three times the combined quadratic mean standard deviation of both measurements ($\sqrt{\overline{SD}_{590\ blank\ t_0}^{\ 2} + \overline{SD}_{590\ blank\ t_{24h}}^{\ 2}}$). The mean of four independent measurements of a isolate minus the combined quadratic standard deviation of the measurements directly after inoculation and after 24 h of incubation ($\overline{\Delta E}_{590\ (t_{24h}-t_0)} - \sqrt{\overline{SD}_{590\ t_0}^{\ 2} + \overline{SD}_{590_{24h}}^{\ 2}}$) equal to or below this threshold was defined as no growth. Values between $E_{590\ cutoff}$ and $2 * E_{590\ cutoff}$ were defined as weak growth, those between $2 * E_{590\ cutoff}$ and $4 * E_{590\ cutoff}$ were defined as moderate growth and those $>4 * E_{590\ cutoff}$ were defined as strong growth.

To validate the biofilm formation potential, cutoff values were used to differentiate between biofilm forming isolates and non biofilm forming isolates. The biofilm forming cutoff for non-biofilm forming organisms ($E_{550\ cutoff}$) was defined as the mean of the

Table 1. Reference isolates tested for growth and biofilm formation [modified according to Timke *et al.* (41) and Diaz *et al.* (21)]

Short number	MRS		MRS 50%		WB		WB 50%		LB		LB 50%		PB		PB 50%		Fingerprint cluster (Figure 1)
	Growth*	Biofilm**	Growth*	Biofilm**	Growth*	Biofilm**	Growth*	Biofilm**	Growth*	Biofilm**	Growth*	Biofilm**	Growth*	Biofilm**	Growth*	Biofilm**	
LB2022	++	–	++	–	–	–	++	–	–	–	–	–	–	–	+++	–	A
LB2790	+++	+++	++	+++	–	+	+	+	–	–	++	–	–	+	–	++	A
LB2580	++	–	++	+	–	–	++	–	–	–	+++	–	–	–	+	–	A
LB2046	+++	–	++	–	–	–	+	–	–	–	+	+	–	–	+	–	B
LB2019	+	–	+	–	–	–	+	–	–	–	+	–	–	–	+	–	B
LB2084	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C
LB2372	+++	+	++	–	–	–	–	–	–	+	+	+	–	–	+	++	C
LB2891	++	+++	+++	+++	–	–	+	+	–	–	–	+	–	–	–	–	D
LB2206	+++	–	+++	–	–	–	–	–	–	–	–	–	–	–	–	–	D
LB2048	++	–	++	–	–	–	+	+	–	–	–	–	–	–	–	–	D
LB2781	+++	+	+++	+	–	–	+	–	–	–	+	–	–	–	++	–	E
LB2936	+++	–	++	–	–	–	+	–	–	–	+	–	–	–	+	–	E
LB2604	+++	–	+++	–	+	–	+	–	–	–	++	–	–	–	+	–	F
LB2786	+++	+	+++	+	–	–	–	–	–	–	–	+	–	–	–	–	F
LB2143	++	+	++	++	–	+	+	–	–	–	+	+	–	–	+	–	
LB2823	+++	–	++	–	–	–	–	–	–	–	–	–	–	–	–	–	
LB2855	–	+	+	++	–	–	+	–	–	–	–	–	–	–	–	–	
LB2020	+	++	+	–	–	–	+	–	–	–	–	–	–	–	–	–	

MRS, DeMan–Rogosa–Sharpe broth.
MRS 50%, DeMan–Rogosa–Sharpe broth, diluted 50:50 with sterile distilled water.
WB, Filtered Bavarian wheat beer.
WB 50% filtered Bavarian wheat beer, diluted 50:50 with sterile distilled water.
LB, Bavarian lager beer.
LB 50%, Bavarian lager beer, diluted 50:50 with sterile distilled water.
PB, Pilsener beer.
PB 50%, Pilsener beer, diluted 50:50 with sterile distilled water.

*–, No growth, $\overline{\Delta E}_{590\,(t_{24h}-t_0)} - \sqrt{\overline{SD}_{590\,t_0}^{\,2} + \overline{SD}_{590\,t_{24h}}^{\,2}} \leq E_{590\,cutoff}$.

+, Weak growth, $E_{590\,cutoff} < \overline{\Delta E}_{590\,(t_{24h}-t_0)} - \sqrt{\overline{SD}_{590\,t_0}^{\,2} + \overline{SD}_{590\,t_{24h}}^{\,2}} \leq 2 * E_{590\,cutoff}$.

++, Moderate growth $2 * E_{590\,cutoff} < \overline{\Delta E}_{590\,(t_{24h}-t_0)} - \sqrt{\overline{SD}_{590\,t_0}^{\,2} + \overline{SD}_{590\,t_{24h}}^{\,2}} \leq 4 * E_{590\,cutoff}$.

+++, Strong growth $\overline{\Delta E}_{590\,(t_{24h}-t_0)} - \sqrt{\overline{SD}_{590\,t_0}^{\,2} + \overline{SD}_{590\,t_{24h}}^{\,2}} > 4 * E_{590\,cutoff}$.

with $E_{590\,cutoff} = \overline{E}_{590\,blank\,t_0} - \overline{E}_{590\,blank\,t_{24h}} + 3 * \sqrt{\overline{SD}_{590\,blank\,t_0}^{\,2} + \overline{SD}_{590\,blank\,t_{24h}}^{\,2}}$.

**–, No biofilm formation $\overline{E}_{550} - \overline{SD}_{550} \leq E_{550\,cutoff}$.
+, Weak biofilm formation $E_{550\,cutoff} < \overline{E}_{550} - SD_{550} < 2 * E_{550\,cutoff}$.
++, Moderate biofilm formation $2 * E_{550\,cutoff} < \overline{E}_{550} - SD_{550} < 4 * E_{550\,cutoff}$.
+++, Strong biofilm formation $\overline{E}_{550} - SD_{550} > 4 * E_{550\,cutoff}$.

measured optical density of four independent negative samples ($\overline{E_{550\ Blank}}$) plus three times the mean standard deviation ($\overline{SD_{550\ Blank}}$) and the mean measurement of the blank value ($\overline{E_{550\ Blank}}$) plus the mean standard deviation ($\overline{SD_{550\ Blank}}$). The mean of four independent measurements of an isolate minus the standard deviation of these measurements $\overline{SD_{550}}$ equal to or below this threshold was defined as no biofilm formation. Values obtained between $E_{590\ cutoff}$ and $2 * E_{550\ cutoff}$ were defined as weak biofilm formation, those between $2 * E_{550\ cutoff}$ and $4 * E_{590\ cutoff}$ were defined as moderate biofilm formation and those $>4 * E_{550\ cutoff}$ were defined as strong biofilm formation according to Diaz *et al.* (21). All wells in the microtitre plate were also checked microscopically for cell adhesion.

Contamination tracking of *L. brevis* fingerprint clusters in a German brewery

To show the practical application of the strain identification and biofilm testing methods, a field study was performed. In order to track the sources of potential contamination, samples were taken from a German brewery with a wide product portfolio including alcohol free, low alcohol, wheat beer, lager beer and strong beer. Additional microbial variation would be anticipated as beers from other breweries are brought on-site for dealcoholisation and packaging.

The sampling took place over two weeks and comprised samples from the production process from cool wort to flash pasteurisation before filling. Samples were also taken of the process water, air in the filling area, process gases and bottled beer. Swab samples were taken from equipment using sterile swabs, pre-moistened with Ringer's solution. The complete list of sample points and processed samples is given in supporting Information for this article. The methods used for sample processing are shown in Table 2. In addition to process samples, samples from continuous sampling before flash pasteurisation were collected of all batches in the sampling period. Negative samples (no microbial growth/without microbiological detection) were not analysed any further. Samples with visible growth were analysed microscopically. Liquid cultures were plated out on NBB agar plates (incubated anaerobically for 7 days at 28°C). DNA was extracted from single colonies and tested for species identification and strain differences as described above.

Results

Genetic differentiation of 20 selected isolates

From the culture bank we were able to differentiate all 20 selected isolates (Figure 1). The $(GTG)_5$ fingerprinting method can be used for all species of beer spoiling bacteria, as shown in Figure 1, including *Lactobacillus casei/paracasei* LC2854 and *Pediococcus damnosus* PD2142. The clustering of these two isolates was in excess of 20% similarity to all applied isolates of *L. brevis*. The investigated isolates of *L. brevis* were divided into groups according to their clusters of high similarity (>70%, A–E). No direct relationship was observed between the isolates within groups A, C and D; however isolates in group B (LB2019 and LB2046) originated from samples of the same brewery from different sample matrices and showed high similarity at 91.2%, which indicates the same strain identity. Isolate LB2019 as well as isolate LB2020, came from the same sample, but LB2020 did not cluster with any of the other differentiated isolates and showed different colony morphology to LB2019. Both findings indicate that this particular brewery had more than one microbiological problem, which is supported by our findings of isolates that were isolated from the same brewery, but clustered in groups A, D and E. Within group E, two isolates with similar slime formation (observed when originally isolated) and isolated from wheat beer but from different breweries, showed high genetic fingerprint similarity. Group F did show a lower similarity (68%) than the threshold of 70%, but these isolates originated from the same brewery and the same sample matrix: unfiltered wheat beer. Only one of the isolates showed visible slime formation in the sample. The slightly higher variation in genetic fingerprint similarity mirrored the varied phenotypical appearance. In general, no fingerprinting cluster groups could be identified, describing isolates of a single habitat.

Table 2. Sample processing for contamination tracking

Method name	Processing type	Medium	Incubation
Low hopped beer	Liquid enrichment	Low hopped beer (MEBAK III 9.3.1)	7–10 days at 28°C, anaerobic
MF, MRS-A + 15 mg/L cycloheximide	Membrane filtration		7–10 days at 28°C, anaerobic
MF, NBB-A	Membrane filtration	NBB agar (Doehler, Darmstadt, Germany)	7–10 days at 28°C, anaerobic
MRS 2× + 30 mg/L cycloheximide + NBB-P-C 5×	Concentrated medium, liquid enrichment	MRS double concentrated, with 30 mg/L cycloheximide and NBB-P-C 5×	7–10 days at 28°C, anaerobic
NBB agar, pour plate	Agar pour plate (1 mL sample)	NBB agar (Doehler, Darmstadt, Germany)	7–10 days at 28°C, anaerobic
NBB-B	Liquid enrichment	NBB boullion (Doehler, Darmstadt, Germany)	7–10 days at 28°C, anaerobic
NBB concentrate	Concentrated medium, liquid enrichment	NBB concentrate (Doehler, Darmstadt, Germany)	7–10 days at 28°C, anaerobic
Nutrient agar, pour plate	Agar pour plate (1 mL sample)	Nutrient agar (DEV) (Sifin Diagnostics Gmbh, Berlin, Germany)	7–10 days at 28°C, anaerobic
Swab samples	Liquid enrichment	NBB-B-AM (Doehler, Darmstadt, Germany)	7–10 days at 28°C, anaerobic

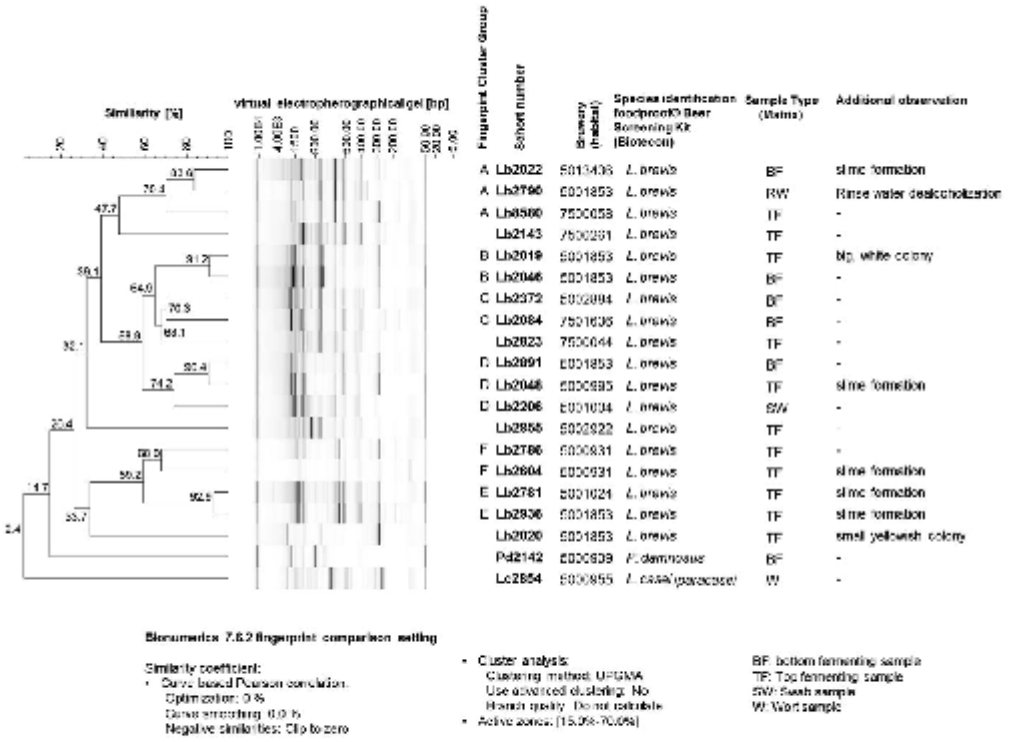


Figure 1. Phylogenetic $(GTG)_5$ comparison of selected *Lactobacillus brevis* isolates subsequently used for biofilm and media growth tests.

Biofilm formation and growth potential of 18 selected isolates in various media

As shown in Table 1, growth and biofilm formation in various media did not correlate with the genetic fingerprint clusters of Figure 1. However, it is worth mentioning that some isolates showed biofilm formation as pure cultures, which has not been reported for *L. brevis* in beer before. LB2372 showed a high ability to form biofilms in lager beer and diluted pilsener beer. A connection can be drawn between the diluted beer and the biofilm formation. In pure wheat beer two isolates could form biofilms (LB2790 and LB2143); in the 50% dilutions there were three isolates that could form biofilms (LB2790, LB2891 and LB2048). In lager beer only one isolate formed a biofilm (LB2372) whereas five isolates could form biofilms in the 50% diluted lager beer. The same trend can be observed for pilsener beer.

Contamination tracking of *L. brevis* fingerprint clusters in a German brewery

Overall about 75% of the bacterial samples isolated from the brewery were identified as *L. brevis* (Figure 2). As the focus of this study was on tracking the source of contamination and on process samples from primary contamination sources, samples directly before flash pasteurisation were not included as they are 'pooled' samples. The distribution of samples that tested positive for beer spoiling *Lactobacillus* sp. during the production process (Figure 3) can be described as heterogenic. While no beer spoiling LAB could be detected in the early process (wort cooling and aeration), the only other samples that tested negative for *L. brevis* within the process flow were the cleaning in place rinses and the harvested yeast tanks. *L. brevis* was found in all the other sampling points and tested positive for beer spoiling LAB, with the exception of the bottom fermenting cylindroconical tanks, which were dominated by *L. backii* and *L. casei*. Genetic fingerprinting was performed for all of the detected species of beer spoiling bacteria, but only data related to the positive detection of *L. brevis* is presented here. A diagram of the process flow with the main sampling points is shown in Figure 5; the detailed sampling points and data on species detection are listed in the supporting information for this article. The genetic fingerprints were processed, using Bionumerics 7.6.2, and resulted in a heterogeneous distribution (Figure 4). In addition to the process samples listed above, isolates of samples taken from the continuous sampling apparatus directly before the flash pasteuriser were analysed as well to demonstrate that the detected

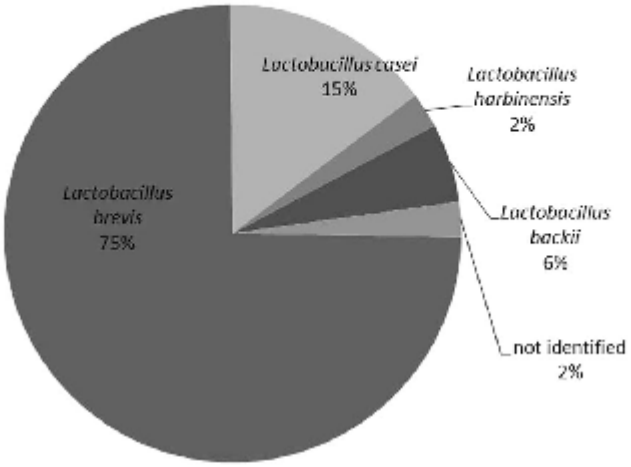


Figure 2. Percentage of identified species, detected in critical sampling point control, before flash pasteurisation (n = 161).

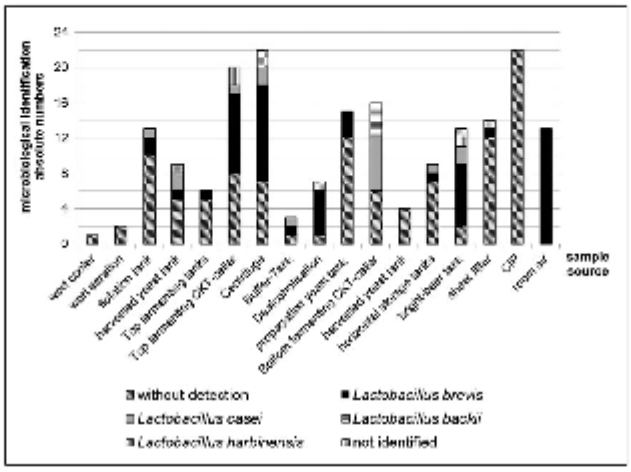


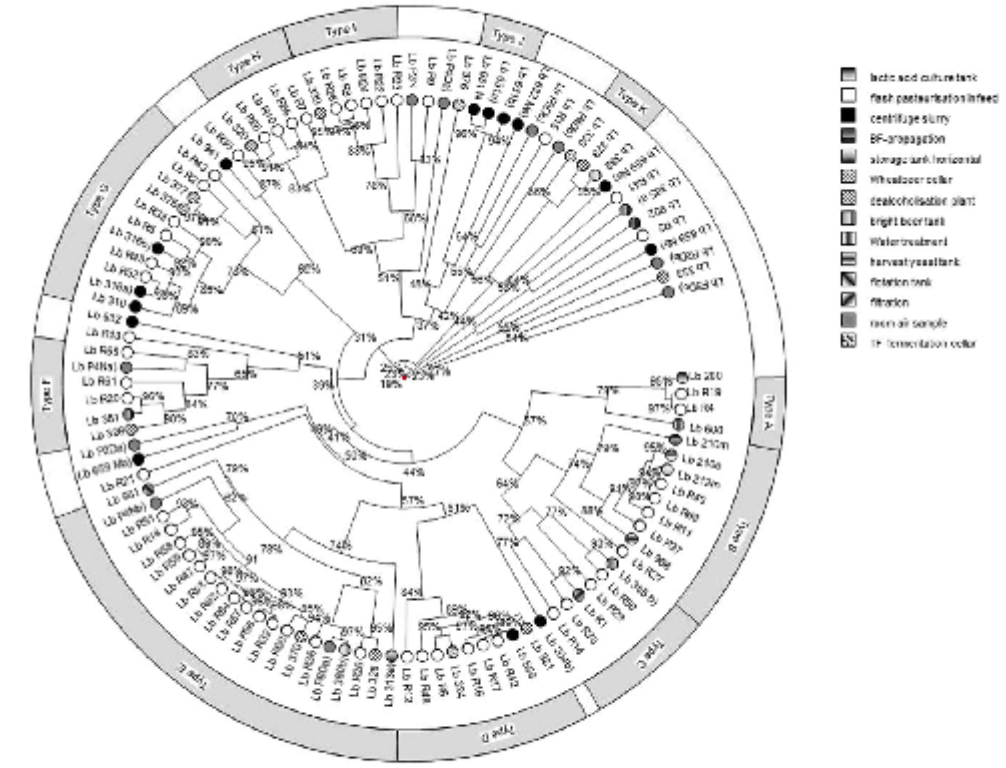
Figure 3. Distribution of beer spoiling *Lactobacillus* sp., in critical sampling point control, before flash pasteurisation to track contamination of *Lactobacillus brevis* fingerprint types in a German brewery.

isolates were also present in the finished product. Isolates that cluster with at least 70% fingerprint similarity were defined as fingerprint types A–K. The distribution of strain types within the brewery showed relationships to specific sampling points as represented in the process flow scheme in Figure 5. This figure also shows how some isolates are found at more than one sampling site, highlighting their widespread distribution throughout the brewery. For example, fingerprint group A was first detected in the horizontal storage tank but also appeared at the filler, bright beer tank, TF CCT cellar and flotation tank.

