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Flavor design by advanced fermentation techniques for lactic acid fermented malt-based beverages

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Great fury, like great whisky, requires long fermentation.
−Truman Capote

Do lactic acid fermented malt-based beverages require also long fermentation?

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“Only one hand cannot fasten a bundle of wood” — African proverb.

Without your hand on this project, the course would have probably been different.

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Preface

This dissertation is submitted for the degree of “Doktor der Naturwissenschaften” at the Technical University of Munich. The research described here was conducted under the supervision of Prof. Dr. Thomas Becker at the Institute for Brewing and Beverage Technology from October 2012 to June 2017.

From the literature review, the functional and technological properties of lactic acid fermented malt-based beverages offer many advantages to beverage industrials to meet diversified consumer’s nutritional needs. However, the market remains in its infancy due to the lack of consumer’s acceptance and quasi-inexistence of production technology. The main problem outlined here is the flavor, which is yet to be improved as per consumer’s request. Therefore, the purpose of this thesis was twofold: first to highlight possible limitations in the production process that may cause poor flavor profile in lactic acid fermented malt wort-based beverages, secondly to design a process for flavor improvement of these beverages for the sake of consumer’s better acceptance. Furthermore, the results of this thesis open a new era in cereal-based beverages allowing food industrials to propose functional beverages with designed flavor for consumers in need such as lactose intolerant and vegan people. The investigations have led to several scientific publications.

Peer-reviewed publications

The following peer-reviewed publications are an entire part of this thesis (in reverse chronological order):

1. **Nsogning Dongmo, S.,** Fischer, S., Kollmannsberger, H., Becker, T. (2018). Exploration of high-gravity fermentation to improve lactic acid bacteria performance and consumer’s acceptance of malt wort-fermented beverages. *International Journal of Food Science & Technology*, 53, 1753-1759.
2. **Nsogning Dongmo, S.,** Fischer, S., Becker, T. (2018). Investigating on the fermentation behavior of six lactic acid bacteria strains in barley malt wort reveals limitation in key amino acids and buffer capacity. *Food Microbiology,* 73, *245-253.*
3. **Nsogning Dongmo, S.,** Sacher, B., Kollmannsberger, H., Becker, T. (2017). Key volatile aroma compounds of lactic acid fermented malt-based beverages – impact of lactic acid bacteria strains. *Food Chemistry,* 229, 565-573.

4. **Nsogning Dongmo, S.,** Procopio, S., Sacher, B., Becker, T. (2016). Flavor of lactic acid fermented malt based beverages: Current status and perspectives. *Trends in Food Science and Technology,* 54, 37-51.

Other peer-reviewed publications generated during this thesis

5. Fritsch, C., **Nsogning Dongmo, S.,** Sacher, B., Becker, T., Osen R. (2017). Development of a high-protein plant-based beverage by using an innovative combination of mashing and fermentation. *Agro FOOD Industry Hi-Tech,* 28, 40-44.
6. **Nsogning Dongmo, S.,** Merz, A., Schönenberg, S., Zarnkow, M., Becker, T. (2015). Use of exogenous enzymes and process management to improve the shelf life of the traditional opaque beer. *Journal of the American Society of Brewing Chemists,* 73, 22-28.
7. **Nsogning Dongmo, S.,** Womeni, H.M., Mbiapo Tchouanguep, F., Linder, M., Fanni, J., Zarnkow, M., Becker, T. (2014). Cooking and drying process optimization of Shea (*Butyrospermum parkii*) butter. *Czech Journal of Food Science,* 32, 578-584.
8. **Nsogning Dongmo, S.,** Niebauer, S., Zarnkow, M., Becker, T. (2012). Different influences on lautering performance and wort quality attributes when brewing with 100% unmalted barley. *Brewing Science,* 55-64.

Sorelle Dongmo Nsogning

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Abbreviations

Abbreviation	Meaning
°	Degree
°C	Degree Celsius
AEDA	Aroma Extract Dilution Analysis
B.	Bifidobacterium
BC	Buffering Capacity
CFDA	Carboxyfluorescein Diacetate
CFDA-SE	Carboxyfluorescein Diacetate - Succinimidyl Ester
CFU	Colony Forming Units
EFSA	European Food Safety Authority
FD factor	Flavor Dilution Factor
GC	Gas Chromatography
GC-MS	Gas Chromatography Equipped with a Mass Spectrometry
GC-O	Gas Chromatography Equipped with an Olfactometry
GC-FID	Gas Chromatography Equipped with a Flame Ionization Detection
HGF	High-Gravity Fermentation
HGW	High-Gravity Wort
HS-SPME	Headspace-Solid Phase Micro-Extraction
LAB	Lactic acid bacteria
LAF	Lactic Acid Fermentation
LAFCB	Lactic Acid Fermented Cereal-based Beverages
LAFMB	Lactic Acid Fermented Malt-based Beverages
L.	Lactobacillus
Lc.	Lactococcus
OAV	Odor Activity Value
OD	Optical Density
PDMS	Polydimethylsiloxane
PDMS/DVB	Polydimethylsiloxane/Divinylbenzene
PI	Propidium Iodide
RI	Retention Index
RT	Retention Time

Summarized overview

The health benefits and functional value of lactic acid fermented cereal-based beverages are well described to replace dairy-based products. However, research has been largely restricted to the laboratory scale, whereby industrial applications and market are quasi-inexistent. As per consumer's request, the flavour remains to be improved and represents the major challenge for the market development. This was the context of the main objective of this thesis: towards a designed process for flavor improvement in lactic acid fermented malt-based beverages (LAFMB) using brewing, fermentation and maturation processes.

So far, extended knowledge gaps on the aroma profile and fermentation process of LAFMB did not allow the understanding of the poor flavor. In this study, the poor flavor was ascribed to the low concentration of defined thirteen key aroma compounds and high organic acid content. Furthermore, significant cell death and metabolic activity reduction of lactic acid bacteria (LAB) occurred in malt wort due to the insufficient key amino acids content (up to 99% depletion) and buffering capacity of malt wort, and the accumulation of lactic acid. Under these conditions, LAB flocculating-like behavior of filamented dead cells clusters was observed.

Thus, the hypothesis that increasing the amino acid content and buffering capacity could extend the fermentation duration and thereby improving the aroma yield and flavor was tested. To this end, high-gravity fermentation (20 %w/w) brought along increased key amino acid content and buffering capacity that significantly improved LAB viability, aroma content, and consumer's acceptance. However, the acceptability remained low and the fermentation duration unchanged. Further, a successful evolutive adaptation of LAB cells from 1.2 g/L to 5 g/L lactic acid did not contribute significantly. Then, through mashing process management, malt wort amino acid content was increased by 48.5%, which further improved LAB growth but not the fermentation duration. Contrary to high-temperature fermentation (28°C), the combination of fed-batch with fermentation temperature reduction to 18°C maintained a high number of cells viable at extended fermentation to up to ten days. The implication was a low lactic acid production, but more esters and aroma formation that were subsequently increased during maturation step. Finally, maturation conditions proved to be decisive for the aroma content, which resulted in distinct flavors.

The results of this thesis develop a process to design and improve the flavor of LAFMB by combining mashing, fermentation, and maturation processes. This process is cost-efficient and simple for industrial applications.

Zusammenfassung

Der funktionelle und gesundheitsbezogene Wert der Milchsäurefermentation malzbasierter Getränke als Ersatz zu Getränken auf Milchbasis ist bereits ausgiebig beschrieben. Der Forschungsschwerpunkt begrenzte sich allerdings bislang auf Laborversuche, industrielle Applikationen sowie eine Etablierung der Produkte im Markt sind quasi nicht vorhanden. Vor allem in Bezug auf den Konsumenten muss der Geschmack deutlich verbessert werden, was die Hauptaufgabe für die Marktentwicklung darstellt. Daher ist es das Ziel dieser Studie, ein Verfahren zur Geschmacksverbesserung malzbasierter Milchsäuregetränke (LAFMB), unter Verwendung von Brau-, Fermentations- und Reifungsprozessen, zu entwickeln.

Derzeit bestehen große Wissenslücken in Bezug auf das Aromaprofil, den Fermentationsstämmen, der Substratzusammensetzung und dem Fermentationsprozess von LAFMB. Wie sich in dieser Studie zeigte, trugen die geringen Konzentrationen dreizehn vordefinierter Schlüssel-Aromakomponenten sowie der hohe Gehalt an organischen Säuren maßgeblich zu dem mangelhaften olfaktorischen Gesamteindruck bei. Die Schlüssel-Aminosäuren wurden bis zu 98 % durch die Milchsäurebakterien (LAB) verstoffwechselt und der Milchsäuregehalt signifikant erhöht, was zu einer verringerten Vitalität und zu einem signifikant schnellen Anstieg des Totzellenanteils führte. Ferner ist die in der Würze vorherrschende Pufferkapazität nicht optimal in Bezug auf die Milchsäurebildung. Unter diesen Bedingungen wurde ebenfalls ein erhöhtes Flokkulationsverhalten beobachtet.

Daraus resultierte die Hypothese, dass eine Erhöhung der relevanten Aminosäuren und Pufferkapazität eine Verlängerung der Fermentationsdauer herbeiführt, was zu einer Verbesserung der Aromabildung führt. Durch eine High-Gravity Fermentation (20 GG-%) konnte ein erhöhter Gehalt an Aminosäuren erzielt und die Pufferkapazität angehoben werden. Folglich wurde eine signifikante Verbesserung der LAB Viabilität, des Aromas sowie der Konsumentenakzeptanz erreicht. Dennoch blieb die Akzeptanz, absolut betrachtet, niedrig und die Fermentationsdauer unverändert. Auch eine evolutive Adaption an eine erhöhte Konzentration an Milchsäure von 1,7 g/l auf 5 g/l resultierte in keiner signifikanten Änderung. Allerdings führte die Erhöhung der freien Aminosäuren um 48,5 %, durch Anpassung des Maischregimes, zu einem erhöhten LAB-Wachstum, wobei wieder die Fermentationsdauer nicht beeinflusst wurde. Durch eine Kombination aus Fed-Batch und der Reduzierung der Fermentationstemperatur von 28 °C auf 18 °C konnte eine längere Fermentationsdauer von bis zu zehn Tagen erzielt werden. Dies trug zu einer

Verbesserung der Aromabildung, vor allem der Ester, bei gleichzeitiger Reduzierung der Milchsäureproduktion, bei. Abschließend zeigte sich deutlich, dass die Reifungskonditionen relevant für die Aromaentwicklung in der LAFMB-Herstellung sind, welche zu individuellen Aromen führten.

Die Dissertation zeigt, dass durch technologische Anpassung des Maisch-, Fermentations- und Reifungsprozesses, der Geschmack von LAFMB signifikant verbessert werden kann. Die Technologie ist kosteneffizient und einfach in industriellen Prozessen integrierbar.

1. Introduction and purpose of the study

In the context of this study, lactic acid fermented cereal-based beverages (LAFCB) are produced from the fermentation of cereal-based substrates by lactic acid bacteria (LAB). Research indicates that LAFCB possess high nutritional and functional values, which confer them a significant market potential. However, the flavor remains to be improved for consumer's acceptance and market development. Yet, the flavor of a food is determinant for its acceptance and consumer's decision to buy. That implies the improvement of LAFCB flavor, as aimed in this study, will ensure its market development and its availability to consumers.