Discussion

Genetic differentiation, biofilm formation and growth of the selected isolate set

It was not possible to link the genetic fingerprint clusters to specific phenotypical properties of the tested isolates. Properties such as antibiotic resistances and tolerances, especially to hop compounds that are partly responsible for growth in beer, are plasmid encoded (horA *(45,46)*, horC *(46,47)*, hitA *(24)*. The rep-PCR system used $(GTG)_5$ only amplifies genomic DNA and does



Similarity coefficient
Pearson correlation
Optimization: 0 %
Curve smoothing: 0.5 %
Negative similarities: Clip to zero
Active zones: [15.0%-70.0%]
Network creation
Network creation method UPGMA

Figure 4 Circular dendrogram, calculated for critical sample point control before flash pasteurisation. [Colour figure can be viewed at wileyonlinelibrary.com]

not represent genetic markers, which makes plasmid located genetic markers virtually invisible with this method. However, the method proved to be useful for the differentiation of the *L. brevis* isolates as well as for the differentiation of other beer-spoiling and potential beer spoiling species, for example the two isolates identified as *Pediococcus damnosus* and *L. casei/paracasei*, showing specific fingerprints that are differentiated from the *L. brevis* isolates.

Based on the results, it is possible to identify stable genetic identities that can be monitored within single or multiple habitats within a brewery (Figure 1). For example groups A and D showed high genetic similarity but were distributed across various habitats. One reason for this might be cross contamination between habitats owing to contract filling by one brewery for the other.

Cluster F does not fulfill the threshold set for this study of 70% similarity but was selected as an exceptional cluster because the similarity value is very close to the threshold (68.0%) and both samples were collected from the same habitat. It is interesting that, within cluster F, despite two isolates coming from the same habitat, only one isolate showed visible slime formation in the beer from which the isolate originated. The difference in this physiological property is mirrored in the genetic fingerprint similarity. The same differences between these isolates can be seen in Table 1, where LB2604 shows higher growth potential, but no observable biofilm formation compared with LB2786. Slime, an essential part of biofilms (48), can enable higher resistance, but does not necessarily enhance biofilm formation. On the contrary, most isolates described as having produced slime in the isolated sample were rather weak biofilm formers.

The analyses of the physiological properties of the *L. brevis* isolate set proved to be very heterogeneous (Table 1). Isolate LB2084 could not show significant growth in any of the tested media. This could have been because of slow growth since the incubation time was limited to 24 h.

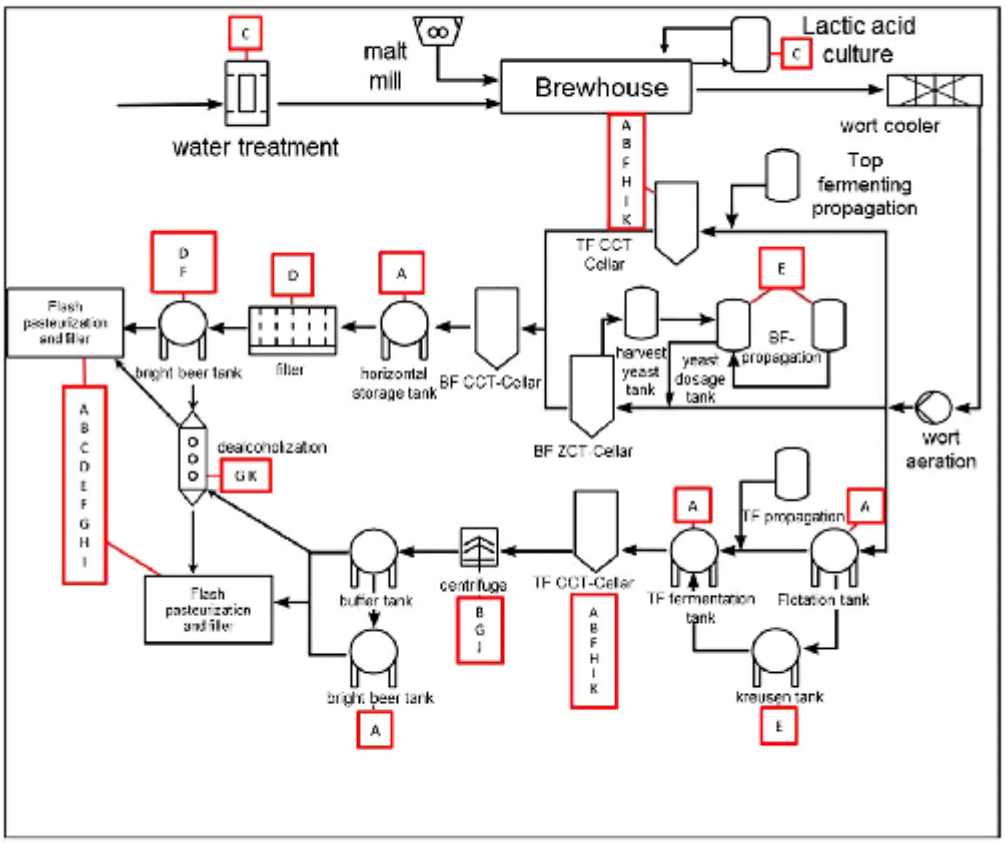


Figure 5 Fingerprint type distribution within the brewery and specific sampling points. [Colour figure can be viewed at wileyonlinelibrary.com]

Some isolates (LB2855, LB2143, LB2786 and LB2790) showed no increase in absorbance at 590 nm, indicating no growth. In contrast the biofilm formation in the same experiment tested positive. The biofilm formation was confirmed by microscopy. These findings indicate that there was no measurable growth, but cells that were inoculated in this experiment started adhering to the surface of the microtitre wells without growth. This effect seems to be a reaction of the tested isolate to the medium. Biofilm formation is also described as being a protective reaction to a hostile environment *(13)*. For this reason, the cell adherence may be a reaction to stress factors in the medium or a lack of essential nutrients. In the case of LB2855, the reaction may be triggered not only by hop compounds but also by osmotic stress, as the effect can be observed in beer as a nutrient as well as in a standard MRS medium, but not in diluted MRS.

In beer, only one isolate – LB2604 – grew significantly within 24 h. Depending on the beer attributes, the forcing test could take up to three months *(7)*. In conclusion, a negative result of the growth potential does not exclude beer spoiling potential.

In general, various *L. brevis* isolates are able to initially form biofilms in various media. Hence, depending on the isolate and substrate, biofilms can build up in different areas of breweries. Their initial creation is not necessarily dependent upon other species, a fact that has not been reported to date. Diluted beer, in particular, as it appears in between process cleaning steps and in the filler environment, seems to be a good medium for biofilm formation. The generally higher biofilm formation with higher nutrients as described by Sutherland *et al. (48)* could not been observed. Generating a growth potential and biofilm formation profile of isolated *L. brevis* isolates can help to identify points in the process that enable persistent biofilm formation (depending on the distribution of diluted process media throughout the process). It can also help to estimate the product spoilage potential. Combined with genetic fingerprinting, identities can be tracked through the process, isolating the most probable contamination source.

Contamination tracking of *L. brevis* fingerprint types in a German brewery

The field study resulted in 11 fingerprint types, or strain types which could be associated with specific sampling points. Each cluster was tracked throughout the process and could be found in

various stages of the brewing process. Nine of the fingerprint clusters could be found in samples before flash pasteurisation and filling. Figure 5 shows that most clusters, while found though the whole process, do not appear in an unbroken line from a specific sampling point in the process flow. There are various reasons for this phenomenon. Firstly, single critical sampling point control provides only a snapshot of the dynamic microbiological situation in the brewery. Secondly, it is very difficult to isolate mixed strains from single samples. Some strains might originally be in the collected samples but could not be isolated because they were overgrown by other species or strains in further processing. It is noteworthy that the appearance of fingerprint type C in the water treatment samples indicated a possible entry of microorganisms into the process through process water. Further inspection of the water treatment equipment showed shortcomings in construction. As this fingerprint type could also be found in the beer directly before flash pasteurisation, it is clearly possible for this fingerprint type to contaminate the product. Inadequate water treatment is a possible contamination source. The diversity of the identified fingerprint types indicates that the microbiological situation in the tested brewery is clearly not the result of a single microbiological contamination event. It is more likely that there are multiple sources of spoilage bacteria entering the process.

In general, the differentiation of *L. brevis* proved to be a valuable tool in hygiene monitoring and determining contamination sources. The high number of different genotypes and therefore potentially variant physiologies that could be found prove the high diversity of *L. brevis* in brewery isolates. Depending on the physiological properties (e.g. growth, slime formation and biofilm formation), it is essential to consider the spoilage potential of this species in terms of the various contributing factors, as well as the potential countermeasures.

Acknowledgements

The authors are very grateful to Mrs Jennifer Koob, Mrs Margit Grammer, Mrs Siglinde Huber and Mrs Osorio Lemus for supporting this project in the Laboratories of the Research Center Weihenstephan for Brewing and Food Quality.

Parts of this study were funded by the Wissenschaftsförderung der Deutschen Brauwirtschaft (Wifö) as part of the basic research project B104 Physiologische und genotypische Lactobacillus brevis-Stammtypisierung – verbesserte Kontaminationsaufklärung (Kurzbezeichnung: L. brevis-Fingerprint) under project management of Fritz Jacob and Mathias Hutzler. The funding source did not have any influence on the study design, the data collection, the analysis and interpretation, the report writing or article submission.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table 2: Publication 2, Supporting Information 1: Sample points field study process samples

department/room	Sample number	sampling point	Isolation method	microscopy	foodproof® Beer Screening
water treatment	600 day 1	permeate pressure pipe 1	NBB-Agar, pour plate	91 CFU short/long rods partly mobile	*Lactobacillus brevis*
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	600 day 2		NBB-A, pour plate	n.d.	n.a.
			MF, NBB-A	2 CFU cocci	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	601 day 1	permeate pressure pipe 2	NBB-A, pour plate	n.d.	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	601 day 2		NBB-A, pour plate	n.d.	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	602 day 1	permeate pressure pipe 3	NBB-A, pour plate	1 CFU short rods partly mobile	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	602 day 2		NBB-A, pour plate	n.d.	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	603 day 1	permeate pressure pipe 4	NBB-A, pour plate	1 CFU short rods partly mobile	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	603 day 2		NBB-A, pour plate	n.d.	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	1 CFU cocci, diplococci	
	604 day 1	overall permeate	NBB-A, pour plate	1 CFU short partly mobile	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	604 day 2		NBB-A, pour plate	1 CFU short rods	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	605 day 1	Brewing water	NBB-A, pour plate	n.d.	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	605 day 2		NBB-A, pour plate	n.d.	n.a.
			MF, NBB-A	n.d.	

			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	606 day 1	raw water after membrane filter (80 µm)	NBB-A, pour plate	n.d.	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	606 day 2		NBB-A, pour plate	n.d.	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	607 day 1	raw water after active coal filter	NBB-A, pour plate	n.d.	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	607 day 2		NBB-A, pour plate	1 CFU short rods	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	608 day 1	concentrate	NBB-A, pour plate	n.d.	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	608 day 2		NBB-A, pour plate	95 CFU short/long rods	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	609	stabilised raw water	NBB-A, pour plate	4 CFU short/long rods	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
city water (below grist mill)	620 day 1	water valve	NBB-A, pour plate	2 CFU short/long rods partly mobile	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+30 mg/l cycloheximide	n.d.	
	620 day 2		NBB-A, pour plate	1 CFU short/long rods	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+30 mg/l cycloheximide	n.d.	
	621 day 1	official control sampling point	NBB-A, pour plate	1 CFU short rods partly mobile	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+30 mg/l cycloheximide	n.d.	
	621 day 2		NBB-A, pour plate	uncountable CFU short/long rods	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+30 mg/l cycloheximide	n.d.	
biological acidification	200	tank 1	NBB-A, pour plate	uncountable CFU, *Lactobacillus* sp., yeasts	*Lactobacillus brevis*
			Low-hopped beer	n.d.	
			NBB-B	n.d.	
	201	tank 2	NBB-A, pour plate	uncountable CFU, yeasts,	n.d.

				short/long rods	
			Low-hopped beer	*Lactobacillus* sp. (culture), wild yeasts	
			NBB-B	*Lactobacillus* sp. (culture),	
	202	tank 3	NBB-A, pour plate	uncountable CFU wild yeasts	n.d.
			Low-hopped beer	*Lactobacillus* sp. (culture), wild yeasts	
			NBB-B	*Lactobacillus* sp. (culture),	
wort cooler	100	cold wort	NBB concentrate	n.d.	n.a.
wort aeration	101	cold wort	NBB concentrate	n.d.	n.a.
	701	wort aeration sterile air in Ringer's solution	NBB-A, pour plate	n.d.	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+30 mg/l cycloheximide	1 CFU cocci	
Top-fermenting fermentation tanks TF tanks)	365	TF tank 1	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	366	TF tank 2	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	367	TF tank 3	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	368	TF tank 4	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	369	TF tank 5	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	370	TF tank 6	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp.	*Lactobacillus brevis*
flotation tanks	105	wort at flotation TF tank 6	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	106	rinse water before flotation TF tank 6	Nutrient agar	1 CFU mold	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+30 mg/l cycloheximide	n.d.	
centrifugation cellar	300	centrifuge infeed	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	301	centrifuge outfeed	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	310	T350 (buffer tank)	NBB concentrate	*Lactobacillus* sp.	*Lactobacillus brevis*
	316	T349 (buffer tank)	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp.	a) *Lactobacillus brevis,* b) *Lactobacillus brevis*
	204	open vat, removed yeast	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	some short rods	*Lactobacillus casei*
			NBB-B	some short rods	a) Mix *Lactobacillus brevis* + *Lactobacillus casei*, b) *Lactobacillus casei*
	631	centrifuge outfeed, thin valve, rinsing before CIP	Nutrient agar, pour plate	ca. 900 CFU short rods	n.a.
			MF, NBB-A	ca. 450 CFU, lactic acid bacteria (non beer spoiling)	*Lactobacillus brevis*
			MF, MRS-A+15 mg/l cycloheximide	ca. 600 CFU *Lactobacillus* sp.	a) *Lactobacillus brevis* b)*Lactobacillus brevis*
	632	centrifuge outfeed, thick valve/yeast outfeed, rinsing before CIP	Nutrient-agar, pour plate	uncountable CFU, short rods partly mobile	n.a.
			MF, NBB-A	short rods, overgrown with yeasts	n.a.
			MF, MRS-A+15 mg/l cycloheximide	uncountable CFU lactic acid bacteria, yeasts	a) *Lactobacillus brevis* b)*Lactobacillus brevis*
	633	centrifuge outfeed, thin valve, bottom,	Nutrient-agar, pour plate	n.d.	n.a.
			MF, NBB-A	n.d.	

		rinsing before CIP	MF, MRS-A+15 mg/l cycloheximide	n.d.	
	659	centrifuge outfeed, thin valve, rinsing after CIP	Nutrient agar	approx..2700 CFU short rods partly mobile	n.a.
			MF, NBB-A	uncountable CFU *Lactobacillus* sp.	a) *Lactobacillus brevis* b) *Lactobacillus brevis*
			MF, MRS-A+15 mg/l cycloheximide	uncountable CFU *Lactobacillus* sp., yeasts	a) *Lactobacillus brevis* b) *Lactobacillus brevis*
	658	centrifuge outfeed, thick valve/yeast outfeed, rinsing after CIP	Nutrient agar	approx. 200 CFU short rods partly mobile	n.a.
			MF, NBB-A	approx. 450 CFU, lactic acid bacteria, (non-beer-spoiling)	
			MF, MRS-A+15 mg/l cycloheximide	approx. 300 CFU, *Lactobacillus* sp., yeast	*Lactobacillus brevis*
	657	centrifuge outfeed, thin valve, bottom, rinsing after CIP	Nutrient agar	1 CFU short rods	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
horizontal maturation tanks	311	T172	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	312	T173	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	313	T174	NBB concentrate	*Lactobacillus* sp	a) *Lactobacillus brevis* b) *Lactobacillus casei*
	314	T175	NBB concentrate	n.d.	n.a.
	315	T176	NBB concentrate	n.d.	n.a.
wheat beer cellar	320	CKT 1	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus brevis*
	321	CKT 2	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus brevis*
	322	CKT 3	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	323	CKT 4	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus casei*
	324	CKT 5	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus brevis*
	325	CKT 6	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	326	CKT 7	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus brevis*
	327	CKT 8	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	328	CKT 9	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus brevis*
	329	CKT 10	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	330	CKT 11	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus brevis*
	331	CKT 12	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	332	CKT 13	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	333	CKT 14	NBB concentrate	*Lactobacillus* sp	*Lactobacillus brevis*
	334	CKT 15	NBB concentrate	n.d.	n.a.
	335	CKT 16	NBB concentrate	n.d.	n.a.
	336	CKT 17	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus harbinensis*
	337	CKT 18	MRS 2x+Cycloheximide +	*Lactobacillus* sp	*Lactobacillus harbinensis*

			NBB-P-C 5x		
	338	CKT 19	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus brevis*
	339	CKT 20	MRS 2x+Cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus brevis*
bottom-fermenting cellar (BF)	345	CKT 21	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	346	CKT 22	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	347	CKT 23	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	348	CKT 24	MRS 2x+Cycloheximide + NBB-P-C 5x	n.d.	n.a.
	349	CKT 25	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus backii*
	350	CKT 26	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	some short rods, *Lactobacillus* sp.	*Lactobacillus backii*
	351	CKT 27	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	some short rods, *Lactobacillus* sp.	*Lactobacillus backii*
	352	CKT 28	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	mold, *Lactobacillus* sp	*Lactobacillus casei*
	353	CKT 29	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	354	CKT 30	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	short rods	*Lactobacillus casei*
	355	CKT 31	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	short rods, *Lactobacillus* sp	Mix a) *Lactobacillus backii*, b*) Lactobacillus casei*
	356	CKT 32	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus casei*
	357	CKT 33	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus casei*
	358	CKT 34	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	359	CKT 35	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus casei*
dealcoholisation	373	buffer tank 4	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus brevis*
	374	buffer tank 1	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	not culturable
	375	T-valve before dealcoholisation	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus brevis*
	376	T-valve after dealcoholisation	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus brevis*
	377	buffer tank 2	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus brevis*
	378	buffer tank 3	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus brevis*
bright beer tanks	380	bright beer tank 1	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	a) *Lactobacillus casei* ,b)*Lactobacillus brevis*
	381	bright beer tank 2	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp	*Lactobacillus brevis*
	382	bright beer tank 3	MF, NBB-A	*Lactobacillus* sp	*Lactobacillus brevis*
			MF, MRS-A+15 mg/l cycloheximide	uncountable short/long rods	n.a.
	383	bright beer tank 4	MF, NBB-A	8 CFU yeasts	n.a.
			MF, MRS-A+15 mg/l cycloheximide	ca. 480 CFU yeasts	n.a.
	384	bright beer tank 5	MF, NBB-A	39 CFU Lactobacilli	n.a.
			MF, MRS-A+15 mg/l cycloheximide	no result	n.a.