Under this chapter, the state-of-the-art in research of LAFCB flavor and existing knowledge gap are reviewed in section 1.1. Following, the approaches that were considered so far for flavor improvement of foods based on lactic acid fermentation (LAF) are described in section 1.2. The purpose of the study including the research question, hypotheses, and main findings is stated in section 1.3.

1.1. State-of-the-art in research and limitations

Under this section, subsections are focused consecutively on the market potential of LAFCB, safety, functional properties and flavor of lactic acid fermented malt-based beverages (LAFMB). The aroma compound formation in lactic acid fermentation is described ultimately.

Consumer's trends and market potential of LAFCB

"A product that does not meet consumer's needs or expectations will fail to develop on the market" (Konsumforschung-GfK, 2017). Therefore, understanding consumer needs ensures the market development. In general, consumers search for healthier and natural foods. In this regard, the actual top consumer's trends in the beverage sector are oriented towards functional beverages with attributes such as naturalness, health functionalities, clean label, with better-quality vital ingredients and simple formulas, and without additives (Watrous 2017, Gilbert 2017, Avery 2016). That is typically observed in: i) the steady increase of the global market of functional beverage around the world (Fortitech 2011, Granato *et al,.* 2010), ii) the continuous decline of carbonated soft drink consumption, and iii) the increase of 5.2% segment volume of plant-based beverages from 2015 to 2016 (Mascaraque 2017, Avery 2016). Furthermore, non-dairy functional beverages are

specifically in demand to meet diversified consumer's choices and health conditions such as lactose intolerance, veganism, low cholesterol and low-fat diet (Granato *et al,.* 2010).

Indeed, the tremendous functionalities of lactic acid fermented cereal-based beverages (LAFCB) as functional beverages to replace dairy products have been well described in research (Angelov *et al,.* 2005, Angelov *et al,.* 2006, Charalampopoulos *et al,.* 2002, Coda *et al,.* 2011, Granato *et al,.* 2010). LAFCB are based on combined functional raw material like cereals and natural ancient processes such as fermentation and brewing. Furthermore, lactic acid fermentation confers to the resulting product a natural preservation through pH lowering and antimicrobial formation that may exclude the need for preservatives use. Thus, LAFCB meet those above-mentioned diversified consumer's expectancies and have therefore a high market potential.

However, the market of LAFCB is quasi non-existing although there exists already a plethora of traditionally produced natural cereal-based beverages. Case studies of LAFCB on the market include probiotic beverages, protein-rich beverages based on malt wort and lupine protein (Fritsch *et al,.* 2017). Another example is a cereal based refreshing-like functional beverage Bionade based on Gluconobacter fermentation of malt wort. Still, consumers requested an improvement of the flavor (Yu and Bogue 2013). Therefore, improving the flavor of LAFCB will increase consumer's acceptance of LAFCB thereby boosting the market development. In this regard, the flavor of LAFCB will be reviewed in the next section but before that, safety concerns and the nutritional and functional values of LAFCB are presented.

Safety concerns of LAFCB

According to the European Food Safety Authority (EFSA), the Qualitative Presumption of Safety (QPS) is assigned as follow: "If the taxonomic group did not raise safety concerns or, if safety concerns existed, but could be defined and excluded, the grouping could be granted QPS status. Thereafter, any strain of microorganism the identity of which could be unambiguously established and assigned to a Qualified Presumption of Safety (QPS) group would be freed from the need for further safety assessment other than satisfying any qualifications specified." (EFSA 2007). Thus, a strain from traditional fermented foods that have been identified and not known to have some pathogenic properties can be attributed a QPS status. Recognised and proposed lactobacillus strains for QPS status from the European Food Safety Authority (EFSA) include, among others, *Lactobacillus plantarum*, *Lactobacillus amylolyticus*, *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus reuteri*, *Lactobacillus acidophilus*, *and Pediococcus pentosaceus*. These

strains are the most used in cereal fermentation for beverage production (see Fig. 1). Besides the QPS status, they also possess the “Generally Recognized as Safe” Status (GRAS-Status) of the U.S. Food and Drug Administration (FDA 2017). That implies LAB strains belonging to the above-mentioned list could be safely used in the traditional production of cereal-based beverages.

Possible drawbacks or safety concerns in LAFMB may regard cereals content in antinutritional compounds. However, processing techniques are known to significantly reduce their content. Indeed, phytic acid, oxalates, trypsin inhibitor, tannins and cyanogenic glucoside are antinutritional factors reported in cereals. Among them, phytic acid and tannins bind to proteins and minerals, thereby reducing their absorption in the intestine (Beta 2003). Oxalates are formed from binding of minerals to oxalic acid. Examples are urinary stones from calcium oxalates that cause hyperoxaluria (Siener *et al,.* 2006). Cyanogenic glucosides durrhin, durrhin-6-glucoside, and amygdalin are reported in *Sorghum bicolor*. Cyanogenic glucosides cause toxicity to humans when hydrolysed to hydrogen cyanide during processing or consumption (Darbani *et al,.* 2016, Selmar *et al,.* 1996). Because processing methods can significantly detoxify or reduce the content of these antinutritional factors, the risk is reduced. Processing steps such as fermentation, germination, soaking or brewing reduced the risk of cyanide poisoning (Bolarinwa *et al,.* 2016, Osuntogun *et al,.* 1989, Kanauchi *et al,.* 2009). Furthermore, LAB significantly reduced cereal phytic acid content during fermentation (Fischer *et al,.* 2014, Reale *et al,.* 2007). For instance, the higher phytic acid content in a barley malt lupine-based lactic acid fermented beverages was reduced to the safe level by a combination of mashing process and LAF (Fritsch *et al,.* 2017). Thus, beverages produced from LAF of malt wort should be considered as safe for consumption.

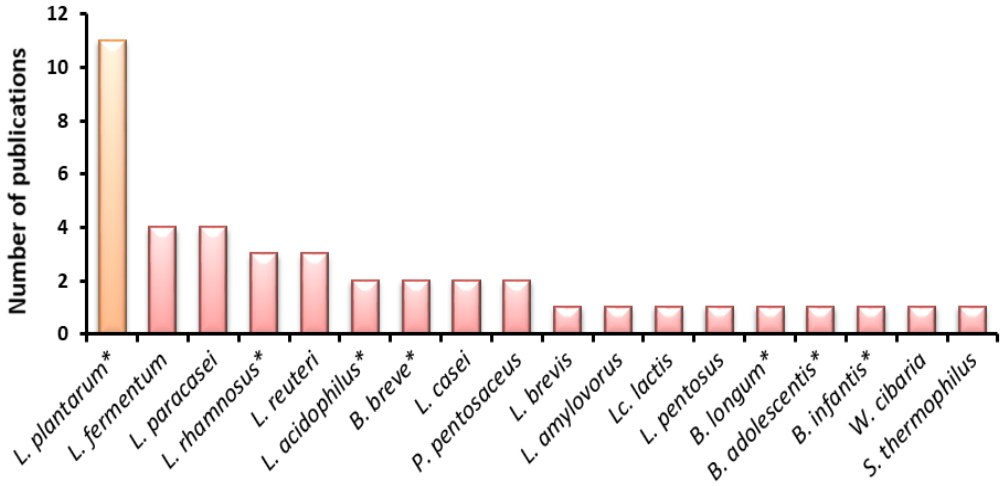


Figure 1. Strains considered in LAFCB and the number of publications in which they were used. *: strains often considered as probiotic. This figure is based on previous research

works (Rivera-Espinoza and Gallardo-Navarro 2010, von Mollendorff *et al,.* 2006, Coda *et al,.* 2011, Angelov *et al,.* 2006, Kedia *et al,.* 2007, Luana *et al,.* 2014, Charalampopoulos *et al,.* 2002, Rathore *et al,.* 2012, Salmeron *et al,.* 2009, Salmerón *et al,.* 2013, Ludena Urquizo *et al,.* 2017, Todorov *et al,.* 2008, Correia *et al,.* 2005).

Nutritional and functional value of LAFCB

In the context of this study, LAFCB are produced from the fermentation of cereal-based substrates by solely lactic acid bacteria (LAB) strains. They are non-alcoholic, containing less than 0.5% abv. (alcohol by volume) or less than 1.2% abv. according to the Food and Drug Administration and the European regulations, respectively. In Africa, most of these beverages are considered more as food than a beverage because of their high nutritional values. The functional and nutritional values of LAFCB are conferred to cereals and LAF.

Cereals are naturally rich raw materials, which possess significant health properties from their high bioactive peptides content. Anticancer, hypotensive, immunomodulating, opioid, anti-Angiotensin Converting Enzyme (ACE), antioxidant and antithrombotic peptides were isolated from cereal protein fractions. Besides, other bioactive compounds such as antioxidants, polyphenols, minerals, vitamins, fibers, prebiotics were found in cereals (Cavazos and Gonzalez de Mejia 2013, Pessione 2012, Hugenschmidt *et al,.* 2010, Coda *et al,.* 2012). Usually, these compounds resist the transformation process to contribute to the nutritional value of the final product. In fact, processing steps also enhance the functional and nutritional value of cereals. For example, germination increases the content of bioactive compounds, protein solubility, and digestibility (Singh *et al,.* 2015). Malting and mashing process improve the nutrients content of malt wort. Accordingly, malt-based substrates proved to better support LAB growth and to provide better sensory quality to the end-product than non-malted cereals. So far, studies proved barley to be the cereal of choice in the production of LAFCB (Salmerón *et al,.* 2013, Salmerón *et al,.* 2014).

LAF enhances the functional and nutritional values of cereal-based substrates through its capacity to produce bioactive compounds and to degrade antinutrients of cereals-based substrates. Bioactive compounds such as antioxidants, B-group vitamins, anti-ACE peptides, as well as anti-viricidal, anti-leukemic and antitumor activities were observed in lactic acid fermented traditional cereal-based beverages (Iwuoha and Eke 1996, Di Cagno *et al,.* 2002, Coda *et al,.* 2011, Capozzi *et al,.* 2011). Detoxification of phytic acid, oligosaccharides, aflatoxins, cyanogenic glucosides and A-gliadin protein fraction contained in cereals by LAB was evidenced (Leroy and De Vuyst 2004, Rizzello *et al,.* 2008, Di Cagno *et al,.* 2002). Furthermore, probiotic potential of diverse LAB strains was

evidenced in cereals fermented substrates as an alternative to relieve the lactose toxicity of dairy products in persons suffering from lactose intolerance (Angelov *et al,.* 2006, Charalampopoulos *et al,.* 2002, Kedia *et al,.* 2007, Rathore *et al,.* 2012, Rozada-Sánchez *et al,.* 2008, Salmeron *et al,.* 2009). The content of cereals in prebiotics such as arabinoxylans and resistant starch makes cereal-based beverages good probiotics carriers. Furthermore, LAB produces diverse antimicrobial substances usually called lantibiotics, that are used in food industry as biopreservatives. According to LAB species, there exists nisin (*Lactococcus*), lactacin, plantaricin (*Lactobacillus*) and pediocin (*Pediococcus*) (Klaenhammer 1993). An antifungal preparate of phenyllactic, OH-phenyllactic and benzoic acids effective against *Fusarium culmorum* spores was evidenced in malt wort fermentation by *L. amylovorus* and *L. reuteri* (Oliveira *et al,.* 2015). Additionally, organic acids produced during LAF and the consequent pH lowering restrict the growth of pathogens.

Summarily, LAFCB possess self-preservation properties, which limit the use of artificial preservatives; are produced from sustainable and clean label process; and possess high nutritional and functional value that meet diversified consumer requirements such as vegetarian, vegan, low-fat, low salt and people suffering from food-related non-communicable diseases. However, the flavor is the preeminent aspect of consumer's choice and will be reviewed in the next subsection.