	385	bright beer tank 6	MF, NBB-A	3 CFU *Lactobacillus* sp n	*Lactobacillus brevis*
			MF, MRS-A+15 mg/l cycloheximide	1 CFU *Lactobacillus* sp	*Lactobacillus brevis*
	386	bright beer tank 7	MF, NBB-A	uncountable CFU short rods	not culturable
			MF, MRS-A+15 mg/l cycloheximide	approx. 300 CFU short rods	n.a.
sheet filter	390	before filter (left bottom)	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
	391	before filter (right bottom)	MF, NBB-A	n.d.	n.a.
			MF, MRS-A+15 mg/l cycloheximide	n.d.	n.a.
	392	before filter (top)	MF, NBB-A	n.d.	n.a.
			MF, MRS-A+15 mg/l cycloheximide	n.d.	n.a.
	400	after filter (direct after filtration)	MF, NBB-A	n.d.	n.a.
			MF, MRS-A+15 mg/l cycloheximide	1 CFU lactic acid bacteria (non-beer-spoiling)	n.a.
	401	after filter (valve at bright beer tanks)	MF, NBB-A	n.d.	n.a.
			MF, MRS-A+15 mg/l cycloheximide	n.d.	n.a.
	402	filter middle	MF, NBB-A	n.d.	n.a.
			MF, MRS-A+15 mg/l cycloheximide	n.d.	n.a.
kieselguhr	K1	freshly opened bag	Low-hopped beer, isolation culture on NBB-A	short rods	*Lactobacillus brevis*
	K2		Low-hopped beer, isolation culture on NBB-A	n.d.	n.a.
	K3		Low-hopped beer, isolation culture on NBB-A	n.d.	n.a.
harvest yeast tanks HYT	205	HY-tank 2	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
			NBB concentrate	n.d.	n.a.
	206	HY-tank 1	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
			NBB concentrate	n.d.	n.a.
bottom fermenting pure culture cellar	210	BF propagation tank 1	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	*Lactobacillus* sp.	*Lactobacillus brevis*
			NBB concentrate	*Lactobacillus* sp.	*Lactobacillus brevis*
	211	BF propagation tank 2	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
			NBB concentrate	n.d.	n.a.
	212	yeast dosage tank	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	short/long rods	*Lactobacillus brevis*
			NBB concentrate	n.d.	n.a.
top fermentation propagation (TF)	220	TF propagation tank 1	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
			NBB concentrate	n.d.	n.a.
	221	TF propagation tank 2	MRS 2x+30 mg/l cycloheximide + NBB-P-C 5x	n.d.	n.a.
			NBB concentrate	n.d.	n.a.
central aeration	710	CO_2 valve before filter in Ringer's solution	nutrient agar	n.d.	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	

	712	sterile air valve in Ringer's solution	nutrient agar	n.d.	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
CIP top-fermenting cellar	635	rinse water tank	Low-hopped beer	yeasts	n.a.
	636	fresh water tank	nutrient agar	n.d.	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	810	acid tank	Low-hopped beer	n.d.	n.a.
	811	caustic tank	Low-hopped beer	n.d.	n.a.
CIP-brewhouse	625	fresh water tank	nutrient agar	n.d.	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	626	rinse water tank	nutrient agar	n.d.	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	uncountable CFU, long rods	
	800	acid tank	Low-hopped beer	n.d.	n.a.
CIP cellar/ CIP5	627	fresh water tank	nutrient agar	n.d.	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	628	rinse water tank	nutrient-agar	38 CFU yeasts	n.a.
			MF, NBB-A	approx. 900 CFU yeasts	
			MF, MRS-A+15 mg/l cycloheximide	uncountable CFU yeasts, 21 molds	
	804	mixed water tank	Low-hopped beer	n.d.	n.a.
	805	acid tank	Low-hopped beer	n.d.	n.a.
CIP bright beer tanks	640	fresh water tank	nutrient agar	4 CFU short rods, yeasts	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	641	rinse water tank	nutrient agar	26 molds	n.a.
			MF, NBB-A	n.d.	
			MF, MRS-A+15 mg/l cycloheximide	n.d.	
	821	acid tank	Low-hopped beer	n.d.	n.a.
	822	acid tank	Low-hopped beer	n.d.	n.a.

Table 3: Publication 2, Supporting Information 2: Sampling points field study swab samples on NBB-B-Am

sample number.	department/room	sample point	acidification in NBB-B-Am	microscopy	isolation culture on NBB-A	foodproof® Beer Screening
901	bright beer tanks	cold water valve	yes	short rods, cocci	a) short rods b) cocci	a) *Lactobacillus casei*, b) mix of *Lactobacillus brevis* and *Lactobacillus harbinensis*
902	bright beer tanks	CO_2-outfeed valve	yes	short rods	short rods	*Lactobacillus brevis*
910	centrifuge cellar	buffer tank 350 T-valve	yes	short/long rods	short rods	*Lactobacillus casei*
911	centrifuge cellar	centrifuge CO_2	yes	n.d.	n.d.	n.a.
941	centrifuge cellar	buffer tank 349 outfeed	yes	long rods	long rods	*Lactobacillus brevis*
912	central aeration	CO_2 after filter	no	n.d.	n.d.	n.a.
915	dealcoholisation	ventilation at carbonisation unit tank 3	no	n.d.	n.d.	n.a.
920	top fermentation propagation	aeration, pressurised air before filter	no	n.d.	n.d.	n.a.
921	top fermentation propagation	aeration, pressurised air after filter	no, sedimentation	some yeasts	n.d.	n.a.
940	centrifuge cellar	buffer tank 349 inside	no	n.d.	n.d.	n.a.
955	horizontal maturation tank	blending apparatus inside, supernatant beer	no	n.d.	n.d.	n.a.
956	horizontal maturation tank	blending apparatus inside, supernatant beer	no	n.d.	n.d.	n.a.
957	horizontal maturation tank	blending apparatus infeed valve	no	n.d.	n.d.	n.a.
961	harvested yeast tank	sample 1,top inspection glass	no	n.d.	n.d.	n.a.
963	harvested yeast tank	manhole cover bottom	yes	yeasts, short/long rods	short rods	*Lactobacillus casei*
965	harvested yeast tank	deflector plate	no, sedimentation	cocci	n.d.	n.a.
966	harvested yeast tank	sample 2,top inspection glass	no, sedimentation	short rods	short rods	*Lactobacillus brevis*
967	harvested yeast tank	tank inside, seal of front inspection glass	no	n.d.	n.d.	n.a.
969	harvested yeast tank	tank inside, bulge wall	no, sedimentation	n.d.	n.d.	n.a.
970	harvested yeast tank	tank inside, bottom outfeed	no, sedimentation	n.d.	cocci	n.d.
971	harvested yeast tank	manhole, inside tank wall	no, sedimentation	short rods	short rods	*Lactobacillus casei*
973	harvested yeast tank	tank inside, deposit behind cleansing jet	no, sedimentation	long rods	n.d.	n.a.
962	flotation tank	tank inside bottom	yes	short rods	n.d.	n.a.
964	flotation tank	bottom manhole under seal	yes	short/long rods	short rods	Mix of *Lactobacillus brevis* and *Lactobacillus casei*
968	flotation tank	seal top inspection glass	no, sedimentation	short rods	n.d.	n.a.
972	flotation tank	overflow	no, sedimentation	short rods	n.d.	n.a.
974	flotation tank	tank inside wall, left of bulge	no, sedimentation	cocci	n.d.	n.a.
975	flotation tank	tank inside wall, right of bulge	no, sedimentation	short rods	n.d.	n.a.
978	flotation tank	tank inside wall at rough spot	no	n.d.	n.d.	n.a.
980	flotation tank	tank inside wall middle	no	n.d.	n.d.	n.a.
981	flotation tank	tank inside wall, welding seam before manhole	yes	long rods	long/short rods	*Lactobacillus brevis*

Table 4: Publication 2, Supporting Information 3: Sampling points field study room air samples

Sample number	Sampling site	Sampling time	Acidification	**foodproof® Beer Screening**
P1 D	wort aeration	sampling day 1	no	*Lactobacillus brevis*
P2 N	wheat beer maturation cellar	sampling day 1	yes	*Lactobacillus brevis*
P2 D a)		sampling day 1	no	*Lactobacillus brevis*
P2 D b)		sampling day 1	no	*Lactobacillus brevis*
P3 D a)	filtration cellar	sampling day 2	no	*Lactobacillus brevis*
P3 D b)		sampling day 2	no	*Lactobacillus brevis*
P3 D c)		sampling day 2	no	*Lactobacillus brevis*
P4 N a)	top-fermenting propagation cellar	sampling day 2	yes	*Lactobacillus brevis*
P4 N b)		sampling day 2	yes	*Lactobacillus brevis*
P4 D a)		sampling day 2	no	*Lactobacillus brevis*
P4 D b)		sampling day 2	yes	*Lactobacillus brevis*
P6 D a)		sampling day 2	no	*Lactobacillus brevis*
P6 D b)		sampling day 2	no	*Lactobacillus brevis*

2.4. Characterization and validation of yeast related biofilms in brewing environment with combined cultivation and specific real-time PCR screening of selected indicator species

Abstract

Microbial spoilage of alcohol-free and low-alcohol beers, beer-mixed beverages, and soft drinks is most commonly caused by yeast. Yeast-related biofilms are therefore a serious problem in the production of these beverages. Fast detection of developing biofilms is a key factor to prevent subsequent spoilage of the product. For fast yeast detection, a new specific medium was developed and combined with real-time polymerase chain reaction (PCR) detection of characteristic beverage-spoiling yeast species. The medium is based on MYPG broth (malt extract, yeast extract, peptone, glucose broth) with resazurin as a redox indicator for cell activity. The growth and biofilm potential of representative strains of commonly present beverage-spoilage yeast species was evaluated using the developed medium. A novel real-time PCR detection system for *Rhodotorula mucilaginosa*, an early biofilm coloniser, was designed and successfully validated. Two field tests of the medium in combination with real-time PCR were performed. One test showed a differentiated hygienic status on a filler, and the other test tracked the contamination source of *Saccharomyces cerevisiae* var. *diastaticus*. The biofilm relevance of the strain set was proven. The modified MYPG proved to be highly sensitive when detecting yeasts. The detection of the selected target species directly in the medium was compatible and can provide detailed hygienic profiles when combined with additional information on the target species. This provides a fast detection method for yeast-related biofilms in brewery environments, differentiated hygienic monitoring, and makes it possible to troubleshoot contamination incidents.

Authors/Authorship contribution:

Riedl R.: Literature search, writing, data creation, study conception and design; **J. Fütterer:** Data analysis and interpretation (culture medium validation), **Goderbauer P.:** Data analysis and interpretation (Biofilm formation), critical review of draft; **Michel M.:** critical content review; **Jacob F.:** Supervised the project; **Hutzler M.:** Creation of the research plan, strain isolation, critical content review

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

ISSN: 0361-0470 (Print) 1943-7854 (Online) Journal homepage: https://www.tandfonline.com/loi/ujbc20

Combined Yeast Biofilm Screening – Characterization and Validation of Yeast Related Biofilms in a Brewing Environment with Combined Cultivation and Specific Real-time PCR Screening of Selected Indicator Species

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To cite this article: R. Riedl, J. Fütterer, P. Goderbauer, M. Michel, F. Jacob & M. Hutzler (2019) Combined Yeast Biofilm Screening – Characterization and Validation of Yeast Related Biofilms in a Brewing Environment with Combined Cultivation and Specific Real-time PCR Screening of Selected Indicator Species, Journal of the American Society of Brewing Chemists, 77:2, 99-112, DOI: 10.1080/03610470.2019.1579036

To link to this article: https://doi.org/10.1080/03610470.2019.1579036

JOURNAL OF THE AMERICAN SOCIETY OF BREWING CHEMISTS
2019, VOL. 77, NO. 2, 99–112
https://doi.org/10.1080/03610470.2019.1579036

Combined Yeast Biofilm Screening – Characterization and Validation of Yeast Related Biofilms in a Brewing Environment with Combined Cultivation and Specific Real-time PCR Screening of Selected Indicator Species

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ABSTRACT

Microbial spoilage of alcohol-free and low-alcohol beers, beer-mixed beverages, and soft drinks is most commonly caused by yeast. Yeast-related biofilms are therefore a serious problem in the production of these beverages. Fast detection of developing biofilms is a key factor to prevent subsequent spoilage of the product. For fast yeast detection, a new specific medium was developed and combined with real-time polymerase chain reaction (PCR) detection of characteristic beverage spoiling yeast species. The medium is based on MYPG broth (malt extract, yeast extract, peptone, glucose broth) with resazurin as a redox indicator for cell activity. The growth and biofilm potential of representative strains of commonly present beverage spoilage yeast species was evaluated using the developed medium. A novel real-time PCR detection system for *Rhodotorula mucilaginosa*, an early biofilm colonizer, was designed and successfully validated. Two field tests of the medium in combination with real-time PCR were performed. One test showed a differentiated hygienic status on a filler, and the other test tracked the contamination source of *Saccharomyces cerevisiae* var. *diastaticus*. Biofilm relevance of the strain set was proven. The modified MYPG proved to be highly sensitive when detecting yeasts. The detection of the selected target species directly in the medium was compatible and can provide detailed hygienic profiles when combined with additional information on the target species. This provides a fast detection method for yeast-related biofilms in brewery environments, differentiated hygienic monitoring, and makes it possible to troubleshoot contamination incidents.

KEYWORDS

Beer; beverage; biofilm; hygienic monitoring; TaqMan real-time PCR; yeast

Introduction

More than 50% of reported microbiological incidents in breweries are associated with secondary contamination during the filling process (e.g., by ambient air, machines, or drops of condensate, and not by input via raw material).[1–5] Since microbiological problems in the filling area do not occur spontaneously but develop over time due to poor hygiene, the hygienic status of brewery filling equipment is subsequently monitored. This action is essential to detect hygienic problems before product-spoiling organisms can settle and start growing as biofilms.[1–4,6] While it is believed that bacteria attach to a surface within minutes, the growth of true biofilms takes much longer.[1,7] Their development is described as a rather complex process with different dominant species over time.[2,6]

The growth of beer-spoilage bacteria in brewery-related biofilms is often related to the later growth steps of biofilms.[6] The greatest priority in hygienic monitoring is to detect organisms that indicate biofilm growth before spoilage organisms can colonize the biofilm. The most important groups detected are aerobic and microaerophilic genera such as *Acetobacter* sp., *Gluconobacter* sp. or other acid-forming bacteria, which are detected via pH color indicators in growth media for hygienic monitoring.[3,4] Most beer-spoilage organisms are microaerophilic or anaerobic bacteria such as *Lactobacillus* spp., *Pediococcus* spp., *Pectinatus* spp., and *Megasphaera* spp., the media used to detect beer-spoilage organisms are optimized for those genera.[3,4,8] Earlier studies showed yeasts as a relevant group in mature biofilms found in the filling department.[9,10] In biofilm development in breweries, yeasts are described as late biofilm colonizers in this model, which may differ according to the substrate.[2–4,6] It was observed that yeasts can create biofilms initially on surfaces, or colonize existing fungal or bacterial biofilms.[11] Yeast biofilm development is similar to that of bacteria biofilms, but it is more complex since some yeasts are able to form pseudohyphae during maturation.[11]

In general, yeasts have a high impact in the beverage industry as starter cultures in the fermentation process and as spoilage organisms in beer, alcohol-free beverages, and beer-mixed beverages. In fruit-based beverages in particular, yeasts cause 90% of spoilage cases.[5,12]

Over the past few decades, the beer market has changed and beer-mixed and alcohol-free beverages are becoming

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more popular worldwide.[13,14] The change in the beer market to a higher variety of low-alcohol and alcohol-free beer types, as well as beer-mixed beverages with compounds that contain sugar, which are produced in a brewery environment, is making it necessary to detect and monitor yeasts in the filling process of breweries. In beverages with a low pH-value, bacteria are not as relevant spoilage organisms as yeasts.[3,4,15] Additionally, the presence of sugar or sweeteners promotes the attachment of first-stage biofilm colonizers.[10]

Most bacteria described in this model of biofilm development cannot grow in beverages with a low pH-value and therefore cannot be used as indicator organisms for biofilm formation in alcohol-free beverages.[3,4] Monitoring biofilm-forming and biofilm-inhabiting yeasts is therefore the most promising solution.