Flavor of LAFCB and limitations

Although the functional and technological properties of LAFMB, its market remains in its infancy. Food flavor was tested as prime criteria in consumer decision to buy or to like a new product (Azzurra and Paola 2009). The flavor of LAFCB is, therefore, a core aspect of its market development.

Olfactory and gustative descriptors that defined the flavor of LAFCB are: acidic/sour, malty, cereal-like, wort-like, roast, astringent, sweet, and fullness (Coda *et al,.* 2011, Salmerón *et al,.* 2013, Salmerón *et al,.* 2015). Descriptors sour, malty, and sweet are dominant. These descriptors are neither expressive nor attractive to consumers who requested an improvement in the flavor profile of LAFCB, the lack of fruitiness was pointed out specifically (Yu and Bogue 2013). In Fig. 2, the typical sensory profile of LAFCB shows the lack of fruitiness as explicitly requested by consumers.

Thus, flavor improvement of LAFCB is imperative for consumer's acceptance and market development. However, there is no study involved in the improvement of the flavor of

LAFCB. So far, to my knowledge, few studies that examined the aroma profile of LAFCB (Salmeron *et al,.* 2009, Coda *et al,.* 2011, Salmerón *et al,.* 2013) focused on diacetyl, acetaldehyde, and acetone, the main aroma compounds of yogurt. Further, studies did not address their contribution to the flavor and did not define the key aroma compounds that contribute to the flavor. Moreover, organic acids lactic and acetic acid produced during LAF exert a negative effect on the sensory aspect of LAFCB through a masking effect on the flavor and astringency. Therefore, the concentration of organic acids in LAFCB should be customized.

Although aroma compound formation by LAB was extensively studied and reviewed (Smit *et al,.* 2005, van Kranenburg *et al,.* 2002), they were mirrored on dairy products yogurt and cheese. Aroma compound formation by LAB in cereal-based substrates remained to be determined for understanding the poor flavor of LAFMB. Then, the aroma compounds formation in LAF is reviewed in the next subsection.

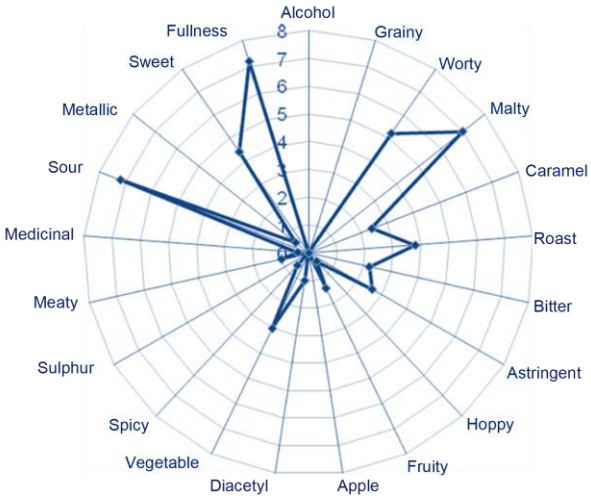


Figure 2. Typical sensory profile of LAFCB showing the lack of flavor (Salmerón *et al,.* 2013).

Aroma compounds formation in lactic acid fermentation of cereal substrates

Fermentation is the exhalation of a substance through the admixture of a ferment which, by virtue of its spirit, penetrates the mass and transforms it into its own nature — Andreas Libavius (1597)

LAB generally transform cereal substrates to their own nature and sensory characteristic, which is dependent on the fermenting strain. In LAFCB production, diverse cereal types and strains have been employed so far. Barley, oat, wheat, emmer and their malts are the considered cereals (Rathore *et al,.* 2012, Salmerón *et al,.* 2014, Coda *et al,.* 2011). As per LAB strains, although eighteen different species have been used so far (Fig. 1), their

choice was rather based on functional properties such as probiotics but less on their ability to flavor formation or the flavor of the finished product.

Lactic acid fermentation is described as a sensory biomodulator of LAFCB (Di Cagno *et al,.* 2011, Peyer *et al,.* 2016, Ogunremi *et al,.* 2017, McFeeters 2004). Main aroma formation pathways in LAF were described in depth (Smit *et al,.* 2005). Amino acids, sugars, and fatty acids are the main precursors of aroma compound formation.

Amino acid catabolism by LAB follows the Ehrlich mechanism that is illustrated in Fig. 3. Main flavor compounds are alcohol, esters and aldehydes. LAB are fastidious micro-organisms that are auxotrophic for several amino acids; either for growth or aroma compounds formation, they must be transported into the cell first. Amino acids are provided to the cell either as peptides or directly as free amino acids by different transport systems (Smit *et al,.* 2005). However, amino acid uptake by LAB was reported to be insufficient (Kunji *et al,.* 1996).

In amino acid conversion as described in Fig. 3, α-ketoglutarate plays a key role because its limitation impairs the conversion of amino acids to the central intermediate metabolite α-keto acid. That was proven by the increased of up to 30% conversion of phenylalanine to phenylacetic acid upon addition of α-ketoglutarate to the fermentation medium (Vermeulen *et al,.* 2006) and significant improvement of amino acid conversion to aroma compounds in cheese (Yvon *et al,.* 1998). However, α-ketoglutarate contribution is dependent on the activity of glutamate dehydrogenase, which catalyses its conversion to glutamate allowing the amino group transfer reaction from amino acids.

Other relevant precursors such as citric acid (related to the NAD/NADH ratio) and pyridoxal-5-phosphate were reported to be involved in amino acid catabolism to aroma compounds too. In sum, three main limitations were found in the aroma formation capability of LAB from amino acid: i) low intracellular pool of the above-mentioned precursors (Vermeulen *et al,.* 2006); ii) insufficient amino acid transport, oligopeptide transport is the main route for nitrogen entry into LAB cells (Kunji *et al,.* 1996); and iii) low aminotransferase and glutamate dehydrogenase activities (Vermeulen *et al,.* 2006). This shows that the poor flavor profile of LAFCB, as presented earlier, is probably due to LAB insufficiency in aroma formation. Therefore, it is necessary to investigate the strategies to allow more aroma formation by LAB from amino acids.

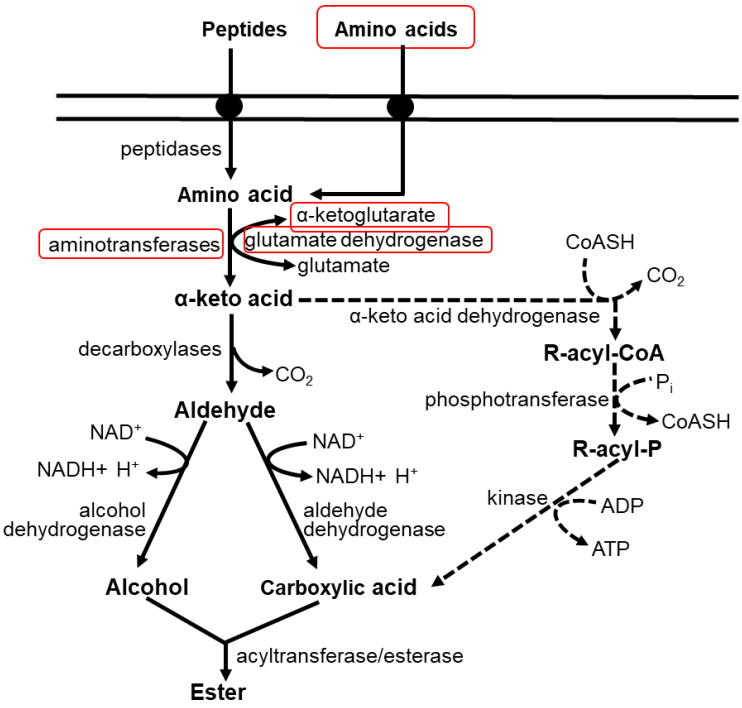


Figure 3. Ehrlich conversion pathway of amino acids to aroma compounds by LAB showing steps of insufficiency in red. Dashed lines represent the branched chain amino acids conversion pathway. This figure is adapted from published works (Smit *et al,.* 2005, De Vuyst *et al,.* 2009, Marilley and Casey 2004).

Sugar metabolism in LAB leads to important aroma compounds such as acetaldehyde, ethanol, acetic acid, and ethyl acetate depending on the fermenting strain type and the environmental conditions as illustrated in Fig. 4. Glycolysis and the pentose phosphate pathway are the two sugar fermentative pathways in LAB. Accordingly, LAB are classified as obligate heterofermentative (pentose phosphate fermentation by means of phosphoketolase) and obligate homofermentative (glycolysis by means of aldolase). A third group exists, the facultative heterofermentative like *L. plantarum* that are in fact homofermentative strains but can switch to the heterofermentative pathways under acidic conditions. This is possible because this group possesses both the aldolase and the phosphoketolase enzymes.

As illustrated in Fig. 4, glycolysis converts sugar mainly to lactate. However, pyruvate is converted to acetate in the presence of oxygen, fructose or acidic conditions, whereas ethanol and acetaldehyde are produced in the absence of oxygen. On the contrary, pentose phosphate conversion leads to lactic acid and acetic acid. Ethanol and acetaldehyde are produced in the absence of oxygen as well. Homofermentation gives rise to two moles ATP whereas only one mole is gained in heterofermentation. Then,

heterofermentative strains tend to metabolise preferentially maltose to gain an additional ATP (Gänzle *et al,.* 2007) since the ATP expenditure requires in the phosphorylation of glucose is saved. Acetic acid production in both case,s either through pyruvate oxidase or acetate kinase, gives rise to additional ATP gain.

In this regard, LAB reroute the pyruvate metabolism to acetic acid not only to gain the extra ATP but also to reduce lactic acid production (Fig. 4) – the main mechanism LAB have developed to cope with lactic acid toxicity. Another mechanism is the conversion of lactate back to pyruvate. This is a result of the induced expression of phosphoketolase and glycolysis enzymes under acid stress (Pieterse *et al,.* 2005) concomitant to oxygen availability. This may explain the long-term survival of LAB under acidic stress at aerobic respiration as compared to anaerobic fermentation (Pedersen *et al,.* 2012, Cesselin *et al,.* 2010, Duwat *et al,.* 2001). In the flavor point of view, respiration reroutes pyruvate conversion to potential aroma compounds acetate, acetoin and diacetyl.

This section shows how sugar metabolism determines the content of ethanol, acetaldehyde, or diacetyl as well as the concentration of lactic acid and acetic acid in lactic acid fermentation. Their concentration in the resulting beverage may modulate the flavor of LAFCB. The ratio of lactic acid to acetic acid is important for the taste, high concentration may have a masking effect on the aroma perception and imparts astringency. Furthermore, lactic acid accumulation is a limitation for aroma compound formation. Indeed, it strongly inhibits LAB growth and viability, which in turn may reduce cell metabolic activity resulting to a low yield of aroma compounds. A significant decrease in the expression of genes involved in amino acid uptake was observed under lactic acid stress (Pieterse *et al,.* 2005). However, literature does not report studies about the potential of other precursors of malt wort to aroma compounds formation. Carotenoids, terpenes or phenolic acids, available in malt wort, may deliver important compounds such as vanillin, β-damascenone, linalool, and geraniol to potentially modulate the flavor of LAFMB.