With the exception of super-attenuating yeasts, such as *Saccharomyces cerevisiae* var. *diastaticus* and *Brettanomyces/Dekkera* spp., product spoilage of beer by yeasts is not common in the brewing industry. Secondary contaminations, especially in the filling process from machines, aerosols, or drops of condensate, are rather rare. Most process steps do not provide the ideal growth conditions (nutrients, oxygen, temperature) for wild yeasts, which are therefore not significant competitors to the highly adapted brewing yeast pitched in greater cell numbers. Spoilage by other species than the mentioned *S. cerevisiae* var. *diastaticus* and *Brettanomyces/Dekkera* spp. is mostly connected with severe production errors (for example contaminated or physiological compromised pitching yeast), resulting in higher concentrations of oxygen or fermentable sugars.[3,4] Nevertheless, yeast can be found in biofilms, isolated from filling equipment in breweries, where they are described as a relevant part of the biofilm, providing growth factors for lactic acid bacteria, therefore making yeasts important hygiene indicator organisms.[1–4,6,9,16,17]

The species *S. cerevisiae* and *Wickerhamomyces anomalus* in particular are described as being prevalent in brewery-associated biofilms. *W. anomalus* is considered to be an early biofilm-colonizing species, with initial biofilm-building potential, and *S. cerevisiae* is considered to be a late colonizer.[9] *S. pastorianus* var. *carlsbergensis* TUM 34/70 and *S. cerevisiae* TUM 68, employed for lager beer types and wheat beers, respectively, are the two strains ordered most frequently from the Yeast Center of the Research Center Weihenstephan for Brewing and Food Quality by breweries worldwide (unpublished data). They were, therefore, selected as model organisms for the *Saccharomyces* brewing strain colonization of biofilms. *Saccharomyces* spp., as predominant culture yeast, make up a high percentage of the microbial cell mass in breweries, but also have relevance in biofilm formation. Bottom-fermenting yeasts, such as the selected strain *S. pastorianus* var. *carlsbergensis* TUM 34/70, are described as being able to form biofilms.[18] Strains of *S. cerevisiae* that are used in rice wine fermentation are described as being able to form biofilms as a monoculture, in a medium with metabolic products of some lactic acid bacteria, or in direct cell-cell contact with those bacteria. These are also found in the vicinity of the brewing and filling areas (e.g., *Leuconostoc mesenteroides* and *Lactococcus lactis*) are also known for their biofilm-forming potential.[19,20] Baker's strains of this species were also reported to form biofilms.[21]

Brewing strains of *S. cerevisiae* are described as rarely forming biofilms. They cannot attach to inert surfaces, but can attach in the presence of biopolymers from auxiliary finings.[22] These can also be present in extracellular polymeric substances (EPS) of biofilm-forming bacteria, such as many strains of *Pseudomonas aeruginosa* (e.g., alginate and other adhesins).[23–25] Starved cells of *S. cerevisiae* also tend to attach to surfaces due to stronger Flo11 expression, which is one of the most described adhesion genes in yeasts.[26] Also, the effect of the biofilm formation of starved cells was observed to be stronger under the influence of alginates.[22] EPS-producing bacteria such as *Pseudomonas* sp., *L. mesenteroides* and *L. lactis* can be found in the filling area in biofilms.[3,4,27] This makes contact with starved *S. cerevisiae* cells from the brewing process, or in the case of EPS of environmental origin such as alginates, plausible. Therefore, biofilms can potentially be colonized by brewing yeasts. Additionally the beer-spoiling variant of *S. cerevisiae*, *S. cerevisiae* var. *diastaticus* is described as a strong biofilm former in beer and sucrose wort.[1,26] *W. anomalus* was reported to be the most important yeast species in the context of biofilm formation in breweries.[9,10] This species is often found as an ubiquitous organism throughout the brewing area.[3–5,28,29] *Dekkera anomala* was reported to form biofilms using beer and wort sucrose broth, with strain-dependent biofilm production.[1] It is one of the most commonly found yeasts connected with product spoilage, and it is resistant to many cleaning and disinfecting solutions, as well as antibiotics.[5,28–30] *Rhodotorula spp.* were found to colonize biofilms in the filling area, especially in the conveyor system near the filler.[1,17] *R. mucilaginosa* can form initial biofilms on surfaces.[11]

Due to their dominance in most breweries and their relevance for yeast biofilms the aforementioned species *D. anomala*, *R. mucilaginosa*, *S. pastorianus* var. *carlsbergensis* TUM 34/70, *S. cerevisiae* TUM 68, *S. cerevisiae* var. *diastaticus*, and *W. anomalus* were selected as test organisms for a novel method to detect yeast biofilms in a combination of enrichment culture and real-time polymerase chain reaction (PCR) identification of the selected species. In the beverage industry, swab samples are widely used for hygienic monitoring, with samples taken from hygienically relevant positions on the equipment. For enrichment, media are used that contain acid indicator dyes to make it easier to detect acid-producing bacteria.[3,6] Since yeasts grow slower than bacteria, this results in a slower detectable pH drop, in comparison to a bacteria targeting medium.

Resazurin is described as a highly sensitive redox indicator of cell activity in culture media, and it can be measured using either colorimetry or fluorimetry, with the latter being the more sensitive method. The oxidized resazurin (blue) is reduced enzymatically by living cells, in two steps, to the fluorescent active resorufin (pink, fluorescent) and to dihydroresorufin. The reduction reaction correlates strongly with

the cell number and is already used to detect microbiological contamination in milk. Further studies have used resazurin as a growth indicator, and they did not show any negative effect on cell growth, which enables the use of cultured cells for further analysis.[31] To improve the detection of yeasts in the brewing environment, a medium based on MYPG was developed. It indicates microbial activity using the redox indicator dye resazurin. In this manner, living yeast cells can be detected independently of pH with a short incubation time and high sensitivity, over the color change from blue to pink/colorless. The modified medium was tested for correlation between detection of cell number and incubation time until the color changed. The results were validated in the laboratory and in field sampling and were compared with common hygienic monitoring media using bacteria as the main target fraction (e.g., NBB-B-AM). To prove the relevance of the strains selected to determine the hygienic status of the brewery environment, the biofilm-forming potential of each strain was tested in MYPG. The specific detection of selected indicator species makes it possible to validate the results of samples that were positive with yeast growth according to the risk level, hygienic status, and product relevance of the sampled position.

For the specific detection and identification of the selected indicator yeasts, real-time PCR assays were used as published for *W. anomalus*, *S. cerevisiae* TUM 68,[28] *S. pastorianus* TUM 34/70,[28,29,32] *D. anomala*,[32] and *S. cerevisiae* var. *diastaticus*.[32,33] TaqMan™ based real-time PCR systems were used as published by Hutzler et al.[28,29] The real-time PCR system for *R. mucilaginosa* was newly developed and validated for this study. The *R. mucilaginosa* real-time PCR system was validated using a strain collection of 108 exemplary strains of typical brewery species. To better interpret the detection results with the specified test species, the initial biofilm-formation potential was tested for a representative strain set of brewery isolates.

The aim of this study was to design a fast detection method for yeasts and yeast biofilms, using a combination of non-specific enrichment culture and specific real-time PCR identification, for the in-depth hygienic monitoring of yeast biofilms on brewing equipment.

Experimental

Modified MYPG broth

The modified medium based on MYPG Universal Yeast Medium was created as a solution of glucose monohydrate (10.58 mg mL^{-1}), yeast extract (2.89 mg mL^{-1}), malt extract (2.89 mg mL^{-1}), and meat-peptone (4.81 mg mL^{-1}) in double-distilled water. To adjust the solution to pH 6.2, 1.0 mL HCl (1 mol l^{-1}) was used, after autoclaving at 121 °C for 15 min. Tetracycline (0.048 µg mL^{-1}) and resazurin (0.019 µg mL^{-1}) were added sterile membrane filtrated (0.2 µm) and presoluted in double distilled water after autoclaving.

Growth control and biofilm-formation potential in stationary phase in modified MYPG

For growth control and to test the biofilm-formation potential of the used microorganisms (Table 1), an adapted test design according to Kolari et al.[34] and modified by Timke et al.[9] in a 96-well microtiter format was used, adapted from a previous study.[35] This test was selected as it is described as being straightforward and useful for quantifying a broad range of microbial biofilms, with the exception of *P. aeruginosa* biofilms.[36] The test was carried out in sterile, black, flat-bottomed polystyrene microtiter 96-well plates, with polymeric optical bottoms for fluorescence applications (Thermo Fisher Scientific, Rochester, NY, U.S.A.). Each well was filled with 250 µl of MYPG broth (malt-yeast-extract-peptone-glucose-broth) with 0.02 µg mL^{-1} of resazurin as a redox indicator for growth. Resazurin is reduced in the presence of cell activity to pink fluorescent resorufin and can be measured fluorometrically at 530-nm extinction and 590-nm emission. Overnight cultures of the test strains were washed, by suspending and centrifuging them in sterile distilled water and adjusted to 10^{-6} cells mL^{-1}. The filled wells were incubated with 2.5 µL of the washed and adjusted cultures with 10^{-6} cells mL^{-1}. Afterward, the microtiter plates were sealed with gas-permeable foil and aerobically incubated at 28 °C, without disturbance, for 24 h. The fluorescence was measured at 530-nm extinction and 590-nm emission (Multi-Detection-Reader Synergy 2, BioTek, Bad Friedrichshall, Germany) directly after sealing the plates, and after incubation, as an indirect growth control. For measuring the biofilm formation potential, the plates were emptied and rinsed afterward, using sterile distilled water. Crystal violet solution, 300 µL, (4 g L^{-1} in 96% vol ethanol) was added to stain residing cells (biofilm) for 5 min at room temperature. All wells were emptied and rinsed 3 times using 400 µL of sterile distilled water. The remaining crystal violet, which was still bound to the cells, was dissolved in 300 µL 96% vol. ethanol at 10 °C overnight and at A_{590nm} was measured using the Synergy 2 Multi-detection reader. Means were calculated using four independent measurements of four biological replicates per strain and normalized against the mean of four independent measurements of four blank samples. The ratings and categorizations of growth and biofilm formation were modified according to Diaz et al.[37] (Table 1). All wells were counter-checked and documented microscopically for adherent cells and trub particles, using a Nikon inverted research microscope Eclipse Ti-E, using a CFI S P-Fluor ELWD ADM 60x C air immersion objective for phase contrast microscopy.

Semi-quantitative validation of the modified MYPG broth validation as a hygienic monitoring medium

Real biofilms are difficult to grow quantitatively and reproducibly, therefore surface dried vital liquid cultures, with defined cell counts per cm^2, were used as biofilm equivalents. For semiquantitative validation, yeast biofilm equivalents, with defined cell counts, were created by spreading 552 µL (an empirically determined volume to completely

Table 1. Growth control and biofilm-formation potential of selected culture yeasts, hygienic indicator yeasts, and obligate spoilage yeasts, in modified MYPG in microtiter format.

		Growth control			Biofilm-forming potential		
Species	Test strain	$\bar{FU}_{Ex530/Em590} - \bar{SD}_{Ex530/EM590}$	Growth detection via resorufin fluorescence[a]	Observable microscopic cell material	$\bar{E}_{590} - \bar{SD}_{590}$	Biofilm-formation potential[b]	Observable microscopic cell adhesion to surface
Dekkera anomala	TUM YP 130610010-3585	2.45E+03	+	yes	0.95	–	no
Dekkera anomala	TUM YP 111219038-3106	2.25E+03	+	yes	1.00	–	no
Rhodotorula mucilaginosa	TUM YP 120806011-7159	4.58E+03	++	yes	2.06	+	yes
Rhodotorula mucilaginosa	TUM YP 111221000-7045	4.26E+03	++	yes	1.51	+	yes
Saccharomyces cerevisiae	TUM 68	3.35E+03	+	yes	1.41	+	yes
Saccharomyces cerevisiae var. *diastaticus*	TUM YP 121113002-1248	(6.30E+02)[c]	+++[c]	yes	2.92	++	yes
Saccharomyces cerevisiae var. *diastaticus*	FZW BLQ B-2-1	(9.94E+02)[c]	+++[c]	yes	1.87	+	yes
Saccharomyces pastorianus var. *carlsbergensis*	TUM 34/70	3.52E+03	+	yes	1.47	+	yes
Wickerhamomyces anomalus	TUM YP 111215057-2047	3.25E+03	+	yes	3.10	++	yes
Wickerhamomyces anomalus	TUM YP 111221000-2042	3.08E+03	+	yes	3.20	++	yes

MYPG: malt extract, yeast extract, peptone, glucose broth; $\bar{FU}_{Ex530/Em590}$: arithmetic mean of four independent fluorescence measures of four independent samples (530 nm extinction, 590 nm emission) after incubation; $\bar{SD}_{Ex530/EM590}$: standard deviation of four independent fluorescence measures of four independent samples (530 nm extinction, 590 nm emission) after incubation; $\bar{E}_{590}$: arithmetic mean of four independent spectral photometric measures of four independent samples (590 nm extinction) after incubation; $\bar{SD}_{590}$: standard deviation of four independent spectral photometric measures of four independent samples (590 nm extinction) after incubation; $FU\ \bar{Blank}_{Ex530/Em590}$: arithmetic mean of four independent fluorescence measures of four independent blank samples (530 nm extinction, 590 nm emission); $SD\ \bar{Blank}_{Ex530/EM590}$: standard deviation of four independent fluorescence measures of four independent blank samples (530 nm extinction, 590 nm emission) after incubation; $E\ \bar{Blank}_{590}$: arithmetic mean of four independent spectral photometric measures of four independent blank samples (590 nm extinction) after incubation; $SD\ \bar{Blank}_{590}$: standard deviation of four independent spectral photometric measures of four independent blank samples (590 nm extinction) after incubation.

[a]Growth in MYPG with resazurin, detected with resazurin reduction, measured fluorometrically (extinction 530 nm/emission 590 nm).

–: no growth $\bar{FU}_{Ex530/Em590} - \bar{SD}_{Ex530/Em590} < FU_{Ex530/Em590}\ cutoff$.

+: weak growth $FU_{Ex530/Em590}\ cutoff < \bar{E}_{590} - \bar{SD}_{Ex530/Em590} < 2{*}E_{590}\ cutoff$.

++: moderate growth $2{*}FU_{Ex530/Em590}\ cutoff < \bar{FU}_{Ex530/Em590} - \bar{SD}_{Ex530/EM590} < 4{*}FU_{Ex530/Em590}\ cutoff$.

+++: strong growth $\bar{FU}_{Ex530/Em590} - \bar{SD}_{Ex530/EM590} > 4{*}FU_{Ex530/Em590}\ cutoff$.

Where: $FU_{Ex530/Em590}\ cutoff = FU\ \bar{Blank}_{Ex530/Em590} - 3{*}SD\ \bar{Blank}_{Ex530/Em590} =$ 1793.71 FU.

[b]Biofilm formation modified for yeasts from Diaz et al. [37].

–: no biofilm formation $\bar{E}_{590} - \bar{SD}_{590} < E_{590}\ cutoff$.

+: weak biofilm formation $E_{590}\ cutoff < \bar{E}_{590} - \bar{SD}_{590} < 2{*}E_{590}\ cutoff$.

++: moderate biofilm formation $2{*}E_{590}\ cutoff < \bar{E}_{590} - \bar{SD}_{590} < 4{*}E_{590}\ cutoff$.

+++: strong biofilm formation $\bar{E}_{590} - \bar{SD}_{590} > 4{*}E_{590}\ cutoff$.

With: $E_{590}\ cutoff = E\ \bar{Blank}_{590} - 3{*}SD\ \bar{Blank}_{590} =$ 1.235.

[c]low fluorescence compared with the other test strains, blue resazurin was completely reduced, high cell numbers visible[c] microscopically. Therefore, contrary to 1, the growth of these two strains is classified as "strong growth."

coat the petri dish) of a dilution series from 10 cells cm^{-2} to 10^5 cells cm^{-2} of liquid cultures of the selected species. The dilution series was created by using sterile Ringer's solution with 0.67% (w/w) Tween 80®. Tween 80® was added to reduce the surface tension of the sample and to enable a minimal liquid layer in the Petri dishes.

The species and dilution steps are given in Figure 1. They were placed in Petri dishes with a diameter of 5.3 cm and dried for 24 h at room temperature in a desiccator. The experimental design is shown schematically in the supplemental information online, Supporting Information 1.

Per dish, four swab samples were taken using sterile moistened swabs from a defined area of 1 cm^2 at the midpoint of the plate radius. One swab was transferred to sterile Ringer's solution and was suspended and homogenized using a Vortex Genie II (Scientific Industries, Bohemia, New York, U.S.A.) for quantification using the Thoma counting chamber (0.1 mm depth); the other swab was aerobically incubated at 28 °C in tubes with the modified MYPG medium. This was performed three times per species and per dilution. All tubes were checked daily for indicator color change from blue to pink.

Field test to compare modified MYPG broth with common media used for brewery hygiene monitoring

Swab samples (hygienic monitoring) were taken as above with sterile swabs, moistened with sterile Ringer's solution, from a sampling area of approximately 1 cm^2 at critical sampling points, in the vicinity of a bottle filler, in an industrial German brewery. The bottling plant filled a bottom-fermented, dark filtered, strong beer with a low relative attenuation (81%) at the time of sampling. The swabs were suspended in 8 mL of sterile Ringer's solution with a

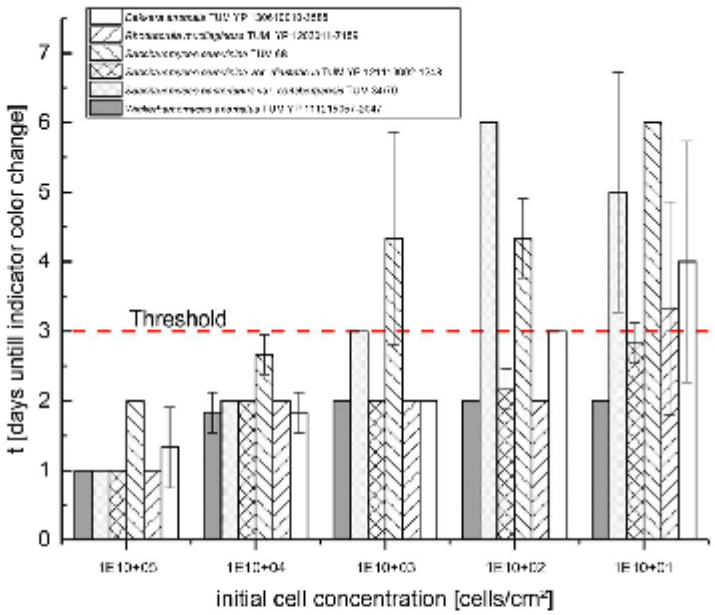

Figure 1. Semi-quantitative validation of modified malt extract, yeast extract, peptone, glucose broth (MYPG) via visual analysis until indicator color change with selected test species on artificial biofilms. The inoculation range was measured using a Thoma cell counter (0.1 mm depth) for the first inoculation, dilution steps were calculated for 1 cm^2 surface, each sample was analyzed daily ($n = 3$ per species and dilution, variance is shown as standard deviation).

vortexer (Vortex-Genie® 2, Scientific Industries, Bohemia, NY, U.S.A.). A 1 mL aliquot of the sample suspension was incubated in 8 mL of the modified MYPG, and another 1 mL aliquot was incubated in 10 mL of the reference medium NBB-B-AM (Döhler, Darmstadt, Germany). NBB-B-AM is typically used for swab samples, to monitor the hygienic status in breweries. Both media were incubated aerobically at 28 °C for 7 days. All samples were checked daily for indicator color change.

DNA extraction for real-time PCR

For DNA extraction, a modified protocol using the InstaGene Matrix™ (Bio-Rad, Hercules, CA, U.S.A.) was used.[38] Hence, 200 µL of a dense liquid enrichment culture was transferred into a 1.5 mL Eppendorf reaction tube and centrifuged for 1 min at 15,114 x g (Hettich MIKRO 200, Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany). The supernatant was discarded and 200 µL InstaGene™ DNA isolation buffer was added to the residing cell pellet and incubated at 56 °C for 30 min (Eppendorf Thermomix comfort). After a 10 s vortex step, the tube was incubated for another 8 min at 95 °C, then centrifuged for 1 min at 15,114 x g. The DNA concentration was measured using a Nanodrop ND 2000 (Thermo Fisher Scientific, Rochester, NY, U.S.A.). For the validation, the DNA concentration of the samples was adjusted with PCR-clean ddH_2O to 5 ng μL^{-1}. The DNA sample material and sample strain set were stored at −20 °C.

Real-time PCR

For the rapid detection of the target species *Rhodotorula mucilaginosa*, a TaqMan® real-time PCR assay was designed (forward primer Rmuc_f194: CCTATTCACTTATAAACACAA AGTCTATGAATG, reward primer Rmuc_r246: ACKTA TCGCATTTCGCTGC, TaqMan®-probe Y58: 6-Fam-CCACATTGGGACTGAGACACGGCC-BHQ-1). The target sequence is the ITS1-5.8S-ITS2 rRNA gene region. To identify usable specific primer binding areas, the rRNA sequences of 99 strains of common brewery microorganisms, collected from the NCBI database (http://www.ncbi.nlm.nih.gov), were aligned (DNASTAR, MegAlign, Lasergene, Version 11). To achieve the greatest possible specificity, primer target areas were selected containing highly specific interspecies polymorphisms. Primers and probes were designed using Primer Express (Primer Express 1.5, Applied Biosystems, Thermo Fisher Scientific, Rochester, NY, U.S.A.). For internal amplification control, a synthetic, random DNA sequence was generated and a specific TaqMan real-time PCR system was designed with this sequence as the target area, as published in a previous study.[35] All primers and probes were tested in silico using BLAST (Basic Local Alignment Search Tool) for homologies with other sites or species. The real-time PCR system was designed by Brandl,[32] Hutzler et al.,[28,29] and Riedl et al.,[35] to be compatible with other systems for spoilage organism detection. As a result, the real-time PCR parameters used for the design were:

- Annealing temperature (Tm) primers: 60 °C
- Annealing temperature (Tm) probes: 10 °C above annealing temperature primers
- Maximum amplicon length: 200 bp.

All nucleotides used are listed in detail in the supplemental information, Supporting Information 1. All nucleotides with the exception of the TaqMan® probe OG-MGB were synthesized by biomers.net GmbH (Ulm, Germany). The OG-MGB, a special TaqMan® probe with minor groove binder (MGB) was synthesized by Thermo Fisher Scientific (Waltham, MA, U.S.A.). All PCR runs were carried out on a LightCycler® 480 Instrument II (Roche Diagnostics Deutschland GmbH, Mannheim, Germany), using 10-µL LightCycler® 480 Probes Master in a 20-µL volume assay with a 5-µL sample. The real-time PCR was performed using 400 nmol l^{-1} of forward and reverse primer and 200 nmol l^{-1} TaqMan probe of the main PCR system. The primers were added in aliquots of 0.8 µL and 10^{-5} mol l^{-1}, the probes were added in 0.4 µL aliquots of 10^{-5} mol l^{-1}.

The internal amplification control IAC135 was added using 250 nM of the primer pair IAC135-f (5′-TGGAT AGATTCGATGACCCTAGAAC-3′) [35] and IAC135-r (5′-TGAGTCCATTTTCGCAGATAACTT-3′) [35] and 200 nM of the TaqMan probe IAC135-S (5′-BHQ1-TGGGAGG ATGCATTAGGAGCATTGTAAGAGAG-HEX-3′).[35] The target DNA of the internal amplification control IAC135 (5′- TGCTAGAGAATGGATAGATTCGATGACCCTAGAA CTAGTGGGAGGATGCATTAGGAGCATTGTAAGAGA-GTCGGAAGTTA-3′) [35] and IAC135-rev (5′-TGCGACACC TTGGGCGACCGTCAATAGGCCACTCGAATGAGTCCA-TTTTCGCAGATAACTTCCGACTCTCTTACAATGCT-3′) [35] was used at a concentration of $5*10^{-20}$ mol l^{-1}. The primers were added in aliquots of 0.5 µL and 10^{-5} mol l^{-1}. The probes were added in 0.4-µL aliquots of 10^{-5} mol l^{-1}. The internal amplification control target DNA was added in 0.1-µL aliquots of 10^{-17} mol l^{-1}. The initial heating at 95 °C was held for 10 min, then 40 cycles were performed at 95 °C for 10 s and at 60 °C for 30 s. The fluorescence was measured at the end of the 60 °C step of each cycle (modified [28,29,32]).

Validation of real-time PCR

To validate the PCR specificity, a strain set of 109 representative species was selected. These species are known to occur in the brewing environment. All of the strains used are listed in the supplemental information, Supporting Information 3, and were grown as dense three-day liquid cultures (MRS, anaerobic for lactic acid bacteria; MIB for strict anaerobic gram-negative bacteria; MYPG, aerobic for yeasts, nutrient broth, aerobic for all other bacteria). DNA was isolated as described and adjusted to 5 ng μL^{-1}. As a reference method, all tested strains used for quantitative real-time PCR validation of the *R. mucilaginosa* identification system were identified by partial sequencing of the ITS1-5.8S-ITS2 rRNA gene and sequence BLAST with the NCBI nucleotide database.

For validation, the relative specificity, relative accuracy, and relative sensitivity were calculated as follows [39]:

$$\textit{relative specificity} = \frac{\textit{number of overall negative results in both methods}}{\textit{number of false positive analysis results versus the reference method} + \textit{number of overall negative results in both methods}} * 100$$

$$\textit{relative accuracy} = \frac{\textit{number of positive analysis results in both methods} + \textit{number of overall negative results in both methods}}{\textit{overall analyses results}} * 100\%$$

$$\textit{relative sensitivity} = \frac{\textit{number of positive analysis results in both methods}}{\textit{number of positive analysis results in both methods} + \textit{number of false negative analysis results versus the reference method}} * 100\%$$

The numbers in the aforementioned formulas were derived from the test results of the reference method (partial sequencing of the ITS1-5.8S-ITS2 rRNA gene) and the validated real-time PCR system. The number of positive analysis results in both methods therefore means the number of species positively tested for the species identity *R. mucilaginosa*. The number of false positive analysis results versus the reference method is the number of strains that tested positive for the species identity *R. mucilaginosa*, but tested negative with the reference method. The number of overall negative results in both methods is the number of species, which tested negatively for the species identity *R. mucilaginosa*, with the validated real-time PCR system. The number of false negative analysis results versus the reference method is

the number of strains that tested negative for the strain identity of *R. mucilaginosa* with the validated method, while the test with the reference method was positive. The overall analysis results are equal to the number of tested strains. For quantitative validation, the *R. mucilaginosa* identification system was tested on a dilution series of the test strain *R. mucilaginosa* TUM YP 120306011-7159 in sterile double distilled water and the Efficiency ($E = 10^{-1/m} - 1$) was calculated of the resulting regression m.[40]

Combination of real-time PCR identification and modified MYPG routine swab samples from a 5-liter can filler

Swab samples were taken from a 5-liter semi-automatic can filler, with sterile swabs moistened with sterile Ringer's solution, at critical sampling points before and after cold water rinsing of the entire filler. This was carried out to check the cleaning efficiency by cold water rinsing on two different sampling days. The reason for the sampling was a previous contamination with *D. anomala* and renovations in the filling hall to improve the hygienic design. The product being filled at that time was filtered lager beer on the first day and unfiltered wheat beer on the second day. Both products were flash pasteurized before filling. The swabs were incubated aerobically at 28°C for 7 days and checked for color change each day. Samples that changed color within 3 days were screened with real-time PCR for the selected indicator yeasts, as previously listed.

Combination of real-time PCR identification and modified MYPG swab samples to identify a contamination source of S. cerevisiae var. diastaticus

Swab samples were taken from a bottle-filler with sterile moistened swabs, at critical sampling points, before and after cleaning and disinfection of the entire filler to check the cleaning efficiency against *S. cerevisiae* var. *diastaticus*. The swabs were incubated at 28°C in modified MYPG for three days. All samples with a positive color indicator change within three days were screened for *S. cerevisiae* var. *diastaticus*.

Results

Growth control and biofilm-formation potential in the stationary phase in modified MYPG

All of the test strains were able to grow in the modified MYPG medium and their growth could be measured by a significant rise in fluorescence and microscopic observable increase of cell material (Table 1).

The two applied strains of *S. cerevisiae* var. *diastaticus* showed a low increase in fluorescence compared with the other strains. The control exhibited a high increase in cell material when examined by microscope. The blue-colored dye resazurin, in all biological replicates, was completely reduced, suggesting cell growth not measurable by fluorescence emission of resorufin.

With the exception of the two applied strains of *D. anomala*, all tested strains showed significant biofilm formation in stationary phase in the 96-well microtiter plates, represented in crystal violet stainable material residing in the microtiter wells. The microscopic analysis of each stainable well confirmed the presence of adherent cells and that there was no staining of noncellular material.

Semiquantitative validation of the modified MYPG broth validation as a hygienic monitoring medium

It was possible to detect all dilutions from the artificial biofilm equivalents of two *W. anomalus* and *S. cerevisiae* var. *diastaticus* within two days via the observable indicator reaction (Figure 1). *R. mucilaginosa* and *D. anomala* could be detected within the 3-day threshold at a calculated cell concentration limit of 10^2 cells cm^{-2}. The tested bottom-fermenting strain *S. pastorianus* var. *carlsbergensis* TUM 34/70 could not be detected below 10^3 cells cm^{-2}, the top-fermenting *S. cerevisiae* culture yeast TUM 68 could not be detected below 10^2 cells cm^{-2} within the 3-day threshold.

Field test to compare modified MYPG broth with common media used for hygienic monitoring in breweries

Both media – NBB-B-AM and the modified MYPG broth – showed a comparable detection pattern of critical sampling points when there was insufficient sanitation (Table 2). With the exception of two swabs, swabs from all sample points that showed an indicator color change in NBB-B-AM within three days, also showed a color change within this time period in MYPG.

Real-time PCR

For real-time PCR identification of the selected species in further work, the identification systems already published and listed in the supplemental information, Supporting Information 2, were used, with the exception of *R. mucilaginosa*, for which a real-time PCR system was designed and validated for this study. The detailed data of the qualitative validation of the newly designed real-time PCR identification system is listed in the supplemental information, Supporting Information 3. In summary, all 18 applied strains of *R. mucilaginosa* were detected with the system, as well as one strain of *R. toruloides*.

Therefore, the relative specificity, relative accuracy, and relative sensitivity [39] are:

$$\textit{relative specificity} = \frac{19}{1+193} * 100\% = 99\%$$

$$\textit{relative accuracy} = \frac{19+193}{212} * 100\% = 100\%$$

$$\textit{relative sensitivity} = \frac{19}{19+0} * 100\% = 100\%$$

The quantitative validation data of the real-time PCR system for *R. mucilaginosa* with strain TUM YP 120306011-7159

Table 2. Comparison of swab sampling in a brewery with modified MYPG and NBB-B-AM at a filler, filling a bottom-fermented, dark filtered strong beer with low relative attenuation.[a,b]

	Modified MYPG							NBB-B-AM						
	24 h	48 h	72 h[c]	96 h	120 h	144 h	168 h	24 h	48 h	72 h[c]	96 h	120 h	144 h	168 h
(1) Filler infeed conveyor rail	–	+	+	+	+	+	+	–	+/-	+/-	+	+	+	+
(2) Filler inlet bottle starwheel	–	–	–	+/- M	+ M	+ M	+ M	–	–	–	–	–	–	–
(3) Filling element	–	–	–	–	–	–	–	–	–	–	–	–	–	+/-
(4) Deflector plate	–	–	–	–	+	+ M	+ M	–	–	–	–	–	–	–
(5) Filler tulip	–	–	–	–	–	–	–	–	–	–	–	–	–	–
(6) Lifting element	–	–	–	–	–	–	–	–	+	+	+	+	+	+
(7) Filler case	–	+/-	+/-	+	+	+	+	–	+	+	+	+	+	+
(8) Filler outlet bottle starwheel	–	–	–	+/- M	+ M	+ M	+ M	+	+	+	+	+	+	+
(9) High pressure injection	–	–	–	–	–	–	–	–	–	–	–	–	–	–
(10) Capper	–	–	+/-	+	+	+	+	–	+	+	+	+	+	+
(11) Filler table	+/-	+	+	+	+	+	+	+	+	+	+	+	+	+
(12) Capper infeed rails	–	+/-	+/-	+	+	+	+	–	+/-	+	+	+	+	+
(13) Capper outlet bottle starwheel	–	–	+/- M	+ M	+ M	+ M	+ M	–	+	+	+	+	+	+
(14) Capper infeed conveyor band	–	+	+	+	+	+	+	+	+	+	+	+	+	+
(15) Capper lifting element	–	–	+/-	+	+	+	+	+	+	+	+	+	+	+
(16) Filler conveyor lubrication	–	–	–	–	–	–	–	–	–	–	–	–	–	–
(17) Filler infeed conveyor band–bottom side	–	+/-	+/-	+/-	+/-	+/-	+/-	–	–	+/-	+	+	+	+
(18) Bottle washing machine conveyor lubrication	–	–	–	–	–	–	–	–	–	–	–	–	–	–
(19) Bottle washing machine outlet conveyor	–	+/-	+	+	+	+	+	–	–	–	–	T	+/-, T	+/-, T
(20) Sewage infeed filler	–	+	+	+	+	+	+	+	+	+	+	+	+	+
(21) Bottle washing machine condense water bottle outlet	–	+/-	+ M	+ M	+ M	+ M	+ M	–	–	–	–	–	M	M
(22) Bottle-inspector	–	–	+/-	+/-	+/-	+	+ M	–	–	–	–	–	T	+/-, T
(23) Filler infeed scroll	–	–	+/-	+/- M	+ M	+ M	+ M	–	–	–	–	–	M	M
(24) Filling valves	–	–	–	–	+/-	+ M	+ M	–	–	–	–	–	–	–
(25) Capper wash down	–	–	+/- M	+/- M	+ M	+ M	+ M	–	–	–	+/- G	+/- G	+/- G	+/- G
(26) Drive shaft capper outlet conveyor band	–	+	+	+	+	+	+ M	–	+/-	+/-	+	+	+	+

MYPG: malt extract, yeast extract, peptone, glucose broth.
[a]Swabs were suspended in 8 mL sterile Ringer's solution with vortexer, 1 mL of suspension was inoculated in modified MYPG and NBB-B-AM, aerobic incubation at 28 °C.
[b]+ positive indicator color change (MYPG: blue-colorless; NBB-B-AM: red-yellow); +/− incomplete color change (MYPG: first reduction step blue-pink; NBB-B-AM: reddish yellow); − negative indicator color change; M: mold; T: visible turbidity/sediment; G: visible gas formation.
[c]Threshold of three days.

are given, with the resulting regression line with $m = -3.43$ regression following an efficiency [40] of

$$E = 10^{-1/m} - 1 = 10^{-1/-3.43} - 1 = 0.96.$$

The regression curve, which results in the described values, is shown in the supplemental information, Supporting Information 4.

Combination of real-time PCR identification and modified MYPG routine swab samples from a 5-liter can filler

Table 3 shows the results of the hygienic monitoring and real-time PCR identification of two different sampling days, before and after rinsing the filler. No direct product-spoilage yeasts could be found. It was possible to detect *W. anomalus* or *R. mucilaginosa* in all samples with positive growth in the modified MYPG.

Combination of real-time-PCR identification and modified MYPG swab samples to identify a contamination source of S. cerevisiae var. diastaticus

As can be seen in Table 3, *S. cerevisiae* var. *diastaticus* could be found at several sampling points, especially at the capper. The contaminant microorganisms survived cleaning and disinfection (using ClO_2 foam disinfectant) at two sampling points and could still be detected in the capper mechanics after cleaning and disinfecting with the ClO_2 foam.

Discussion

Growth control and biofilm-formation potential in the stationary phase in modified MYPG

The fluorescence method for validating cell growth appears to be too sensitive for higher cell densities or cell activity, as seen in Table 1, in the example of the two used strains of *S. cerevisiae* var. *diastaticus*. The colorimetric method (reduction of the blue resazurin dye) appears to be applicable. Since this test can also be performed visually, the modified MYPG medium, with resazurin as a redox indicator for cell activity, is highly applicable as an easy-to-use method for detecting yeast growth.

Contrary to previous studies, the tested strains of *D. anomala* showed no observable biofilm formation in the stationary phase. Biofilm production has previously already been described as being strain dependent for this species, and both species may lack any biofilm-forming potential.[1] As both strains were isolated from a brewing environment and the experiment settings include a rather complex and nutrient-rich medium, biofilm formation in a beer-like matrix might be different. For *Saccharomyces* yeast, biofilm formation is stronger with glucose-starved cells.[21,26] A similar effect is possible with *D. anomala*. It has also been reported that multi-species biofilms with yeasts and bacteria

Table 3. Combination of real-time PCR identification and modified MYPG swab samples to identify a contamination source of *Saccharomyces cerevisiae* var. *diastaticus*.

	Before cleaning and disinfection with CO_2			After cleaning and disinfection with CO_2		
	Indicator color change of modified MYPG[a]	Microscopy	Real-time PCR for *Saccharomyces cerevisiae* var. *diastaticus*[b]	Indicator color change of modified MYPG[a]	Microscopy	Real-time PCR for *Saccharomyces cerevisiae* var. *diastaticus*[b]
Infeed star wheel filler, inner side	–	Without detection	n.a.	+	Yeasts	n.d.
Infeed starwheel filler surface	–	Without detection	n.a.	+ (Surface film)	Yeasts	n.d.
Plastic rail filler infeed	–(Mold)	Yeasts, molds	n.a.	–	Without detection	n.a.
Filler table	+ (Surface film)	Yeasts	n.d.	+ (Surface film)	Yeasts	n.d.
Capper infeed starwheel	+ (Surface film)	Yeasts, short-rod bacteria	+	+ (Surface film)	Yeasts	n.d.
Capper starwheels	+ (Surface film)	Yeasts, short-rod bacteria	+	+ (Surface film)	Yeasts	n.d.
Capper casing	–	Molds	n.a.	–	Without detection	n.a.
Capper stamps	+ (Surface film)	Yeasts, short-rod bacteria	+	+ (Surface film)	Yeasts	+
Capper elements	+ (Surface film)	Yeasts	n.d.	+ (Surface film)	Yeasts, molds	n.d.
Capper sealer	+ (Surface film)	Yeasts, short-rod bacteria	+	+	Yeasts	+
Crowner caps infeed rails	–Molds	Molds	n.d.	–	Yeasts	n.a.
Crowner cap transfer	–	Molds and spores	n.a.	–	Molds	n.a.