Subsequently, this section shows that the aroma compounds formation by LAB from sugar metabolism is dependent on the fermenting strain, oxygen availability, and acidic stress. On the contrary, aroma formation from amino acid was observed to be dependent on the amino acid type and transport, enzyme activity and precursor availability in the medium. Fig. 8 describes in detail the dependence of aroma formation on the interconnection of starter culture performance and type as well as to the environmental conditions and fermentation parameters. Also, major actors and important aspects to be considered regarding flavor improvement of cereals-based beverages are described.

Then, the main limitations that may exist in the aroma compound formation ability of LAB in malt wort are insufficient amino acid uptake, low precursors pool, insufficient key enzyme aminotransferase and glutamate dehydrogenase, availability of oxygen and lactic acid toxicity.

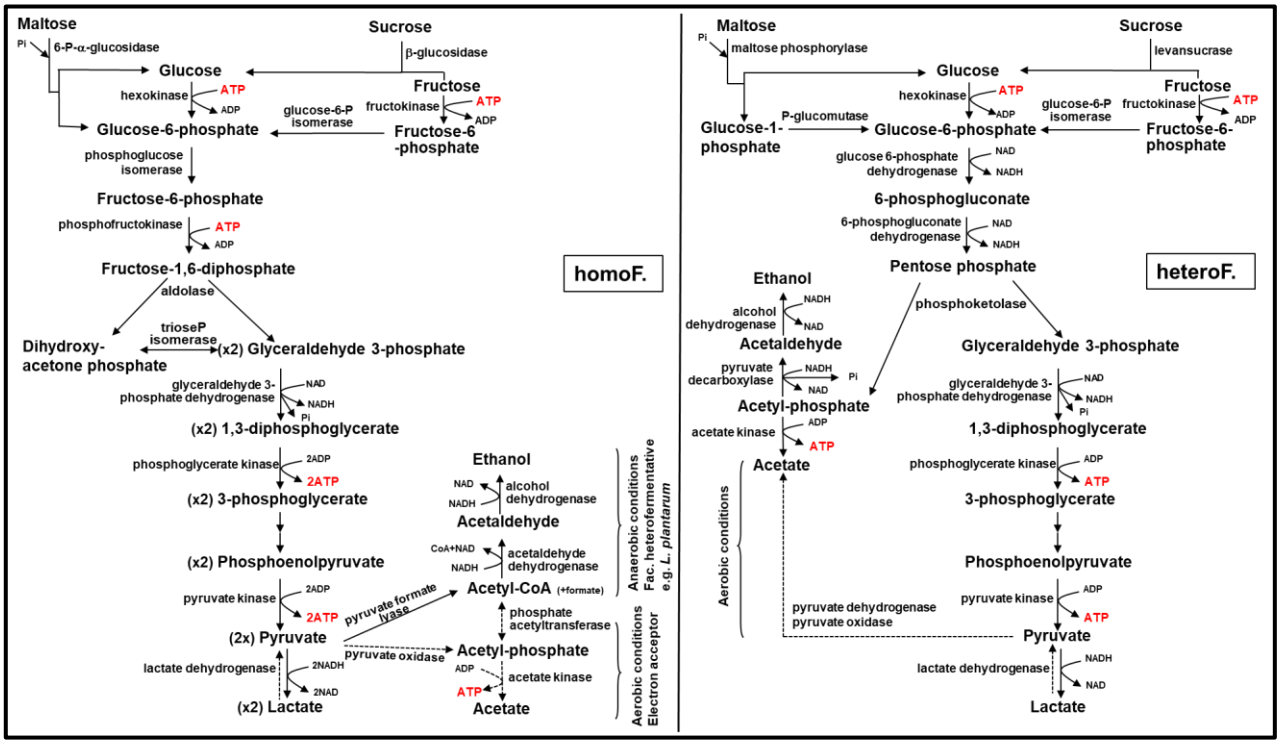


Figure 4. Proposed malt wort sugar metabolic pathways in LAB showing the effect of anaerobic and aerobic conditions as well as electron acceptor on the product and energy yield; homoF: Homofermentative (Embden-Meyerhof-Parnas pathway) and heteroF: heterofermentative (pentose phosphate pathway). Dashed lines are aerobic conversion of pyruvate and lactate to acetate under acidic stress; this figure was constructed based on the results of previous studies (Ehrmann and Vogel 1998, Gänzle *et al,.* 2007, Pessione *et al,.* 2010, Guo *et al,.* 2017, Chen *et al,.* 2015).

1.2. Approaches for flavor improvement in lactic acid fermentation

Metabolic engineering tools are widely used for aroma compound development or to increase the yield of a single aroma compound by LAB (Papagianni 2012, Hugenholtz *et al,.* 2000). However, genetical engineering was not the scope of this thesis because of the set goal to have in the end a natural beverage with clean label, besides consumer's skeptic belief about genetically modified foods (Sybesma *et al,.* 2006).

In the previous section, insufficient aroma formation capacity by LAB was reported in the availability of precursors, insufficiency in amino acid transport and enzymatic activities.

Further, the dependence of aroma formation on the interconnected effect of strain type and performance, environmental conditions and substrate composition is well described in Fig. 8. Either the fermenting strain, medium composition or fermentation conditions affect directly and indirectly the aroma compound formation. The strain performance is dependent on medium composition and fermentation conditions. How the fermenting strain, medium composition or fermentation conditions can be customized to improve the aroma formation in cereal-based substrate fermentation is reviewed in the next sections.

Strain selection and starter culture design

Starter culture is defined as “a microbial preparation of large numbers of cells of at least one microbial species to be added to a raw material to produce a fermented food by accelerating and steering its fermentation process” (Leroy and De Vuyst 2004). Strain selection and definition play a major role in fermented product development and is determinant for the flavor. To define and keep a good starter, it is essential to know the expectations in terms of flavor and aroma. Further, the knowledge of the biochemical pathways leading to the formation of targeted aroma compounds is of benefit in the choice of the starter. Most often, screening tests are based on autochtonous bacteria, occurring in the natural raw material. Screenings for aroma compound production seems a very difficult task due to the extensive steps involved in aroma analysis and the necessary large sample volume.

Then, dairy LABs were successfully screened for their ability to formed 3-methylbutanal, 3-methylpropanal and benzaldehyde, important aroma compounds of yoghurt (Smit *et al,.* 2004). Also, a tailored made starter culture combination of two screened LAB strains based on their proteolytic and decarboxylase activity successfully increased the amount of 3-methylbutanal in yoghurt resulting to a yoghurt with targeted enhanced chocolate-like flavor (Ayad *et al,.* 2001).

Another successful approach was the consideration of non-growing strain of *Lactococcus lactis* to significantly enhance the production of aroma compounds in cheese ripening (van de Bunt *et al,.* 2014). Further, attenuated nonstarter *L. plantarum* cell is consistently considered as an adjunct culture to accelerate flavor development during cheese ripening (Gobbetti *et al,.* 2015). Major aspects to consider when designing, screening or selecting a strain of LAB for aroma formation is summarized in Fig. 5.

To date, there is no defined starter culture to produce LAFCB; a wide range of strains have been considered as summarized in Fig. 1. It might be difficult to define the starter culture

of LAFCB since strains used so far were based on their functionalities, mainly as probiotics. It might be very useful to consider a mixture of strains in the starter culture development for LAFCB, which includes a strain providing the desired flavor as the basic starter culture and a strain providing functional or nutritional properties. Starter culture made of strains combination are widely used when it concerns lactic acid fermentation. Furthermore, it is important for these strains to meet the GRAS or the QPS status described in section 1.1.

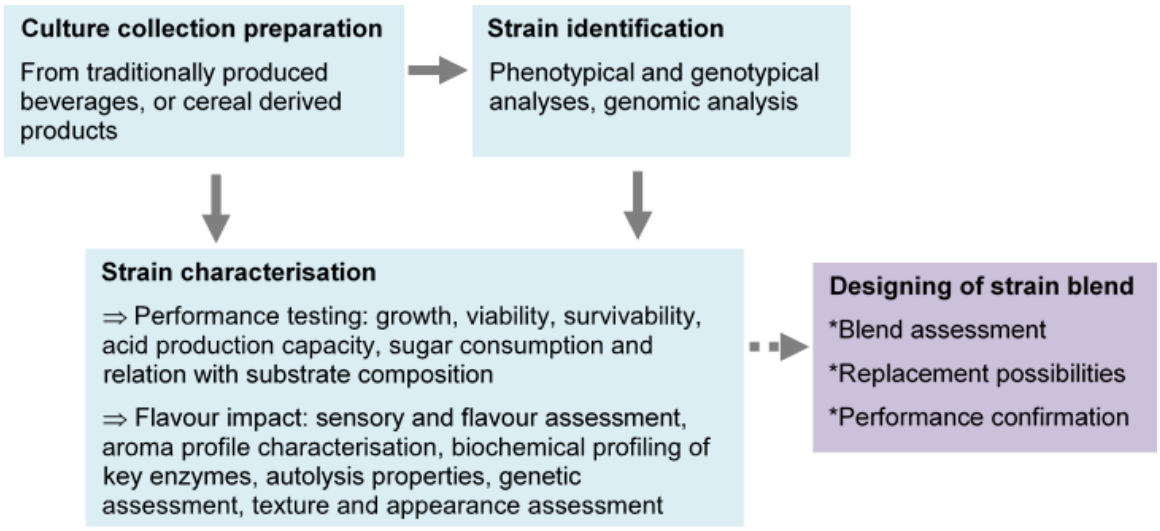


Figure 5. Important steps in starter culture screening, selection or design

Medium composition management

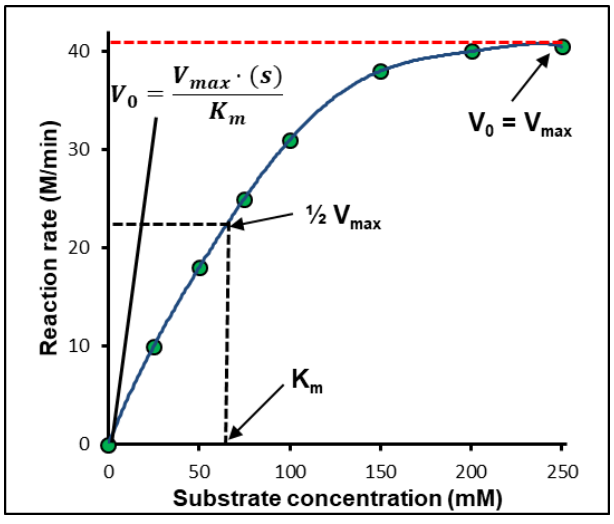
Medium composition affects the aroma compound yield directly through the provision of aroma precursors and indirectly through its contribution to LAB fermentation performance. The concentration of precursors determines the yield in aroma compound formation, provided required enzyme and conditions are available. The dependence of product formation on the substrate concentration is well demonstrated by Michaelis-Menten kinetics in Fig. 6 and Eq. 1. It shows that the maximum enzymatic reaction rate (V_{max}) is achieved at substrate saturation, whereas at low concentration the reaction rate is relatively low. However, it considers the assumption that no product inhibition occurs. This shows how important it is to have sufficient substrate in the medium for expected high yield in aroma compounds.