PCR = polymerase chain reaction; MYPG: malt extract, yeast extract, peptone, glucose broth; n.a.: not applicable; n.d.: not detectable.
[a]+: indicator color change of resazurin after 3 days of incubation; –: no indicator color change of resazurin after 3 days of incubation.
[b]PCR was performed after 3 days of incubation with growth positive samples on modified MYPG.

are more stable than biofilms that consist of single species, which may result in *D. anomala* having a stronger biofilm-forming potential in the presence of bacteria.[20] Swab samples of beverage production environments that test positive for this species should be viewed very critically due to the spoilage potential of this species in beer and other beverages. The weak initial biofilm-forming potential means that finding this species in biofilms may indicate mature biofilms, which have already been colonized by weaker or nonbiofilm-forming organisms.

R. mucilaginosa shows the potential to form biofilms initially. This species is not considered to be a product-spoilage organism for nonalcoholic carbonated beverages and is considered to be a potential spoilage organism for noncarbonated beverages.[2,3,5] The tested strains were collected in the brewing environment from surfaces associated with biofilm formation. The detection of this species in biofilms is an indicator of young biofilm formation and inefficient cleaning, as it can build biofilms *de novo* given sufficient nutrients.

S. cerevisiae TUM 68 and *S. pastorianus* var. *carlsbergensis* TUM 34/70 are exemplary brewing yeasts. Due to their use as starter cultures, these strains are omnipresent in breweries and can spoil non-alcoholic beverages as strong fermenters. It is quite possible to find these yeasts during hygienic monitoring in the filling department, since it is possible for cells to slip through filtration or be introduced to the filling department when filling unfiltered unpasteurized beer. The weak initial biofilm potential of both strains indicates that the detection of brewing yeast strains in the filling area is a sign of mature biofilms that have already been colonized by later-stage biofilm-colonizing organisms. The tendency of starved *S. cerevisiae* cells to build stronger biofilms under the influence of polysaccharides, such as alginates, indicates a strong ability to colonize existing bacterial or fungal biofilms and supports this theory. This would also explain why *Saccharomyces* yeasts could not be found in young biofilms in earlier studies.[41] As the test for biofilm formation is conducted in a glucose-rich medium, the biofilm formation of these strains in a medium with minimal glucose suggests a result in a stronger biofilm formation due to a stronger expression of the Flo11 gene.[26] This might be an important issue for breweries that fill both beer and other beverages using the same equipment.

The two tested strains of *S. cerevisiae* var. *diastaticus* showed biofilm formation in various strengths. The detection of this species in hygienic monitoring samples should be regarded as highly critical. The high biofilm-forming potential and the high spoilage potential in beer represent a high risk to product safety if this yeast is found in the filling department. Being able to initially form biofilms, this variety may settle persistently within the process. As this species can also spoil fermented beer with a high attenuation, this species has a high spoilage potential for nearly all beer types.

W. anomalus has already been described as a biofilm-forming species [9] and this was confirmed in this study. As one of the most commonly found yeast species in brewery environments, together with its high initial biofilm

Table 4. Combination of real-time PCR identification and modified MYPG routine swab samples from a 5-liter can filler.

Day 1

	Before rinsing							After rinsing						
		PCR Screening[b]							PCR Screening[b]					
	mod. MYPG[a]	*Dekkera anomala*	*Rhodotorula mucilaginosa*	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae var. diastaticus*	*Saccharomyces pastorianus*	*Wickerhamomyces anomalus*	mod. MYPG[a]	*Dekkera anomala*	*Rhodotorula mucilaginosa*	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae var. diastaticus*	*Saccharomyces pastorianus*	*Wickerhamomyces anomalus*
Filling element 1	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Filling element 2	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Filling element 3	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Filling element 4	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Filling element 5	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Can lid before stopper opener	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Can stopper	+	n.d.	n.d.	n.d.	n.d.	n.d.	positive (ct. 30)	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Infeed table	+	n.d.	positive (ct. 30)	n.d.	n.d.	n.d.	positive (ct. 17.44)	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Holding plate top	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Holding plate bottom	+	n.d.	n.d.	n.d.	n.d.	n.d.	positive (ct. 30)	+	n.d.	n.d.	n.d.	n.d.	n.d.	positive (ct. 18.55)
Can lid after filler	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Sealer stamp	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Beer residue on can	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Swab sample disinfection bath	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Empty can inner wall	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Rinse water filler	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Conveyor band before filler	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Conveyor band after filler	+	n.d.	n.d.	n.d.	n.d.	n.d.	positive (ct. 19.65)	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Rinser nozzle	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Day 2

	Before rinsing							After rinsing						
		PCR Screening[b]							PCR Screening[b]					
	mod. MYPGa	*Dekkera anomala*	*Rhodotorula mucilaginosa*	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae var. diastaticus*	*Saccharomyces pastorianus*	*Wickerhamomyces anomalus*	mod. MYPGa	*Dekkera anomala*	*Rhodotorula mucilaginosa*	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae var. diastaticus*	*Saccharomyces pastorianus*	*Wickerhamomyces anomalus*
Filling element 1	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Filling element 2	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Filling element 3	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Filling element 4	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Filling element 5	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Can lid before stopper opener	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Can stopper	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Infeed table	+	n.d.	n.d.	n.d.	n.d.	n.d.	positive (ct. 16.6)	+	n.d.	n.d.	n.d.	n.d.	n.d.	positive (ct. 21.62)
Holding plate top	+	n.d.	n.d.	n.d.	n.d.	n.d.	positive (ct. 16.94)	+	n.d.	n.d.	n.d.	n.d.	n.d.	positive (ct. 20.73)
Holding plate bottom	+	n.d.	n.d.	n.d.	n.d.	n.d.	positive (ct. 17.25)	+	n.d.	n.d.	n.d.	n.d.	n.d.	positive (ct. 22.27)

Can lid after filler	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Sealer stamp	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Beer residue on can	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Swab sample disinfection bath	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Empty can inner wall	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Rinse water filler	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Conveyor band before filler	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Conveyor band after filler	+	n.d.	n.d.	n.d.	n.d.	n.d.	positive (ct. 20.53)	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Rinser nozzle	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

PCR = polymerase chain reaction; MYPG: malt extract, yeast extract, peptone, glucose broth; n.a.: not applicable; n.d.: not detectable.
[a]+indicator color change of resazurin after 3 days of incubation; –: no indicator color change of resazurin after 3 days of incubation.
[b]PCR was performed after 3 days of incubation of samples with positive growth in modified MYPG.

formation potential, the detection of this species can be seen as an indicator of biofilm formation and insufficient cleaning. The sole detection of this species indicates young biofilms, while in combination with other species that are known to solely colonize biofilms, this finding might indicate mature yeast biofilms. The risk of product spoilage is low, as this species is described as a weak fermenter.

Generally, the test for biofilm formation potential is only a short term test in a nutrient rich medium and in stationary phase. As biofilm formation is also influenced by other factors, such as sheer forces and nutrient shortages,[24] the information gained about biofilm forming potential can only provide orientation.

Semiquantitative validation of the modified MYPG broth as a hygienic monitoring medium

The brewing yeast strains (*S. cerevisiae* TUM 68 and *S. pastorianus* var. *carlsbergensis* TUM 34/70), selected for this study, grew much slower than the wild yeast strains applied (Figure 1). Due to the slow growth of brewing yeasts, a color indicator reaction within the three-day threshold can be linked either to the presence of non-*Saccharomyces* wild yeasts or to higher cell numbers of *Saccharomyces* brewing yeasts. A scheme is proposed that identifies risk levels that correspond to the time until indicator change.

The data supports a high potential of the modified MYPG broth as a hygienic indicator medium for yeast biofilms in a brewing environment. The point when the color changes can be an estimate of the level of contamination on the tested surface. The yeast physiology is not compromised by the indicator dye and further analyses are possible, especially real-time PCR analyses as described later.

Field test to compare modified MYPG broth with common media used for hygienic monitoring in breweries

The most important sampling points are those with direct contact to the product (e.g., filling element, filler tulip, high pressure injection [HPI], and filling valves). All of those sampling points showed comparable low contamination levels in both media. The next important sample points are those without direct contact with the bottled product and possible contact to product residues due to the production process. These can be niches from which the product spoiling organisms take root and spread. At some of those, differences between the results of both media appear (e.g., both filler starwheels, the filler deflector plate, lifting elements, filler infeed scroll, capper wash down). The modified MYPG medium detects mold growth at those sampling points that are considered unproblematic while filling beer, or other carbonated beverages. While filling noncarbonated beverages, molds may be problematic. The same can be observed at sampling points without product contact, the bottle washing machine, and the bottle inspector.

In general, the modified MYPG seems to be comparable to NBB-B-AM within a 72h incubation period (Table 2). Of

the sampling points, 16 of the 26 showed positive growth in the modified MYPG, while with NBB-B-AM 13 of 26 sampling points showed positive growth. The differences in the distributions of sample points with positive microbiological findings may be a result of the media composition and the filled product. The modified MYPG contains tetracycline to suppress bacterial growth. While NBB-B-AM favors bacterial growth, MYPG promotes fungal growth, resulting in different detection patterns.

Overall, MYPG proved to be a useful medium for swab samples in the brewery environment, especially when handling beverages, sensitive for fungal spoilage.

Real-time PCR

The simultaneous detection of *R. toruloides*, the only species which was also detected with the real-time PCR system developed for this study, does not prevent the use of the system to validate hygienic monitoring samples. Both species *R. mucilaginosa* and *R. toruloides* have the same significance for breweries, resulting in the same interpretation if *R. toruloides* is wrongly detected as *R. mucilaginosa*. The calculated relative specificity, relative accuracy, and relative sensitivity and efficiency are comparable to real-time PCR systems developed by Hutzler et al[28,29] and Brandl.[32] The real-time PCR system developed for detection of *R. mucilaginosa* proved to be compatible to the systems developed by both authors and were therefore used combined in this study.

Combination of real-time PCR identification and modified MYPG routine swab samples from a 5-liter can filler

All findings were noncritical (no *D. anomala* or *S. cerevisiae* var. *diastaticus*), and there was no imminent risks to the product. None of the tested species were detected at the most critical sampling positions (e.g., with direct product contact), especially the filling elements and the cans.

Every investigated sampling point that showed a positive detection of yeast had no direct product contact. However, these spots were known to be locations where biofilms could form due to their complex construction, cleanability, and possible product residues. The results give an overview of plant hygiene and the cleanability of the tested sampling points. The detection of *W. anomalus* and *R. mucilaginosa* indicated young biofilm formation at the affected sampling points. The infeed table, for example, is one of the most common places with microbiological growth in the filling environment. The constant product leakage from the filling overflow, which remains on the surfaces, creates a perfect environment for microorganisms, as is demonstrated by the low ct values (Cycle threshold, first PCR cycle with measurable signal) of *W. anomalus* (Table 4). The increase in the ct values (decrease of target microorganism load) after rinsing indicates some cleaning or dilution effect, but no perfect sanitation. Detecting this species in this position is not problematic due to the low spoilage potential. Subsequently, the machine parts of the sampled position are in no direct contact with the filled product, which impedes contamination from machine equipment. Nevertheless, mature biofilms with product spoilers may indicate a risk, as biofilm particles may be transferred indirectly by aerosols or spray water.

The effect of rinsing the filler between sampling can be demonstrated by the analysis results (Table 4). On day one, the sample positions with positive yeast detection were reduced from four positions before rinsing, to one position after rinsing. On day two, the number of positions with detectable yeast growth was reduced from four to three. At the positions with positive yeast growth, *W. anomalus* could be detected at all positions and *R. mucilaginosa* at one position. The cell numbers, or the growth rate in the modified MYPG, was reduced after rinsing, as can be seen in the increasing ct values after rinsing and the complete disappearance of *R. mucilaginosa* (Table 4).

Combination of real-time PCR identification and modified MYPG swab samples to identify a contamination source of S. cerevisiae var. diastaticus

The capper sealer and stamps, which still showed contamination with *S. cerevisiae* var. *diastaticus*, were in direct contact with bottle openings, and biofilm particles are easily transferred by aerosols and spray water. The capper is known to be critical for biofilm formation.[6] The complex mechanics are likely to have dead spaces and product aerosols from high pressure injection and friction dust from the cap conveyors can merge into a nutrient-rich sludge, which settles in dead spaces. Some sampling points also showed findings with yeast after the rinsing step, but without detection of *S. cerevisiae* var. *diastaticus*. This indicates transfer of yeast species, more robust to the rinsing conditions from other areas by the rinsing step.

After these findings, the capper was completely dismantled and heavy biofilm formation was found within the inner mechanics of the capper. After thorough cleaning and sanitation of the caper mechanics, *S. cerevisiae* var. *diastaticus* was no longer detected at the capper mechanics.

The combination of modified MYPG swab samples and real-time PCR identification proved to be useful for microbiological stage-by-stage control in the context of hygienic problems with yeasts in the filling area.

Conclusions

The colorimetric method of resazurin as indicator dye for yeast growth was tested successfully for the semiquantitative detection of biofilm relevant yeasts *in vitro*. Yeast species classified with a high spoilage potential in beer and other non-*Saccharomyces* yeasts were easily detected. A risk level scheme depending on the time of indicator color change was postulated. The biofilm relevance of the used strain set was confirmed by the results of biofilm forming potential. The medium and colorimetric test was further combined with selected, species specific real-time PCR detection

systems and proven to be highly applicable for hygienic control and specific contamination tracing in breweries.

Acknowledgments

The authors are very grateful to Jennifer Koob, Alexandra Gonsior, Margit Grammer, Siglinde Huber, and Franziska Elisath for supporting this project in the Laboratories of the Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität.

Conflict of interest

No commercial or other relationships had any influence on the study design, the data collection, the analysis, interpretation, the report writing, and article submission. No conflict of interest is declared within this study.

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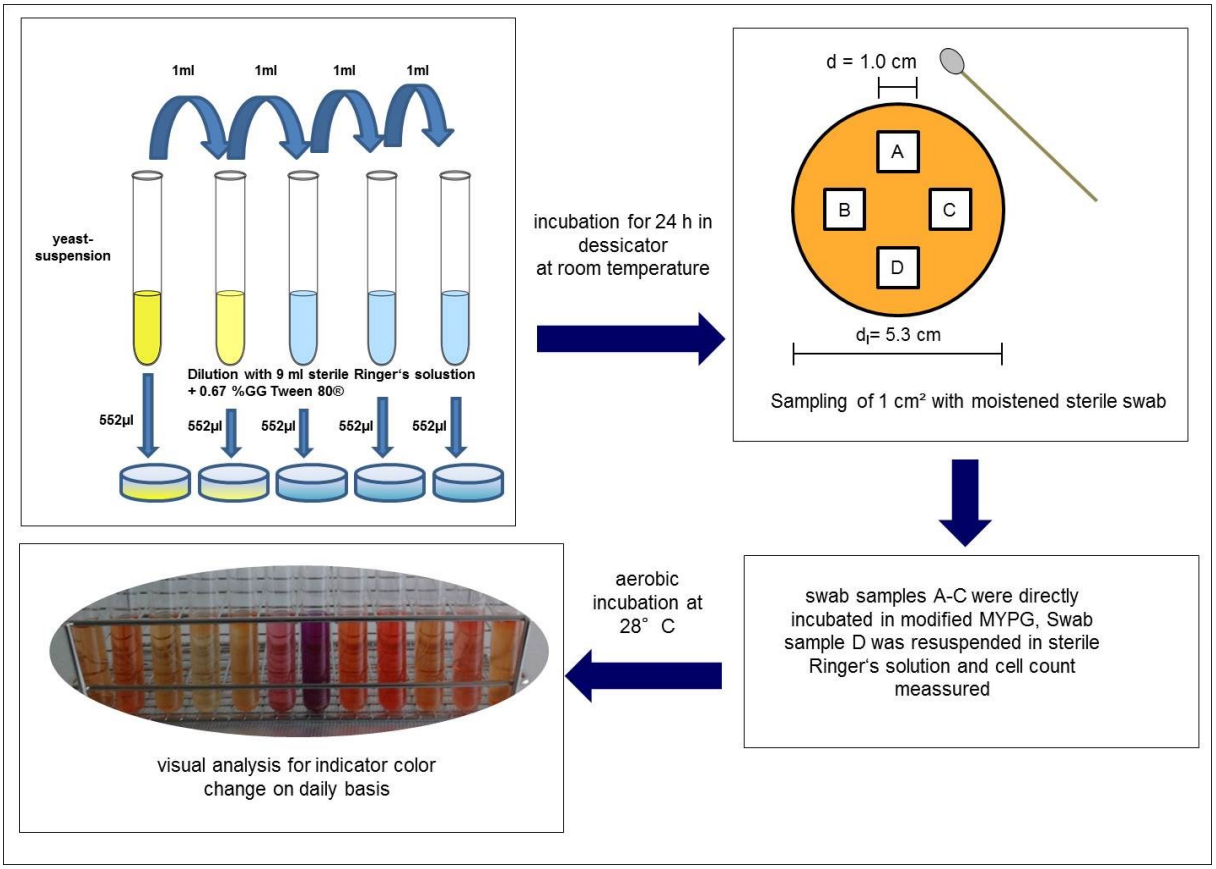

Figure 1: Publication 3, Supporting Information 1: Schematic of the experimental design for semi-quantitative validation of the modified MYPG broth validation as a hygienic monitoring medium

Table 5: Publication 3Supporting Information 2:, TaqMan® based real-time PCR detection system for selected indicator yeasts