Amino acids, carbohydrates, and fatty acids are major aroma precursors of lactic acid fermentation reported so far. Previous work on lactic acid fermented products such as sourdough or yogurt did not focus on the supplementation of medium for flavor improvement. The reason may be that amino acids were sufficiently provided during fermentation or the corresponding key aroma compounds are not derived from amino

acids. In sourdough, for instance, studies focused on more understanding the proteolysis and liberation of amino acids during fermentation (Gobbetti *et al,.* 1994) than its implication on fermentation itself and on the bread flavor. Furthermore, yeast in sourdough is a provider of free amino acids through proteolysis; a 400% increase in total free amino acids was observed upon yeast addition to sourdough (El-Dash and Johnson 1970). In lactic acid fermentation of cucumber, yeasts are added as nitrogen provider to remedy the insufficiency in branched chain amino acids and vitamins for LAB growth (Paramithhiotis *et al,.* 2017).

Furthermore, a five-fold increase in acetaldehyde production was observed upon supplementation of 10 mM threonine in milk fermentation by *S. thermophilus* (Hugenholtz *et al,.* 2000). The addition of proline and sugars to the dough prior to baking could enhance the content of 2-acetyl-1-pyrroline and pyrazines (Pacyński *et al,.* 2015), the key aroma compounds of gluten-free bread and bread crust. Amino acid supplementation, however, could lead to the formation of off-flavors and should therefore, be customised. Off-flavors pyrrolizines and azepinones formation in sourdough upon proline addition is a case (Bredie *et al,.* 2006).

These considerations for medium optimization in nutrients were all based on laboratory trials with external addition whereas, no interest was given to the raw material processing for medium nutrients enrichment. To date, there is no consideration of increasing the amino acid content of cereal-based substrates for flavor improvement.



$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

$$K_m = \frac{k_{-1} + k_2}{k_1}$$

Km: Michaelis-Menten constant, substrate concentration at which the reaction velocity is 50% of V_{max}

k_1: Rate constant for ES complex formation

k_{-1}: Rate constant for ES complex dissociation

k_2: Rate constant for product P formation

V_{max}: Maximum reaction rate achieved at substrate saturation

S : Substrate concentration

P : Product

E : Enzyme

Figure 6. Schematic representation of the Michaelis-Menten saturation curve (kinetics) of the enzyme reaction rate depending on the substrate concentration.

Mashing process management as a tool to increase amino acid content

Malt wort was described previously as the most suitable medium for lactic acid fermentation because it provides high nutritional value for LAB growth. Malted cereal substrates promote LAB growth and aroma compounds formation quite better than non-malted cereals (Coda *et al,.* 2011, Salmerón *et al,.* 2015). The production of malt wort in brewery starts by malting, which includes steeping, germination and kilning, the aim is to develop necessary enzymes for the mashing steps. The malted grains are thereafter ground and mixed with water to render proteins and carbohydrates available for malt enzymes during mashing process. In breweries, mashing process is well standardized and well defined according to the end quality of the desired beer as seen in Fig. 7. Rest temperature of 62°C and 72°C for 30 min each are defined as optimum for barley β- and α-amylase activity, respectively. For protein conversion, a defined temperature of 50 - 52°C is employed as optimum temperature for malt endopeptidases. As such, the extract yield is dependent on the temperature and time exposure of malt enzymes. However, one should consider the half-life of the enzymes. The half-life, the time for which, an enzyme lose its half activity, is dependent on the temperature. At optimum temperature, an enzyme half-life is shorter whereas it is longer at a lower temperature. Based on that, the overall endoproteolytic activity of malt mash, for instance, remained constant during 55 min protein rest at 40°C as compared with the reduction at conventional temperature of 52°C (Jones and Marinac 2002).

Thus, mashing process has the power to adjust the extract yield and free amino nitrogen content in the finishing malt wort by customizing the time and/or temperature of enzyme exposition. Furthermore, speeding up or lengthening the germination stage proved to increase the soluble protein content (Jones and Marinac 2002). It is known that 33% of the soluble protein of a typical malt wort come from non-germinated barley whereas 45% is solubilized during malting and the remaining 22% are released during mashing (Jones and Budde 2005). The sugar conversion step proved to have no effect on protein yield because of the rapid denaturation of endopeptidases at high temperature employed.

Several studies have focused on the optimization of mashing process to produce substrates from diverse cereals such as millet, oat, buckwheat or teff intended for functional beverages (Gupta *et al,.* 2010, Muñoz-Insa *et al,.* 2011, Zarnkow *et al,.* 2010, Wijngaard and Arendt 2006). However, these studies did not consider either LAB nutritional requirements such as amino acids and sugars content nor the intended quality of the resulting product. Here, by customizing the mashing process at the protein rest

duration, a profound action of malt endopeptidases can be exploited for more free amino nitrogen and amino acids.

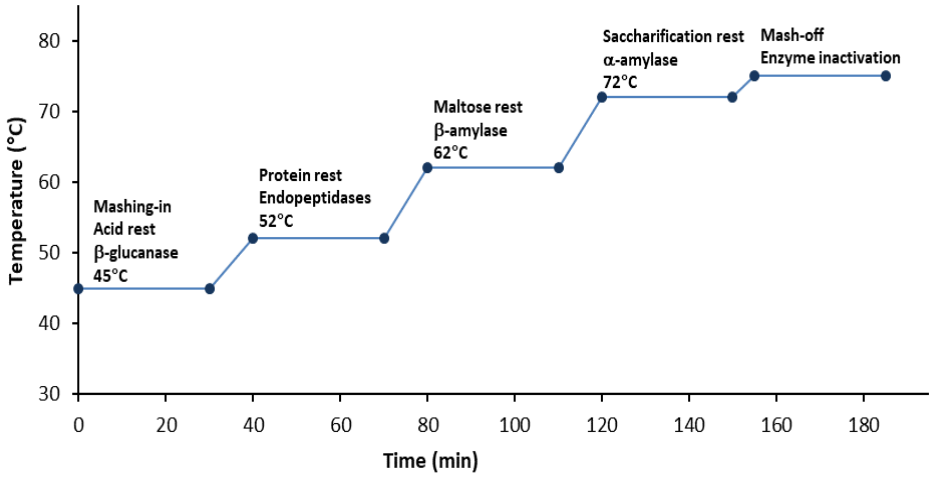


Figure 7. Schema of conventional barley step-mashing program, enzymes involved and required optimum temperature.

Exploitation of environmental stresses

LABs are exposed during fermentation to constant fluctuations in their growth environment. Therefore, they are forced to develop responses to adapt and survive to the fluctuations they are subject to in their growing environment. The main stress responses in malt wort concern acidic, oxidative and osmotic stress.

Environmental stresses that LABs are subject to or that arise during fermentation are often deleterious to the cell and therefore to the quality of the resulting product. However, recently, attention has been given to the exploitation of these stresses for the delivery of aroma compounds of interest (Serrazanetti *et al,.* 2009). The acidic stress is the most encountered during lactic acid fermentation, it is reported on one side to be deleterious to LAB cells but on the other side, it induces the expression of genes involved in metabolic activities. A typical example is the rerouting of pyruvate metabolism to acetic acid, diacetyl and acetoin production at the expenses of lactic acid. Further, relevant accumulation of higher alcohols and acetic acid were observed in LABs following acidic stress exposure (Guerzoni *et al,.* 2007). There, genes involved in branched chain amino acids catabolism were overexpressed leading to seven times overproduction of aroma compounds. Most importantly, it was proved that under acidic stress conditions, LABs respond by switching from sugar metabolism to amino acid catabolism (Serrazanetti *et al,.* 2011), which is favorable for aroma formation.

The oxidative stress encounters also in lactic acid fermentation. The advantages of aerobic respiration on the growth and viability of LABs were reported in the previous section, the consequent formation of acetoin and diacetyl through pyruvate rerouting as well. However, the presence of oxygen generates free radical hydrogen peroxide (H_2O_2). When the concentration becomes higher than the capacity of LAB cell to detoxify them, cells are exposed to the oxidative stress. Implications of oxidative stress exposure to the aroma compound formation by LAB was described. γ-decalactone, 2(5)-furanones, aldehydes and 3-methyllbutanoic acid were over accumulated as a consequence of oxidative and osmotic stress exposure of *L. sanfranciscensis* (Guerzoni *et al,.* 2007, Serrazanetti *et al,.* 2013). This section shows that it is possible to adjust the aroma compound formation by LAB through the exploitation of the environmental stresses. However, the aroma precursors availability and starter culture performance are the prerequisites.

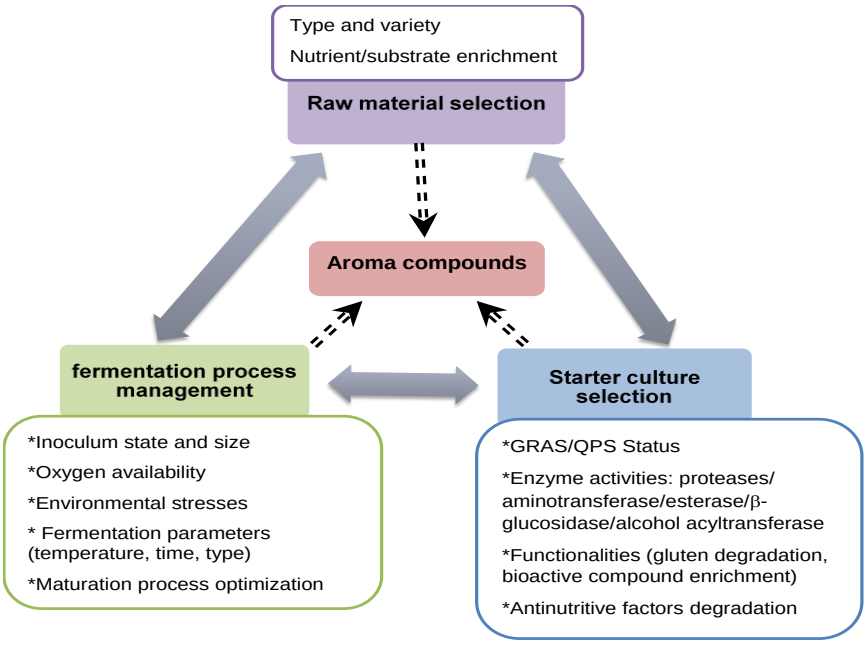


Figure 8. Basic representation of the possible interconnection between the major actors of aroma compound formation during lactic acid fermentation of malt wort.

Based on the approaches proposed in Fig. 8, three major actors that are involved in the aroma compound formation of lactic acid fermentation in cereals are raw material composition, starter culture potential and performance, and fermentation conditions. As previously reviewed, aroma compound formation by LAB is limited by several factors such as low amino acid uptake, insufficient enzymatic activities, low precursors pool, in addition to lactic acid toxicity.

Whether cereal substrates support efficiently aroma formation by LAB is still to be determined. The approaches considered so far for flavor development in lactic acid fermentation give very less attention to the fermentation process management or the medium improvement. A possible explanation might be that well-known lactic acid fermented products are based on well-defined processes and starter culture. For a newly developed product like LAFCB, where no starter culture, substrate or fermentation process is defined yet, the flavor development should start from these basic steps.

1.3. Purpose of the study and research hypotheses

It is well described in research that LAFCB possess high potentials as functional beverages to replace dairy-based products (Angelov *et al,.* 2005, Angelov *et al,.* 2006, Charalampopoulos *et al,.* 2002, Coda *et al,.* 2011, Granato *et al,.* 2010). Indeed, non-dairy functional beverages are specifically in demand to meet diversified consumer's choices and health conditions (Granato *et al,.* 2010, Fortitech 2011). However, the market of LAFCB is quasi-inexistent. Flavour improvement was requested by consumers, who pointed out the lack of fruitiness (Yu and Bogue 2013). Therefore, **the problem** dealt with in this study is that the flavor of LAFCB is poor, which causes poor consumer's acceptance and therefore low market development.

Yet, food flavor is determinant for its acceptance. For functional beverages, the flavor is the first criteria for consumer's decision to buy or not, but not the nutritional value (Azzurra and Paola 2009). Furthermore, the taste was paramount but health and nutritional content of limited interest in a study with college students on beverage consumption (Block *et al,.*). Therefore, improving the flavor of LAFCB is a real issue and constitutes the major challenge to boost consumer's acceptance and the market development.

So far, there was no attempt for flavor improvement of LAFCB. Although a study showed an improved acceptance of LAFCB when using a lactic acid bacteria (LAB) strain (Salmerón *et al,.* 2015). Still, the study did not focus on flavor improvement. Furthermore, thirteen strains have been used in LAFCB research (Fig. 1), most of them not commercially available. Consideration of flavorings for flavor enhancement is however not valuable in this study regarding the set goal and consumer's demand for natural foods without artificial ingredients (section 1.1). For Bionade, a successful malt-based refreshing fermented beverage on the market, fruit extracts are added to enhance the flavor (Wittberg and Vieselmaier 2009). Also, fruits extract addition to LAFCB has been proposed (Tenge and Geiger 2001) but this will lead to the loss of LAFCB flavor authenticity and naturalness.

Then, flavor improvement based on existing process customization will further enhance the market value of LAFCB.

In this respect, **the aim of this dissertation** is twofold: to understand limitations on the flavor of LAFMB and to develop a process for flavor improvement of LAFCB based on existing processes brewing, fermentation and maturation customization. Strategies for customization used to improve the aroma formation in lactic acid fermented foods are based on strain selection, medium composition management and exploitation of environmental stresses as described in section 1.2. However, none of these strategies was considered so far for flavor improvement of LAFCB. Thus, **the main research question** addressed in this thesis was: *How to improve and design the flavor of LAFCB by using existing processes and technologies?*

To answer this research question, essential is to outline the knowledge gap that exists in the flavor of LAFCB and in lactic acid fermentation of malt wort itself. To date, few studies have focused on the aroma composition of LAFCB (Salmerón *et al,.* 2013, Coda *et al,.* 2011) not allowing to understand the poor flavor of LAFCB. The flavor is determined by the concentration of key aroma compounds. Therefore, the elucidation of LAFCB aroma composition and definition of key aroma compounds are prerequisites for flavor improvement.

Further, understanding the limitations occurring in LAB fermentation in malt wort will orientate in process design for flavor improvement. Indeed, amino acids are essential for LAB growth (Terrade and Mira de Orduña 2009), and strongly involved in aroma compound formation (Smit *et al,.* 2005). In lactic acid fermentation, the buffering capacity is important to reduce the pH lowering effect of lactic acid production. Lactic acid reduces LAB viability (Pieterse *et al,.* 2005). Therefore, reducing lactic acid production is necessary for the sake of LAB performance. Whether malt wort composition is optimal to support LAB growth and aroma compounds formation is still to be determined. Substrate limitation and product inhibition are successfully solved in industrial applications by continuous medium feeding (Stanbury *et al,.* 1995). Moreover, maturation step is vital for flavor development in lactic acid fermentations (Gobbetti *et al,.* 2015). Whether it can impact LAFCB flavor is worth to determine.

Thus, following **research hypotheses** were tested:

1. *Lack of literature knowledge on the flavor of LAFCB does not allow to understand the limitations that exist for flavor improvement.*

2. *Low content of key aroma compounds in LAFCB could deliver a poor flavor profile. Furthermore, organic acid production reduces the acceptance.*
3. *Amino acid content and buffering capacity of malt wort could be limited to cause poor LAB fermentation performance and low aroma yield in conjunction with lactic acid.*
4. *Increasing the amino acid content and the buffering capacity will extend the fermentation duration thereby to improve the aroma yield and consumer's acceptance.*
5. *Temperature reduction can lower lactic acid production and medium feeding can continuously provide amino acid for extended LAB viability and increased aroma yield.*
6. *Under optimized malt wort composition and fermentation conditions, maturation step inclusion will lead to significant aroma development and improvement of LAFCB flavor.*

1.4. Thesis Highlights

The stated hypotheses were investigated to achieve the set goal to develop a process for flavor improvement of LAFCB based on brewing, fermentation, and maturation that is described in Fig. 15. The findings were presented partly as publications.

Paper 1 shows that the main knowledge gap in LAFCB flavor is the understudied aroma profile and the quasi-non-existence of process technology.
Published as: Flavor of lactic acid fermented malt-based beverages: Current status and perspectives. *Trends in Food Science and Technology.*

In **Paper 2,** it was found that the low concentration of key compounds is responsible for the poor flavor of LAFMB and the aroma concentration is related to the starter culture.
Published as: Key volatile aroma compounds of lactic acid fermented malt-based beverages – impact of lactic acid bacteria strains. *Food Chemistry.*

Paper 3 shows that LAB fermentation performance in barley malt wort is abridged through significant reduction of cell viability and metabolic activity. Key amino acids exhaustion, low wort buffering capacity and lactic acid accumulation are the limitations.
Published as: Investigating on the fermentation behavior of six lactic acid bacteria strains in barley malt wort reveals limitation in key amino acids and buffer capacity. *Food Microbiology.*

In **Paper 4**, high-gravity fermentation (HGF) provided greater malt wort amino acids and buffering capacity that significantly improved LAB fermentation performance, increased the aroma yield and consumer's acceptance of LAFMB**.**

Published as: Exploration of high-gravity fermentation to improve lactic acid bacteria performance and consumer's acceptance of malt wort-fermented beverages. *International Journal of Food Science & Technology.*

Protein rest extension during mashing process further increased malt wort amino acid content and buffering capacity. The inclusion of fed-batch and fermentation temperature reduction at 18°C to HGF prolonged LAB growth and viability, increased the aroma content and reduced lactic acid production. Finally, application of maturation step significantly increased the aroma content.

These findings bring significant and original advances to the research in LAFCB by providing a strong scientific understanding and essential information for flavor improvement that has been lacking so far. Further, these results open new lines of research in LAFCB. This information is beneficial to researchers or food industrials dealing with lactic acid fermentation or LAFCB to improve the fermentation performance of their strains or the flavor of their lactic acid fermented products. Furthermore, this study is particularly of benefit to beverage industrials as it provides not only a process to produce LAFMB with improved flavor but also important information to customize their needs. The process *per se* will undoubtedly heighten the opportunity to introduce a LAFCB on the market that is likely to have significant development. Finally, this study will benefit the society mainly people suffering from the lactose intolerance or those attached to food choices such as vegan and low-fat that could not drink dairy-based product to easily find a replacement in LAFCB.

2 . Results

This section presents a summary of the publications of this study based on the hypotheses stated in section 1.3 including strategies and red threads. Full copies of the publications are embedded hereinafter.

2.1. Summary of publications

Flavor of lactic acid fermented malt based beverages: Current status and perspectives. *Trends in Food Science and Technology*, Paper 1, Page 28.

Consumer's healthy choices are driving the beverage market towards those with added health value, fewer additives and preservatives. This is observable in the steady increase of the functional beverage market. Beverages produced from lactic acid fermentation of cereals have been lastly of great interest as functional beverages to replace dairy-based beverages. Lactic acid fermented cereal-based beverages (LAFCB) are rich in bioactive compounds derived from cereals and lactic acid fermentation. However, the quasi-non-existence of their markets was questioned in this review.

Poor sensory profile, low consumer's acceptance, and lack of production technology were found to be the challenges. Food flavor is the first criterion for consumer's choice before nutritional value. Accordingly, consumers requested an improvement of the flavor of LAFCB. Common sensory descriptors sour, sweet, cereal-like, and malty are not regarded as positive by consumers. It is therefore of great necessity to optimize the flavor of these beverages when aiming to improve consumer's acceptance. The question remains how? The aroma profile of LAFMB is understudied although it is the main aspect of any attempt for flavor improvement. Considerations are given to very few compounds such as acetone, acetaldehyde, acetoin, and diacetyl. Furthermore, there is neither a report on their implication to the flavor nor key aroma compounds of LAFCB were defined. Lactic acid fermentation of cereals was reported as biomodulator of sensory characteristics, but the study remains superficial. Furthermore, lactic acid fermentation process of malt wort is not developed yet and no starter culture and substrate are defined yet.

Summarily, the flavor of LAFMB should be studied extensively for attempts of flavor improvement. Strategies that can be exploited are proposed; potential aroma formation routes from malt wort lactic acid fermentation, so far not reported, were proposed.

Key volatile aroma compounds of lactic acid fermented malt based beverages – impact of lactic acid bacteria strains. *Food Chemistry,* Paper 2, Page 43.

Flavor improvement of LAFCB being of prime importance, volatile aroma characterization of LAFMB is a prerequisite for understanding the poor sensory profile. In that sense, this Paper defined the volatile composition of LAFMB, key aroma compounds, and the related impact of LAB strains. Firstly, the screening test allowed to select six positive (*L. plantarum*), neutral (*L. amylolyticus*) and negative (*L. brevis*) control strains out of sixty strains. Four different aroma extraction techniques allowed to identify fifty-six volatile compounds, belonging to ten different groups. By means of aroma extract dilution analysis, and odor activity values (OAV) calculations, thirteen key aroma compounds of LAFMB were defined. Among them was the fruity ester ethyl 2-methylbutanoate and other fruity odor compounds such as β-damascenone, furaneol, vanillin, nor-furaneol, and acetaldehyde. Most of them, are reported for the first time in lactic acid fermentation of cereals. However, their concentrations were found to be low and strongly related to LAB strain although LAB strain did not significantly affect the aroma composition. To study the implication of these results to the flavor, significant differences in the flavor profile were observed. Beverages produced with strains of the same fermentative pathways delivered similar flavor. *L. plantarum* Lp.758 strain that delivers the most pleasant flavor was proposed as a starter culture. Comparison with the aroma profile shows higher flavor dilution factor and OAV values of key aroma compounds for this strain.

Thus, the main conclusion is that the low concentration of important aroma causes poor flavor profile of LAFMB, the fermenting strain plays a significant role.

Investigating on the fermentation behavior of six lactic acid bacteria strains in barley malt wort reveals limitation in key amino acids and buffer capacity. *Food Microbiology,* Paper 3, Page 52.

The aim was to understand the fermentation behavior of six selected LAB strains in malt wort considering the low aroma yield recorded. For that purpose, it was found that LAB growth in malt wort is very short ranging from 24-48h depending on the strains. Coupled prompt cell death with a significant increase in death rate and a decrease in the metabolic activity were responsible. A flocculation-like behavior of filamented dead LAB cells was observed under these conditions. From these results, it was observed that a significant proportion of LAB cells are continuously dying during malt wort fermentation, thereby reducing the viable count. The proposed behavior of living, dead and total LAB cells during malt wort fermentation is schematized in Fig. 9. Furthermore, fermentable sugars were found not limiting, except fructose. Amino acids were significantly utilized with lysine, glutamic acid, arginine, and leucine limiting. The buffering capacity was low, which caused rapid pH drop at the beginning of fermentation. There, lactic acid production played a double role in the buffering capacity change during fermentation: it reduces the pH value to its pKa value, which becomes favorable to the formation of buffer agents (lactic acid/lactate). Consequently, the accumulation of lactic acid/lactate buffer then increased the buffering capacity at the late-stage fermentation. Lactic acid was accumulated significantly as well as acetic acid and succinic, whereas malic acid was degraded. The lactic acid concentration of 3.9 g/L obtained in this study was higher than the reported growth inhibitory concentration (≤ 1.7 g/L). Lactic acid was therefore critical for LAB cell viability and growth. Furthermore, malt wort individual supplementation with relevant amino acids resulted in significant improvements in LAB cell growth and viability, the effect was further improved in combination with buffer agents supplementation. However, wort buffering alone improved cell growth but not the viability possibly because of the greater lactic acid accumulation in a buffered medium. As per fructose, a non-significant impact could be drawn.