Target organism	System Name	Nucleotide	Name	Target Area	Nucleotide sequences (5' -> 3')	Reference
internal amplification control	IAC135	forward primer	IAC135-f	IAC135	TGGATAGATTCGATGACCCTAGAAC	[103]
		reward primer	IAC135-r	IAC135	TGAGTCCATTTTCGCAGATAACTT	[103]
		TaqMan®-probe	IAC135-S	IAC135	HEX-TGGGAGGATGCATTAGGAGCATTGTAAGAGAG-BHQ-1	[103]
		synthetic DNA forward sequence	IAC135	-	TGCTAGAGAATGGATAGATTCGATGACCCTAGAACTAGTGGGAGGATGCATTAGGAGCATTGTAAGAGAGTCGGAAGTTA	[103]
		synthetic DNA reward sequence	IAC135-rev	-	TGCGACACCTTGGGCGACCGTCAATAGGCCACTCGAATGAGTCCATTTTCGCAGATAACTTCCGACTCTCTTACAATGCT	[103]
Dekkera anomala	Dan	forward primer	Da-f	ITS1-5.8S-ITS2 rRNA gene	ATTATAGGGAGAAATCCATATAAAACACG	[13]
		reward primer	Da-r	ITS1-5.8S-ITS2 rRNA gene	CACATTAAGTATCGCAATTCGCTG	[13]
		TaqMan®-probe	Y58	ITS1-5.8S-ITS2 rRNA gene	6-Fam-CCACATTGGGACTGAGACACGGCC-BHQ-1	[13]
Rhodotorula mucilaginosa	*Rmuc_246*	forward primer	Rmuc_f194	ITS1-5.8S-ITS2 rRNA gene	CCTATTCACTTATAAACACAAAGTCTATGAATG	this study
		reward primer	Rmuc_r246	ITS1-5.8S-ITS2 rRNA gene	ACKTATCGCATTTCGCTGC	this study
		TaqMan®-probe	Y58	ITS1-5.8S-ITS2 rRNA gene	6-Fam-CCACATTGGGACTGAGACACGGCC-BHQ-1	[13]
Saccharomyces cerevisiae	OG-COXII	forward primer	OG-f	*COXII*	TTCGTTGTAACAGCTGCTGATGST	[53, 54]
		reward primer	OG-r	*COXII*	ACCAGGAGTAGCATCAACTTTAATACC	[53, 54]
		TaqMan®-probe	OG-MGB	*COXII*	Fam-ATGATTTTGCTATCCCAAGTT-MGB-BHQ-1	[53, 54]
Saccharomyces cerevisiae var. *diastaticus*	Sdi	forward primer	Sd-f	*STA1*	TTCCAACTGCACTAGTTCCTAGAGG	[13, 111]
		reward primer	Sd-r	*STA1*	GAGCTGAATGGAGTTGAAGATGG	[13, 111]
		TaqMan®-probe	Sdia	*STA1*	6-Fam-CCTCCTCTAGCAACATCACTTCCTCCG -BHQ-1	[13, 111]
Saccharomyces pastorianus/ *Saccharomyces bayanus*	UG300	forward primer	UG300E	random subtractive hybridisation sequence by Scherer [111]	CTCCTTGGCTTGTCGAA	[13, 111]

		reward primer	UG300M	random subtractive hybridisation sequence by Scherer [111]	GGTTGTTGCTGAAGTTGAGA	[13, 111]
		TaqMan®-probe	UG	random subtractive hybridisation sequence by Scherer [111]	6-Fam-TGCTCCACATTTGATCAGCGCCA-BHQ-1	[13]
Wickerhamomyces anomalus	Pan	forward primer	Pa-f	ITS1-5.8S-ITS2 rRNA gene	AATGTTAAAACCTTTAACCAATAGTCATG	[13]
		reward primer	Pa-r	ITS1-5.8S-ITS2 rRNA gene	ACGTATCGCATTTCGCTGC	[13]
		TaqMan®-probe	Y58	ITS1-5.8S-ITS2 rRNA gene	6-Fam-CCACATTGGGACTGAGACACGGCC-BHQ-1	[13]

Table 6: Publication 3, Supporting Information 3: Validation of real-time PCR identification system for Rhodotorula mucilaginosa

Species	Strain	Real-time PCR-identification *R*
Acetobacter aceti	TUM BP 000-1991	negative
Acetobacter pasteurianus	TUM BP 000-1990	negative
Asaia lannensis	TUM BP 000-0994	negative
Bacillus subtilis	TUM BP 000-0980	negative
Candida boindinii	TUM YP 000-6007	negative
Cryptococcus laurentii	TUM YP 000-0011	negative
Debaryomyces hansenii	TUM YP 000-0006	negative
Dekkera anomala	TUM YP 000-3040	negative
Dekkera bruxellensis	TUM YP 000-3096	negative
Enterobacter sp.	TUM BP 000-6088	negative
Enterococcus sp.	TUM BP 111206005-0075	negative
Escherichia coli	TUM BP 000-0981	negative
Gluconacetobacter liquefaciens	TUM BP 000-0105 (DSM 5603 (BS 279))	negative
Gluoconobacter oxydans	TUM BP 000-0078	negative
Hafnia alvei	TUM BP 000-0993	negative
Hanseniaspora uvarum	TUM YP 000-0054 (CBS 5074)	negative
Kazachstania exigua	TUM YP 000-337	negative
Kluyvera ascorbata	TUM BP 131213038-0099	negative
Kluyveromyces marxianus	TUM YP 000-0005	negative
Kocuria kristinae	TUM BP 000-0083 (DSMZ 22032)	negative
Lactobacillus acetotolerans	TUM BP 120706025-2967	negative
Lactobacillus acidophilus	TUM BP 000-2081 (DSMZ 20079)	negative
Lactobacillus alimentarius	TUM BP 000-2979	negative
Lactobacillus amylolyticus	TUM BP 000-2969	negative
Lactobacillus amylophilus	TUM BP 000-2068	negative
Lactobacillus amylovorus	TUM BP 000-2080 (DSMZ 20531)	negative
Lactobacillus backi	TUM BP 140407001-2242	negative
Lactobacillus bifermentans	TUM BP 000-2014 (DSMZ 20003)	negative
Lactobacillus brevis	TUM BP 120711011-2578	negative
Lactobacillus brevis (formerly *Lactobacillus brevisimilis*)	TUM BP 000-2976	negative
Lactobacillus buchneri	TUM BP 000-2060	negative
Lactobacillus casei	TUM BP 120509129-2360	negative
Lactobacillus collinoides	TUM BP 000-2061	negative
Lactobacillus coryniformis	TUM BP 000-2978	negative
Lactobacillus curvatus	TUM BP 000-2977 (BS 218)	negative
Lactobacillus delbrueckii ssp. *delbrueckii*	TUM BP 000-2968	negative
Lactobacillus dextrinicus	TUM BP 000-2987	negative
Lactobacillus fermentum	TUM BP 000-2069	negative
Lactobacillus frisingensis	TUM BP 130919043-2789	negative
Lactobacillus fructivorans	TUM BP 000-2038	negative
Lactobacillus gasseri	TUM BP 000-2970	negative
Lactobacillus ghanensis	TUM BP 000-2931	negative
Lactobacillus harbinensis	TUM BP 120906016-2993	negative
Lactobacillus helveticus	TUM BP 000-2971	negative
Lactobacillus hilgardii	TUM BP 000-2975	negative
Lactobacillus johnsonii	TUM BP 000-2972 (BS 224)	negative
Lactobacillus kefiri	TUM BP 000-2037	negative
Lactobacillus lindneri	TUM BP 121213056-2397	negative
Lactobacillus malefermentans	TUM BP 000-2974	negative
Lactobacillus parabrevis T	TUM BP 000-2080 (DSMZ 20531)	negative
Lactobacillus parabuchneri	TUM BP 121008043-2282	negative
Lactobacillus paracollinoides	TUM BP 150113003-2371	negative
Lactobacillus perolens	TUM BP 130000240-2596	negative
Lactobacillus plantarum	TUM BP 121121170-2217	negative
Lactobacillus reuteri	TUM BP 000-2055 (BS 227)	negative
Lactobacillus rhamnosus	TUM BP 000-2996	negative
Lactobacillus rossii	TUM BP 130806019-2754	negative
Lactobacillus salivarius	TUM BP 000-2997	negative
Lactobacillus sanfranciscensis	TUM BP 000-2982	negative
Lactococcus lactis	TUM BP 000-8973	negative
Leuconostoc mesenteroides	TUM BP 000-0983	negative
Megasphaera cerevisiae	TUM BP 121011015-5986	negative

Meyerozyma guilliermondii	TUM YP 000-0041	negative
Micrococcus luteus	TUM BP 000-0995	negative
Oenococcus oeni	TUM BP 000-0013	negative
Pantoea dispersa	TUM BP 000-0992	negative
Pectinatus cerevisiiphilus	TUM BP 120919033-4402	negative
Pectinatus frisingensis	TUM BP 000-4327	negative
Pectinatus haikarae	TUM BP 120919239-4404	negative
Pediococcus clausenii	TUM BP 000-3986	negative
Pediococcus damnosus	TUM BP 140313142-2243	negative
Pediococcus inopinatus	TUM BP 000-3984	negative
Pediococcus pentosaceus	TUM BP 000-3985	negative
Pichia membranifaciens	TUM YP 000-2009	negative
Pseudomonas poae	TUM BP 000-7057	negative
Pseudomons fluorescens	DSM 50090 (BS236)	negative
Rhodosporidium toruloides	TUM YP 000-0110 (DSMZ 70398)	positive (ct 27.45)
Rhodotorula mucilaginosa	BLQ 16-D-2	positive (ct 28.34)
Rhodotorula mucilaginosa	BLQ 16-F-2	positive (ct 28.22)
Rhodotorula mucilaginosa	BLQ 16-L-2	positive (ct 29.04)
Rhodotorula mucilaginosa	BLQ 17-A-3	positive (ct 27.81)
Rhodotorula mucilaginosa	BLQ 17-H-9	positive (ct 29.62)
Rhodotorula mucilaginosa	BLQ 17-J-9	positive (ct 28.83)
Rhodotorula mucilaginosa	BLQ 15-F-6	positive (ct 28.89)
Rhodotorula mucilaginosa	TUM YP 120306011-7159	positive (ct 27.77)
Rhodotorula sloffiae	BLQ 17-F-2	negative
Saccharomyces bayanus	TUM YP 000-1999	negative
Saccharomyces cerevisiae* var. *diastaticus	TUM YP 000-1042 (DSM 70487)	negative
***Saccharomyces cerevisiae* TUM 184**	TUM YP 000-1001	negative
***Saccharomyces cerevisiae* TUM 66/70**	TUM YP 000-1044	negative
***Saccharomyces cerevisiae* TUM 68**	TUM YP 000-1045	negative
Saccharomyces paradoxus	TUM YP 000-1043 (BS 11 (WYSC 63))	negative
Saccharomyces pastorianus	TUM YP 000-1010	negative
***Saccharomyces pastorianus* TUM 34/70**	TUM YP 000-1008	negative
***Saccharomyces pastorianus* TUM 34/78**	TUM YP 000-1010	negative
Saccharomyces uvarum	TUM YP 000-1090	negative
Saccharomycodes ludwigii	TUM YP 000-0046 (SL17)	negative
Schizosaccharomyces pombe	TUM YP 000-0039	negative
Selenomonas lacticifex	TUM BP 000-0998	negative
Torulaspora delbrueckii	TUM YP 000-0003	negative
Weissella paramesenteroides	TUM BP 000-0988	negative
Weissella viridescens	TUM BP 000-0989 (BS 198)	negative
Wickerhamomyces anomalus	TUM YP 000-2004	negative
Zygosaccharomyces bailii	TUM YP 000-5094	negative
Zygosaccharomyces rouxii	TUM YP 000-5092	negative
Zymomonas mobilis	TUM BP 000-0036 (DSMZ 424)	negative

TUM strains: strain collection of spoilage organisms of Research Center Weihenstephan for Brewing and Food Quality, TUM

BLQ strains: strain-collection of Yeast Center, Research Center Weihenstephan for Brewing and Food Quality, TUM

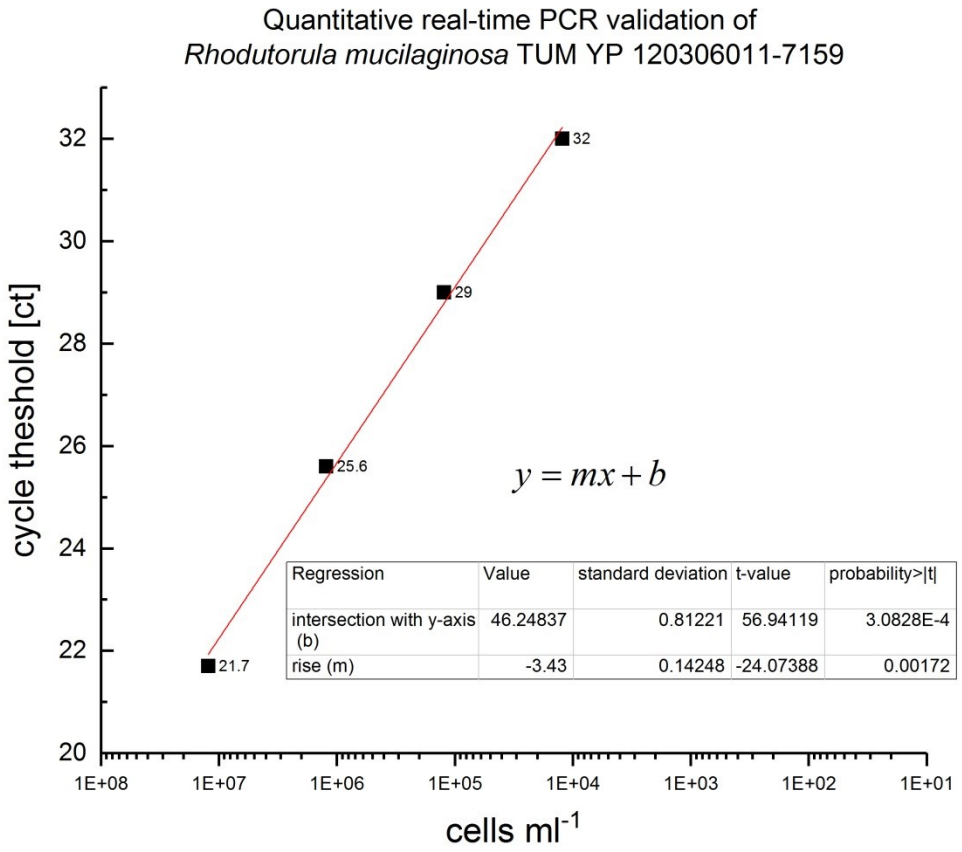

Figure 2: Publication 3, Supporting Information 4: Quantitative validation of real-time PCR system for Rhodotorula mucilaginosa

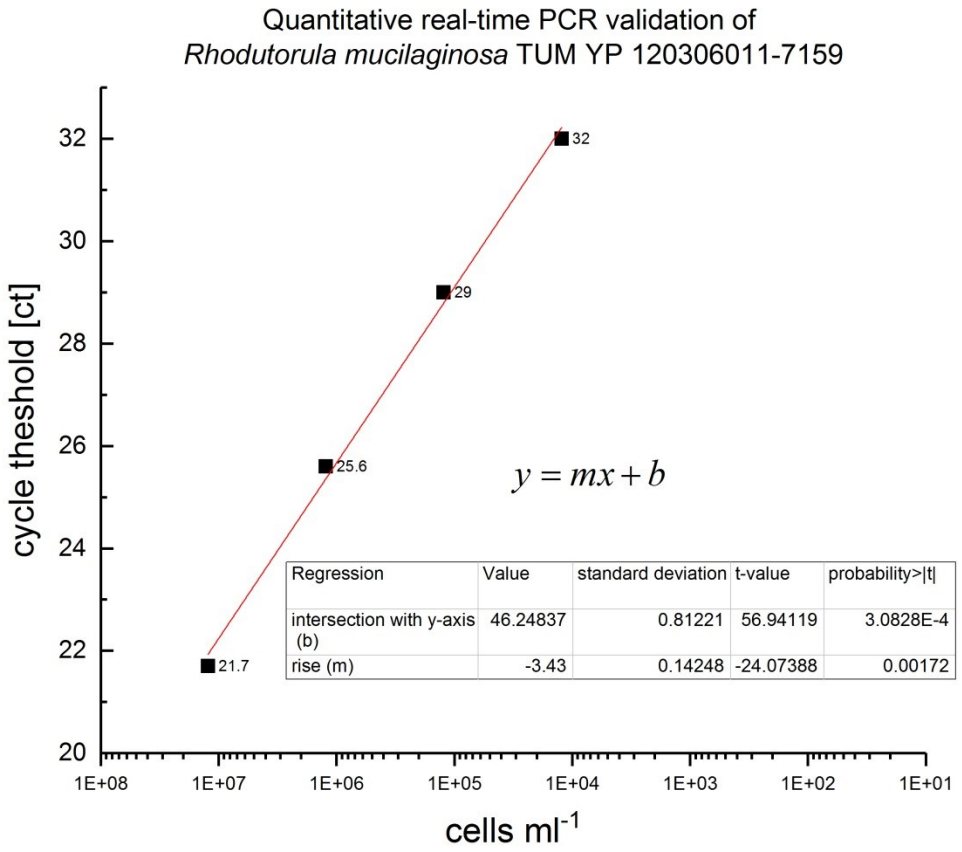

Figure 3: Publication 3, Supporting Information 4: Quantitative validation of real-time PCR system for *Rhodotorula mucilaginosa*

3. Discussion

Biofilms are a serious problem in breweries and are the main reason for secondary microbial contaminations in the filling department. Biofilms present a habitat and breeding ground for beer-spoiling bacteria [2, 3, 5, 6, 115, 117, 119].

The main goal of this dissertation was to develop a fast modular method for monitoring brewery-bound biofilms using selected indicator species. The method is composed of a classical swab culture method as already established in breweries with NBB-B-AM [2, 3, 5, 6], a species-specific Taq-Man® based real-time PCR detection. Species variation within biofilms according to different products (e.g. low-hopped beer types, alcohol-free beer or beer mix beverages) were considered by choosing different enrichment media and choosing different indicator species for real-time PCR detection. The developed and used media are designed to meet the growth requirements of species expected to grow in the product and on equipment surfaces.

For this dissertation, two scenarios for products that were more sensitive to microbial spoilage were selected. Acetic acid bacteria are reported as being a representative group in young biofilms bound to brewing equipment [2, 3, 5, 6], subsequent studies were not able to confirm these findings [115, 118, 130-132]. For this reason, the group of acetic acid bacteria was not further considered as indicator organisms in this dissertation.