These results led to conclude that the limitation in key amino acids in combination with low buffering capacity are the key limitations to LAB fermentation performance in malt wort causing low aroma yield. There, lactic acid content is problematic.

Exploration of high-gravity fermentation to improve lactic acid bacteria performance and consumer's acceptance of malt wort-fermented beverages. ***International Journal of Food Science & Technology,*** **Paper 4, Page 61.**

Flavor improvement of LAFCBs is determinant for its market development and consumer's acceptance. Increasing the content of key aroma compounds in LAFMB is essential for the flavor. It was hypothesized that the increase of malt wort amino acid content and buffering capacity could significantly improve LAB fermentation performance, thereby increasing the aroma yield. Consideration of HGF provided greater free amino acid and sugar content. The buffering capacity was greater, which resulted in low pH change during fermentation although lactic acid production was higher. The implication was a significant improvement of LAB fermentation performance at 20 %w/w wort. Furthermore, the content of higher alcohols, 2-phenylethanol, acetaldehyde and β-damascenone in HGF beverages were significantly increased by up to +0–161%, +11–147%, +27–44%, and +25–66%, respectively. The main outcome was, therefore, the high preference of HGF beverages by consumers over low gravity fermented beverages. There, the higher sugar content of high-gravity wort might have balanced the sugar to acid ratio for a better harmony.

These results bring significant input to the efforts to improve LAFMB flavor and acceptance. However, the acceptance remained under 50% highlighting the necessity for further investigations. In that perspective, further investigations were done: i) evolutive cell adaptation to lactic acid stress from 1.7 g/L to 5 g/L did not bring significant change, ii) extending protein rest time during mashing process increased the amino acid content by 48.5%, iii) fermentation temperature reduction to 18°C combined with fed-batch fermentation of optimized malt wort could maintain high proportion of viable cells at extended fermentation (up to ten days), and iv) maturation of the resulted beverages significantly increased the aroma compound content, mainly esters. Maturation was decisive for the content of relevant aroma compounds and the final flavor.

From these results, the process for flavor design and improvement of LAFMB was developed and proposed in Fig. 15. It describes a simple and economical process that is easily applicable in industry. Further, these results open new lines of research in LAFCB.

2.2. Flavor of lactic acid fermented malt based beverages: Current status and perspectives.

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Review

Flavor of lactic acid fermented malt based beverages: Current status and perspectives

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ABSTRACT

Background: Although several research studies have described potentialities of lactic acid fermented cereal beverages as functional beverages, their market and industrial applications are quasi non-existing. Poor sensory quality, low acceptance, and lack of production technology seem to be the challenges. Sensory characteristics, commonly described as sour, sweet, cereal-like, and malty, are not always regarded as positive by consumers and represent therefore an important hurdle for their acceptance. Neither their aroma composition has been studied in depth for overall aroma understanding, nor has an attempt for sensory profile improvement been done. Aroma type and quantity might depend on several factors like starter culture, substrate, and fermentation process.

Scope and approach: In this review, we discussed the potential of cereal malt wort as a precursor medium for aroma compound formation during lactic acid fermentation; sensory characteristics and aroma-active compounds of lactic acid fermented cereal beverages are also described; strategies that can be exploited for flavor improvement are proposed with focus on existing technologies. Case studies based on existing products are included for technological innovation in order to meet increasing consumer's demands for new tastes.

Key findings and conclusions: Further works on characterization of aroma compounds, elucidation of key aroma compounds that contribute to the overall aroma, flavor impact of the interactions aroma - organic acids, consumer's needs investigation, lactic acid bacteria starter culture selection and fermentation process management will provide significant advances towards the flavor improvement of cereal beverages for a promising market.

1. Introduction

The increasing society quest for healthier and natural foods, new tastes and food ingredients is oriented, in beverage sector, towards non-alcoholic and functional beverages with preference towards those containing no additives and preservatives (Roethenbaugh, 2007; Vartanian, Schwartz, & Brownell, 2007). The global market for functional foods in general and functional beverage in particular has steadily increased over the past decade all over the world. Furthermore, nondairy functional beverages are in demand due to vegetarianism, milk cholesterol content, and lactose intolerance (Fortitech, 2011; Granato, Branco, Cruz, Faria, & Shah, 2010; Granato, Branco, Nazzaro, Cruz, & Faria, 2010). Thus, one of the challenges facing food industries is to innovate in order to adapt to changing consumer's demands and maintain market leadership. Investigations on new fermented foods are the emerging trends to be applied in the food Technology. Beverages already available on the market and consideration of existing resources such as cereals, combined with current technologies and processes are valuable bases for further innovation.

Cereals have recently retained a lot of attention as raw material for non-alcoholic and functional beverages production. High fiber, whole or multi-grain containing beverages represented in 2012 and 2013 the greater part of the better-for-you new foods and beverages with desirable benefits (IRI, 2014). Desirable benefits derived from cereals are their high nutritional value and bioactive compound content (Adil, Wani, Masoodi, & Gousia, 2012; Alvarez-Jubete, Arendt, & Gallagher, 2009; Fardet, 2010; Sarwar, Sarwar, Sarwar, Qadri, & Moghal, 2013; Thompson, 1994). Lactic acid bacteria (LAB)

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have been extensively used since many decades for the production of cereal based beverages. Their tremendous potentialities as cell factories for the delivery of functional biomolecules in cereal based beverages to fulfill broad range of consumers life style nutrition have been explicitly highlighted (Hassani, Procopio, & Becker, 2015; Singh, Rehal, Kaur, & Jyot, 2013; Waters, Mauch, Coffey, Arendt, & Zannini, 2013).

Lactic acid fermented malt based beverages are non-alcoholic, with low pH value (3.5–4.5) and produced by the fermentation of cereals, cereal substrates or blends by LAB strains. Available lactic acid fermented cereal beverages include yogurt-like cereal functional beverages and traditional cereal fermented beverages (Corbo, Bevilacqua, Petruzzi, Casanova, & Sinigaglia, 2014; Müller-Auffermann, Thormann, Hutzler, & Jacob, 2013) (Table 1). Barley, wheat, oat, rye, rice, sorghum, quinoa and amaranth were demonstrated as potential growth medium substrate for LAB. Furthermore, malted cereal substrates promote LAB growth and aroma compound generation better than non-malted cereals (Charalampopoulos, Pandiella, & Webb, 2003; Coda, Rizzello, Trani, & Gobbetti, 2011; Gebremariam, Hassani, Zarnkow, & Becker, 2015; Kedia, Wang, Patel, & Pandiella, 2007; Salmerón, Rozada, Thomas, Ortega-Rivas, & Pandiella, 2014; Salmerón, Thomas, & Pandiella, 2015; Zannini et al., 2013).

Despite the acknowledged health benefits of lactic acid fermented cereal beverages, they are poorly accepted by consumers. The most important attributes for functional food choice was taste in 59% of cases whereas nutritional aspects were considered as important in only 36% of cases (Azzurra & Paola, 2009). In the same line, consumers requested an improvement of the flavor of lactic acid fermented cereal based beverages as the most important attribute (Yu & Bogue, 2013). Therefore, the key issue for future viability and success of these beverages on the market is consumer acceptance (Granato, Branco, et al., 2010) which is driven by the flavor and nutritional aspects. Their flavor is generally characterized as sour, malty, sweetish, cereal-like, and worty-like (Coda et al., 2011; Gebremariam et al., 2015; Salmerón, Rozada, et al., 2014; Salmerón et al., 2015). Due to consumer diversity to sour food acceptance, the sourness on account of intrinsic organic acids may also impact sensory perception. Recently, a cereal beverage fermented with a *Lactobacillus plantarum* strain was better accepted by consumers. It was tentatively attributed to acetaldehyde content (Salmerón et al., 2015). Flavor improvement is therefore a key step for the development of these beverages. Considerations on their overall aroma composition are thus very crucial although little attention has been given.

Aroma-active compound groups were identified in lactic acid fermented cereal beverages. Quantitation studies on acetaldehyde, acetone, acetoin, and ethanol revealed concentrations of acetaldehyde and acetoin higher than their odor detection threshold (Salmerón et al., 2015). There are no studies yet that described their contribution to the flavor or which attempt to enhance the aroma profile. Furthermore, attractive aroma compounds such as esters were quasi absent.

Aroma compound formation in lactic acid fermentation involves different pathways, precursors and enzymes (Gänzle, Vermeulen, & Vogel, 2007; Smid & Kleerebezem, 2014). The occurrence and activity of enzymes, esterases for instance, are strain dependent in LAB (Kim et al., 2013; Liu, Holland, & Crow, 2004; Rojas, Gil, Piñaga, & Manzanares, 2003). In addition, enzyme activity rate are well-known to rely on fermentation parameters such as temperature, pH, substrate, and cofactors availability. On the other hand, environmental stresses improved the formation of particular aroma compounds in sourdough lactic acid fermentation (De Angelis, Bini, Pallini, Cocconcelli, & Gobbetti, 2001; Serrazanetti, Guerzoni, Corsetti, & Vogel, 2009; Serrazanetti et al., 2011). Therefore, aroma type and concentration may be determined by substrate composition, starter culture and environmental conditions and process.

This review presents research advances on lactic acid fermented cereal beverages focusing on sensory characteristics and aroma-active compounds. The potential of malt wort as a basis for lactic acid fermentation and source of aroma compound precursors is discussed as well. Existing limitations and future research strategies for flavor improvement of lactic acid fermented cereal beverages are proposed, especially for new design of processes, selection of more suitable LAB and strain consortia, or by optimization of raw materials.

2. Some examples and case studies for technological innovation

Innovation in lactic acid fermented malt based beverages is oriented towards production of probiotic beverages which follows the general wellness claim trend. Table 1 lists existing commercially available and traditionally produced lactic acid fermented cereal beverages. Commercially available beverages are made from probiotics LAB strains and intended to be consumed as probiotic beverages. Sometimes fruit extracts are added to enhance the flavor as with Jovita Probiotisch beverage. Mageu was successfully fermented with probiotic strains of *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* and the sensory acceptance was similar to that of traditionally produced beverage (Nyanzi, Jooste, Abu, & Beukes, 2010). Fortification of Mahewu with iron for nutritional value increase was successfully developed (Salvador, 2015). Obiolor is associated with health properties such as attenuation of high-fat diet, dyslipidemia, protein oxidation, lipid peroxidation as observed in rats, and antioxidant properties (Ajiboye et al., 2016). Grainfields Wholegrain probiotic Liquid® is rich in probiotics, vitamins, amino acids and enzymes.