The first scenario focuses on low-hopped beer types, represented by Bavarian Wheat Beer (2.2). Due to the lower concentration of hop bitter acids and the richer substrate (especially in unfiltered wheat beer), lactic acid bacteria, normally of no spoilage relevance in beer and categorised as potential spoilers may be able to spoil the product [2, 6, 55, 57]. Selected species for this scenario were *Lactobacillus acetotolerans* and *Lactobacillus rossiae* as representatives of bacteria already reported to have specific beer-spoilage potential in low-hopped beer and wheat beer [27, 55, 57, 58, 100]. *Lactobacillus rossiae*, originally isolated from sourdough [20] often appears in breweries as a persistent biofilm and is considered to be relevant to biofilm due to its slime-forming potential [57]. *Lactococcus lactis* and *Leuconostoc mesenteroides*, being described as potential beer-spoiling bacteria and strongly biofilm-forming species [2, 6, 30, 78] with spoilage incidents only reported in alcohol-free beer [2, 6], were selected as indicator species for early lactic acid bacteria biofilms [2, 6, 30, 93]. An enrichment medium was developed, providing comparable growth factors as Bavarian Wheat Beer may provide, taking into account the specific oxygen quencher L-cysteine monohydrochloride to overcome the viable, but putatively non-culturable state (VPNC) described in detail for

Lactobacillus acetotolerans [27, 28, 100] (Wheat Beer media by Hutzler and Riedl (WBM-HR)). The medium was validated successfully as broth for hygienic monitoring, as well as agar plate medium, using a selection of isolates of target and non-target germs. The developed TaqMan® based real-time PCR systems proved to be highly specific and worked compatibly with the developed medium. The relevance for biofilm monitoring of the specified lactic acid bacteria species was confirmed by the biofilm-formation potential in the nutrient-rich medium MRS, already described as a standard medium to investigate the biofilm-forming potential of lactic acid bacteria [30]. *Lactococcus lactis* and *Leuconostoc mesenteroides* proved they were capable of forming at least weak biofilms, making them perfect indicator germs for lactic acid biofilms yet to spoil the product. Given that a biofilm provides a habitat for other species, high cell counts of these species on brewing equipment may indicate a serious hygienic problem in the build-up. *Lactobacillus acetotolerans* did not show measurable biofilm formation, sustaining the thesis that this species mostly originates from the brewery sections that contain yeast such as fermentation tanks and vessels in the case of contamination cases as described by Deng [27]. *Lactobacillus rossiae* showed high variances in its biofilm-formation potential. The three tested isolates showed a heterogenic biofilm-formation potential, ranging from no potential at all to moderate biofilm potential. Heterogenic phenotypes with regard to biofilm-formation potential could be observed in different strains of this species. *Lactobacillus rossiae* has already been described as having a wide intra-species variation of biochemical properties and RAPD genotypes [29], which may also include biofilm formation. The tested isolates were isolated from the brewery environment, possibly leading to a different biofilm-formation potential using media more similar to the substrate used in the brewery or leading to biofilm formation generated by the interaction of different species as already observed [43, 72]. The same heterogeneity of biofilm-formation potential was observed with the tested isolates of *Lactobacillus brevis*, which were originally only used as a target germ for positive control with expected high growth in the used medium. *Lactobacillus brevis* was not anticipated to be an initial biofilm former, as this species was described as the sole biofilm coloniser in earlier publications [2, 3, 5, 6].

As *Lactobacillus brevis* is the most frequently found spoilage species in beer, the finding that some isolates of this species displayed an initial biofilm-formation potential was quite concerning. A more in-depth study was conducted (2.3) on the variance of this species in biofilm-formation potential, beer spoilage (e.g. growth in different selective media) and genetic rep-PCR Fingerprint ($(GTG)_5$ Primer set) [32, 140]. The rep-PCR fingerprinting was considered to be a second level of identification within the modular hygienic monitoring based in this dissertation. Therefore the rep-CR primer set $(GTG)_5$

was used, as this set is described as highly differentiating for a wide range of species [23, 32, 45, 110, 126]. A set of isolates from the brewing environment, identified as *Lactobacillus brevis* was differentiated, using the REP PCR primer set GTG_5, as well as phenotypes, represented by growth in different types of selective media (e.g. various strong hopped beers) and biofilm formation in the same. It was not possible to make a direct correlation between the fingerprint profile and the growth profile. Properties such as antibiotic resistances and tolerances, especially to hop compounds that are partly responsible for growth in beer, are plasmid encoded (horA [60, 125], horC [60, 61], hitA [50]. The REP-PCR system used $(GTG)_5$ only amplifies genomic DNA and does not represent genetic markers, which makes plasmid-located genetic markers virtually invisible with this method. It was possible to differentiate stable genetic identities of *Lactobacillus brevis* that can be monitored within the brewing process or between different brewing sites. Non-target species were also tested and it was possible to clearly differentiate those (e.g. *Pediococcus damnosus*, *Lactobacillus casei*/*paracasei*) from the fingerprints of the tested isolates of *Lactobacillus brevis*. Interestingly, isolates recorded as forming slime in bottled beer were not automatically strong biofilm formers. On the contrary, most isolates described as having produced slime in the sample they were isolated from were weak biofilm formers. Slime, a form of exopolymeric substance (EPS) may be an essential part of biofilms and can enable higher resistances [121], but does not necessarily enhance biofilm formation. It was also very interesting that four isolates showed no measurable rise in optical density by cell material in any of the tested media, while the biofilm test (adsorbed and spectral photometrically measured crystal violet) was positive. The biofilm formation was confirmed by direct light microscopy. These findings indicate that there was no measurable growth, but cells that were inoculated in this experiment started adhering to the surface of the microtiter wells without growth. This effect seems to be a reaction of the tested isolate to the applied medium. Biofilm formation is also described as being a protective reaction to a hostile environment [38].
For this reason, the cell adherence may be a reaction to stress factors in the medium or a lack of essential nutrients. This may be observed by hop compounds as well as by osmotic stress, as this effect was not only seen in stronger hopped beer, but also in the medium MRS. In beer, only one isolate grew significantly within 24 hours. Depending on the beer attributes, what is known as the forcing test could take up to three months [107]. In conclusion, a negative result for growth potential does not exclude absolute beer-spoiling potential. In general, various *Lactobacillus brevis* isolates are able to initially form biofilms in various media. Hence, depending on the isolate and substrate, biofilms can build up in different areas of breweries. Their initial creation is not necessarily dependent upon other species, a fact that has not been reported to date.

Diluted beer, in particular, as it appears in between process cleaning steps and in the filler environment, seems to be a good medium for biofilm formation. The generally higher biofilm formation with higher nutrients as described by Sutherland et al [121] could not be observed. Generating a growth potential and biofilm formation profile of isolated *Lactobacillus brevis* isolates can help to identify points in the process that permit persistent biofilm formation (depending on the distribution of diluted process media throughout the process). It can also help to estimate the product spoilage potential.

Combined with genetic fingerprinting, identities can be tracked through the process, isolating the most probable contamination source. To prove this, a field study was conducted in a brewery with persistent contamination of *Lactobacillus brevis*. Tracking fingerprint types, 11 stable clusters could be found, which were associated with specific sampling points. Each cluster was tracked through the brewing process and the earliest specific sampling point was identified as a potential contamination source. Most clusters, while found throughout the process, did not appear in an unbroken line from a specific sampling point in the process flow. There are various reasons for this phenomenon. Firstly, single critical sampling point control provides only a snapshot of the dynamic microbiological situation in the brewery. Secondly, it is very difficult to isolate mixed strains from single samples. Some strains might originally be in the collected samples but could not be isolated because they were overgrown by other species or strains in the further sample processing. It is worth mentioning that the appearance of one fingerprint type in the water treatment samples indicated a possible entry of microorganisms into the process through process water. Further inspection of the water treatment equipment showed shortcomings in construction, which could be resolved after the investigation. As this fingerprint type could also be found in the beer directly before flash pasteurisation, it is clearly possible for this fingerprint type to contaminate the product. The insufficient water treatment is one possible contamination source. The diversity of the identified fingerprint types indicates that the microbiological situation in the tested brewery is clearly not the result of a single microbiological contamination event. It is more likely that there are multiple sources of spoilage bacteria entering the process. In general, the differentiation of *Lactobacillus brevis* proved to be a valuable tool in hygiene monitoring and determining contamination sources. The high number of different genotypes and therefore potentially variant physiologies that could be found proves the high diversity of *Lactobacillus brevis* in brewery isolates. Depending on the physiological properties (e.g. growth, slime formation and biofilm formation), it is essential to consider the spoilage potential of this species in terms of the various contributing factors, as well as the potential countermeasures.

The second scenario of a more sensitive product includes alcohol-free and beer-mix beverages (2.4). In addition to bacterial spoilage, these product groups are susceptible to yeast spoilage. In brewery biofilms, yeasts are often described as late colonisers and a relevant part of mature biofilms [2, 3, 5, 6, 115, 117, 133]. As they are not only able to colonise existing fungal or bacterial biofilms, but can also form those initial biofilms [115, 117, 133] and also due to the high relevance of yeasts in the beverage industry [31, 59], a yeast-specific hygiene monitoring was developed. This focused particularly on yeasts that can form initial biofilms or are ubiquitous in the production process. Hygienic monitoring media such as NBB-B-AM do contain pH indicator dyes to indicate the growth of biofilm-relevant bacteria [2, 3, 5, 6]. Since yeasts grow slower than bacteria, this results in a drop in pH that is slower to detect compared with a media that targets bacteria. Resazurin is described as a highly sensitive redox indicator of cell activity in culture media, and it can be measured using either colorimetry or fluorimetry, with the latter being the more sensitive method. The oxidised resazurin (blue) is reduced enzymatically by living cells, in two steps, to the fluorescent active resorufin (pink, fluorescent) and to dihydroresorufin. The reduction reaction correlates strongly with the cell number and is already used to detect microbiological contamination in milk. Further studies have used resazurin as a growth indicator, and they did not show any negative effect on cell growth, which enables the use of cultured cells for further analysis [89]. A medium based on MYPG was therefore developed, using resazurin as an indicator dye for hygienic monitoring in a similar way to NBB-B-AM. Tetracycline was added to suppress uncontrolled bacterial growth. Using resazurin in a fluorescence assay proved to be too sensitive with higher cell densities or cell activity, only the visually colorimetric method (reduction of the blue resazurin dye) appeared to be an easy-to-use method for detecting yeast growth. The medium was successfully tested as a medium for hygienic monitoring using a setup that simulates biofilms with a defined cell density. The species *Wickerhamomyces anomalus*, *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae* TUM 68, *Saccharomyces pastorianus* var. *carlsbergensis* TUM 34/70, *Dekkera anomala* and *Saccharomyces cerevisiae* var. *diastaticus* were selected as indicator species for yeast biofilm. The reason for this selection was either omnipresence of the species in the brewing process, already described initial biofilm formation or high spoilage potential. Compared with NBB-B-AM, the medium was also able to semi-quantify the amount of cells via the time needed for the indicator dye to change colour. The used culture yeast strains (*Saccharomyces cerevisiae* TUM 68 and *Saccharomyces pastorianus* var. *carlsbergensis* TUM 34/70) grew much slower than the wild yeast strains used. A colour indicator reaction within the three-day threshold can therefore be linked either to the presence of non-*Saccharomyces* wild yeasts or to higher cell numbers of

Saccharomyces brewing yeasts. A scheme is proposed that identifies risk levels that correspond to the time until indicator change. The data supports a high potential of the modified MYPG broth as a hygienic indicator medium for yeast biofilms in a brewing environment. The time at which the colour changes can help to estimate the level of contamination on the tested surface. The yeast physiology is not compromised by the indicator dye and further analyses are possible, especially real-time PCR analyses as described later. For the specific detection and identification of the selected indicator yeasts, real-time PCR assays were used as published for *Wickerhamomyces anomalus*, *Saccharomyces cerevisiae* TUM 68 [53, 54], *Saccharomyces pastorianus* var. *carlsbergensis* TUM 34/70, [13, 53, 54] *D. anomala* [13] and *Saccharomyces cerevisiae* var. *diastaticus* [13, 111] TaqManTM-based real-time PCR systems were used as published by Hutzler and Brandl. [13, 53, 54] The real-time PCR system for *Rhodotorula mucilaginosa* was newly developed and validated for this dissertation. The validation of the real-time PCR system for *Rhodotorula mucilaginosa* showed the simultaneous detection of *Rhodotorula toruloides*. This does not prevent the use of the system to validate hygienic monitoring samples, since the interpretation of the hygienic monitoring results is the same for both species. The calculated relative specificity, relative accuracy, and relative sensitivity and efficiency are comparable to real-time PCR systems developed by Hutzler [53, 54] and Brandl [13]. The developed real-time PCR system for the detection of *Rhodotorula mucilaginosa* proved to be compatible to the systems developed by both authors and are therefore used in combination in this study.

To confirm biofilm relevance, isolate sets of these species were tested for biofilm formation in MYPG in the stationary phase. Contrary to previous studies, the tested strains of *Dekkera anomala* show no observable biofilm formation in the stationary phase. Biofilm production is already described as being strain dependent for this species; both species might lack any biofilm-forming potential [115]. As both strains were isolated from a brewing environment and the experiment settings include a rather complex and nutrient-rich medium, biofilm formation in a beer-like matrix might be different. For *Saccharomyces* yeast, biofilm formation is stronger with glucose-starved cells [33, 101]. A similar effect is possible with *Dekkera anomala*. It is also reported that multi-species biofilms with yeasts and bacteria are more stable than biofilms that consist of single species, which may result in *Dekkera anomala* having a stronger biofilm-forming potential in the presence of bacteria [43, 72]. Swab samples of beverage production environment that tested positive for this species should be viewed very critically due to the spoilage potential of this species in beer and other beverages. The weak initial biofilm-forming potential means that finding this species in biofilms may indicate mature biofilms that have already been colonised by weaker or non-biofilm-

forming organisms. *Rhodotorula mucilaginosa* shows the potential to form biofilms initially. This species is not considered to be a product-spoilage organism for non-alcoholic carbonated beverages and is considered to be a potential spoilage organism for non-carbonated beverages [2, 3, 6, 59]. The tested strains were collected in the brewing environment from surfaces associated with biofilm formation. The detection of this species in biofilms is an indicator of young biofilm formation and inefficient cleaning, as it can build biofilms *de novo* given sufficient nutrients. *Saccharomyces cerevisiae* TUM 68 and *Saccharomyces pastorianus* var. *carlsbergensis* TUM 34/70 are exemplary brewing yeasts. Due to their use as starter cultures, these strains are omnipresent in breweries and can spoil non-alcoholic beverages as strong fermenters. It is quite possible to find these yeasts during hygienic monitoring in the filling department, since it is possible for cells to slip through filtration or be introduced to the filling department when filling unfiltered, unpasteurised beer. The weak initial biofilm potential of both strains indicates that the detection of brewing yeast strains in the filling area is a sign of mature biofilms that have already been colonised by later-stage biofilm-colonising organisms. The tendency of starved *Saccharomyces cerevisiae* cells to build stronger biofilms under the influence of polysaccharides such as alginates indicates a strong ability to colonise existing bacterial or fungal biofilms and supports this theory. This would also explain why *Saccharomyces* yeasts could not be found in young biofilms in earlier studies [116]. As the test for biofilm formation is conducted in a glucose-rich medium, the biofilm formation of these strains in a medium with minimal glucose would result in a stronger biofilm formation due to a stronger expression of the Flo11 gene [33]. This might be a real issue for breweries that fill both beer and other beverages using the same equipment. The two tested strains of *Saccharomyces cerevisiae* var. *diastaticus* show biofilm formation in various strengths. The detection of this species in hygienic monitoring samples should be regarded as highly critical. The high biofilm-forming potential and spoilage potential represent a high risk to product safety if this yeast is found in the filling department. Being able to initially form biofilms, this variety may settle persistently within the process. As this species can also spoil fermented beer with a high attenuation, this species is also relevant to regular beer types. *Wickerhamomyces anomalus* has already been described as a biofilm-forming species [134], which was confirmed in this study. As one of the most commonly found yeast species in the brewery environment, together with its high initial biofilm-formation potential, the detection of this species can be seen as an indicator of biofilm formation and insufficient cleaning. The sole detection of this species indicates young biofilms, while in combination with other species that are known to solely colonise biofilms, this finding

might indicate mature yeast biofilms. The risk of product spoilage is low, as this species is described as a weak fermenter.

Three field studies were conducted applying the described yeast hygienic monitoring. The first compared swab samples in MYPG with resazurin and the common medium NBB-B-AM on a bottle filler that filled strong German beer. MYPG with resazurin was able to detect comparable biofilm maturation levels. In general, the modified MYPG seems to be more sensitive than NBB-B-A. The differences in the distributions of sample points with positive microbiological findings may be a result of the media composition and the filled product. The modified MYPG contains tetracycline to suppress bacterial growth, while NBB-B-AM contains a yeast inhibitor, not described in the following. While NBB-B-AM favours bacterial growth, MYPG promotes fungal growth. Overall, MYPG proved to be a useful medium for swab samples in the brewery environment, especially when handling beverages that are sensitive to fungal spoilage. Adapting the yeast hygienic monitoring with real-time PCR detection of the selected indicator species on a 5l can filler, it was possible to detect yeast biofilms in the filling environment. The biofilms were found at locations with no direct product contact, but were typical sites with complex construction, cleanability and possible product residues. The results give an overview of plant hygiene and the cleanability of the tested sampling points. The detection of *Wickerhamomyces anomalus* and *Rhodotorula mucilaginosa* indicated young biofilm formation at the affected sampling points. Positions with constant product leakage from the filling overflow showed low ct, meaning high cell numbers. This demonstrates the correlation between real-time PCR findings and the suspected perfect environment for biofilm build-up. After the rinsing step, the reduction of samples with positive findings and the increase in the ct at the locations still giving positive findings, mirrors the decrease in cells at the same time, attesting a thinning but not completely cleansing effect through this step. Detecting *Wickerhamomyces anomalus* in this position is not problematic due to the low spoilage potential, as well as the fact that it is not in direct contact with the product. Nevertheless, mature biofilms with product spoilers may indicate a risk, as biofilm particles may be transferred by aerosols or spray water.

The third field study combined the proposed hygienic monitoring using the MYPG with resazurin and tetracycline with the specific detection using real-time PCR in a brewery that had an actual problem with contamination of *Saccharomyces cerevisiae* var. *diastaticus*. It was possible to isolate the capper, sealer and stamps as possible contamination sources of this species. The capper is known to be critical to biofilm formation [5]. The complex mechanics are likely to have dead spaces and product aerosols from HPI (high pressure injection) and friction dust from the cap conveyors can merge into a nutrient-rich sludge, which settles in dead spaces. Some sampling points

also showed findings with yeast after the rinsing step, but without detection of *Saccharomyces cerevisiae* var. *diastaticus*. This indicates a transfer of yeast species that are more robust to the rinsing conditions from other areas by the rinsing step.

After these findings, the capper was completely dismantled and heavy biofilm formation was found within the inner mechanics of the capper. After thorough cleaning and sanitisation of the caper mechanics, it was no longer possible to detect *Saccharomyces cerevisiae* var. *diastaticus*. The combination of modified MYPG swab samples and real-time PCR identification proved to be useful for the microbiological stage-by-stage control in the context of hygienic problems with yeasts in the filling area.

In summary, the combination swab samples, further incubated on biofilm microorganism-specific media, and specific, semi-quantitative real-time PCR for selected indicator species was successful as detailed hygienic monitoring in breweries. The selected species are not a complete list of biofilm-relevant species and additions to this list may be made. The real-time PCR methods used in this dissertation are designed to be compatible with those of Hutzler [53, 54] and Brandl [12], enabling the addition of spoilage-relevant species at any time and to run the new setup in the same real-time PCR assay. Differentiation of the detected species at a deeper than species level proved to be a very useful and powerful tool to identify the spoilage source within the production process.

For further research it might be interesting to include screenings for biofilm-relevant genes such as the biofilm-relevant version of FLO11 in *Saccharomyces cerevisiae* var. *diastaticus* to obtain information on biofilm potential faster and more easily.

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RIEDL, R., DÜNZER, N., MICHEL, M.;JACOB, F., HUTZLER, M. (2019): **'Beer enemy number one: genetic diversity, physiology and biofilm formation of Lactobacillus brevis'**. J INST BREW 2019, DOI 10.1002/JIB.553.

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