Further innovation trend could be directed towards the development of beverages with health related benefits such as gluten-free, low-cholesterol and high bioactive compound content that could be suitable for diverse nutrition lifestyles such as veganism, vegetarianism, low cholesterol nutrition, and people suffering from nutrition related non-communicable diseases and cardiovascular diseases. A case study is the development of a high content cereal based beverage produced from pseudo cereal quinoa, lupine (*Lupinus albus* L.), and mesquite (*Prosopis chilensis (Mol.) Stunz*) with a final protein content of 1.36% that was acceptable for drinking (Cerezal Mezquita, Acosta Barrientos, Rojas Valdivia, Romero Palacios, & Arcos Zavala, 2012). This beverage could be a suitable product for vegan nutrition lifestyle or high protein nutrition lifestyle. However, the high content of lupine in flatulescence oligosaccharides and phytate should be a serious concern. Lactic acid fermentation by selected LAB strains could not only reduce the content with non-significant change on the net protein ratio (Camacho et al., 1991) but also improve the flavor for a more appealing beverage. Besides the richness of lupine in plant protein, it has also functional properties such as hypoglycemic effect and body weight reduction as observed in humans and rats (Bertoglio et al., 2011; Capraro et al., 2014). Therefore, the production of a protein rich beverage based on malt wort, lupine and lactic acid fermentation will be an innovation in cereal based beverage production. However, the flavor being the most important criteria for functional food choice before nutritional aspect (Azzurra & Paola, 2009), a focus should be given to the flavor profile.

Table 1
Some examples of commercially available and traditionally produced fermented cereal based beverages from lactic acid fermentation. i) Commercially available cereal based beverages produced from lactic acid fermentation.

Brand	Producer, Country	Cereal	LAB strains	Reference
Whole Grain Probiotic Liquid®	Grainfields, Australia	malted oats, maize, wheat, barley, millet, rice, rye grains	*L. acidophilus*, *L. delbruekii* subsp. *bulgaricus* *S. boulardii* *S. cerevisiae*	a
Proviva®	Skane Dairy, Sweden	oatmeal gruel, malted barley	*L. plantarum* 299v	a
SOYosa®	Bioferme, Finland	oat	*L. acidophilus*, *Bifidobacterium lactis*	b
Yosa®	Bioferme, Finland	oat	*L. acidophilus*, *Bifidobacterium lactis*	b
Jovita Probiotisch®	H &J Bruggen, Germany	blend of cereals	n.p.	b

ii) Traditionally produced cereal-based beverages from lactic acid fermentation.

Name	Country	Cereal	LAB strains	Reference
Obiolor	Nigeria	sorghum, millet malt	*L. plantarum* *Streptococcus lactis*	c
Mahewu	South Africa,	maize meal, millet/sorghum malt or wheat	*L. delbrueckii*	d
Mageu	Zimbabwe	flour	*L. brevis* *Leuc. mesenteroides*	
Bushera	Uganda	millet, sorghum	*L. plantarum*, *L. paracasei*, *L. fermentum*, *L. brevis*, *L. delbrueckii*, *Streptococcus thermophilus*	e
Oskikundu	Namibia	cooked pearl millet, sorghum malt	n.p.	f
Sobia	Saudi Arabia	wheat, malt flour	spontaneous fermentation	g

n.p.: not provided.
[a] (Soccol et al., 2012).
[b] (S. Liu & Han, 2014).
[c] (Achi, 1990).
[d] (Gadaga, Mutukumira, Narvhus, & Feresu, 1999; Holzapfel & Taljaard, 2004).
[e] (Muyanja, Narvhus, Treimo, & Langsrud, 2003).
[f] (Taylor & Emmambux, 2011).
[g] (Gassem, 2002).

3. Malt wort as a suitable medium for the LAB growth

Fermentation performance is decisive for metabolic activities. Medium composition affects not only cell growth but also metabolite formation. Thus, fermentation medium should fulfill nutritional and physical requirements of LAB. LAB growth in cereal substrates is unambiguous although strain dependency on nutrient requirements. Furthermore, malt derived substrates were chosen as most suitable substrates for LAB growth and aroma compound development (Charalampopoulos et al., 2003; Coda et al., 2011; Gebremariam et al., 2015; Kedia et al., 2007; Salmerón, Rozada, et al., 2014; Salmerón et al., 2015; Zannini et al., 2013). The malting and brewing process involves several chemical and enzymatic reactions that generate a nutrient-rich medium, the so-called malt wort. A lot of attention has been devoted to the optimization of malting and brewing of wide range of cereals as raw materials for functional beverage production (Hassani, Zarnkow, & Becker, 2013; Steiner, Gastl, & Becker, 2011; Zarnkow et al., 2008; Zarnkow, Keßler, Back, Arendt, & Gastl, 2010). Table 2 presents wort composition in regard to growth factors and aroma precursors.

Growth behavior of LAB in malt based substrates is similar to the common bacteria growth trend. Fermentation duration generally does not exceed 72 h until the stationary phase is reached. Subsequent decrease of the pH value is observed due to organic acids production (Charalampopoulos, Pandiella, & Webb, 2002; Charalampopoulos et al., 2003; Rozada-Sánchez, Sattur, Thomas, & Pandiella, 2008; Rozada, Vázquez, Charalampopoulos, Thomas, & Pandiella, 2009; Salmerón, Rozada, et al., 2014). Growth cessation was tentatively attributed to the accumulation of organic acids but not to substrate limitation (Rozada-Sánchez et al., 2008). Besides nutrient availability, growth promoting factors in malt wort include its pH value of 5.2–5.6 suitable for LAB, high buffering capacity of 7.2–27.7ml/0.1N HCL (Krüger & Anger, 1990), and the presence of non-digestible materials like dextrins, and β-glucan. Dextrins and β-glucan, in addition to sugars, serve as prebiotics and cell preservative from acidic stress (Charalampopoulos et al., 2002; Rathore, Salmeron, & Pandiella, 2012). Amino acids, vitamins and minerals are also important for LAB growth and metabolic activities (Hayek & Ibrahim, 2013). In contrast, it is important to emphasize the inhibiting effect of hops, usually added to wort in breweries, on the growth of some LAB (Simpson, 1993).

Further studies to elucidate growth limiting factors and

Table 2
Malt wort predominant nutrients in relation to LAB nutrient requirements and aroma compound precursors.

Groups	Elements	References
Carbohydrates	glucose (10–15%), fructose (1–2%), sucrose (1–2%), maltose (50–60%), maltotriose (15–20%, dextrins (20–30%)	(Stewart, 2006)
Nitrogenous materials	peptides, proteins, ammonia, amino acids (glutamic acid, valine, glycine, proline, aspartic acid, methionine, phenylalanine, asparagine, leucine, tyrosine, glutamine, isoleucine, tryptophan, serine, histidine, alanine, threonine, ammonia, lysine, arginine, hydroxyprolin, cysteine, ornithine, citrullin, sarcosin)	(Jones & Pierce, 1964; Krüger & Anger, 1990)
Vitamins	thiamin, riboflavin, nicotinamide, pantothenic acid, pyridoxine, cobalamin, folic acid, inositol	(Priest & Stewart, 2006)
Minerals	phosphat, sodium, magnesium, calcium, manganese, zink, potassium, copper, iron, chloride, nitrate, silicate	(Krüger & Anger, 1990)
Fatty acids	myristic acid, palmitic acid, palmitoleic acid, heptadecanoic acid, stearic acid, oleic acid, linoleic acid, linolenic acid	(Bravi, Benedetti, Marconi, & Perretti, 2014)
Organic acids	lactic acid, citric acid, fumaric acid, acetic acid	(Rozada-Sánchez et al., 2008)
Phenolic acids	*p*-coumaric acid, caffeic acid, protocatechuic acid, *m*-coumaric acid, gallic acid, ferulic acid, vanillic acid, homovanillic acid	(Floridi, Montanari, Marconi, & Fantozzi, 2003; Szwajgier, 2009)

essential nutrients of LAB in cereal fermentation with the aim of extending fermentation duration are needed. As such, the richness of wort in aroma precursors like sugars, amino acids, fatty acids, glycosides and phenolic acids make it suitable matrix for aroma compound generation during fermentation.

4. Contribution of malt and wort aroma composition to the flavor

Malt wort aroma is crucial for the derived beverage flavor as it is for beer flavor. While some aroma compounds survive the fermentation process to further contribute to the final beverage flavor, others may be converted to potent aroma compounds during fermentation. Malt wort production process induces the formation of various aroma compounds which define its typical flavor. As a result, the sensory quality of malt derived substrates is more attractive than unmalted cereal substrates. Aroma compounds are formed from maillard reactions, strecker degradation, fatty acid oxidation, glycosides hydrolysis and oxidation, and miscellaneous reactions (Buckee, Malcolm, & Peppard, 1982; De Schutter et al., 2008b).

Table 3 summarizes a total of ninety-five aroma compounds reported in malt and un-hopped wort. They are formed from different precursors, and impart different flavor qualities. Alcohols, aldehydes, esters, furans, heterocyclic compounds, ketones, nitrogenous compounds, sulfur compounds, terpenoids, lactones, and acids are the compound groups. Some of them may derive from malt whereas others are formed during boiling process. 3-Methylbutanal, 2-methylpropanal, (E,E)-2,4-decadienal, diacetyl, 1-octen-3-one, 2-and 3-methylbutanoic acid, 4-hydroxy-2,5-dimethyl-3-(2H)-furanone, dimethylsulfide, methional and vanillin are the most important odorants of malt (Fickert & Schieberle, 1998).

Wort boiling is of great complexity in regard to aroma generation. It induces the formation of aroma compounds but at the same time it leads to the lost by evaporation. Strecker aldehydes and furans for example are generated during wort boiling whereas lipid oxidation products hardly develop. Furthermore, concentration of norisoprenoid β-damascenone during wort boiling significantly increased at low pH value (De Schutter et al., 2008b). Ethanol, acetic acid, acetaldehyde, 2-methyl-2-butenal, (E)-2-octenal, (E)-2-nonenal, 2-heptanone, 3-hydroxy-2-butanone, ethyl-3-methyl butanoate, ethyl acetate, ethyl-2-methyl propanoate, methyl acetate, isoamylacetate, methylgeranate, γ-nonalactone, α-terpineol, 3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolon), 3-methoxy-4-hydroxybenzaldehyde (vanillin) were detected in wort but not in malt (Buckee et al., 1982; De Schutter et al., 2008b; Dong et al., 2013; Fickert & Schieberle, 1998), they might be lost during boiling process.

Pyrazines, pyrroles, furans and sulfur-containing compounds are products of maillard reactions occurring at high temperature processing of wort. Fat degradation products are alkanals, 2-alkenals, and 2,4-alkadienals (Parker, Hassell, Mottram, & Guy, 2000). Strecker aldehydes are mainly formed from leucine, isoleucine and especially methionine (Perpète & Collin, 1999) even though significant concentration change was observed when wort lipid content was modified (Gallardo et al., 2008). Therefore, the significant impact of boiling on wort aroma composition can be exploited in wort production for the removal of targeted undesirable aroma compounds.

Linalool, a terpene derivative with strong sensory quality, was detected in malt wort. Yet, there is no evidence of glycosides in unhopped malt wort although they are naturally occurring in cereals like barley, wheat, oat (Blessin, 1962; Elena Mellado-Ortega & Hornero-Méndez, 2015). Furthermore, linalool, geraniol and α-terpineol can be synthesized from precursors by LAB glucosidases and arabinosidases during fermentation or by cleavage at acidic conditions (Michlmayr et al., 2012). 3-hydroxy-damascone, detected in wort, can be converted to β-damascenone during lactic acid fermentation under favorable acidic conditions (Gijs, Chevance, Jerkovic, & Collin, 2002).

Although scarce research data reported on wort flavor, those brewed with dark malts were described as bitter, burnt, husky, sweet, caramel and bread-like (Coghe, Martens, D'Hollander, Dirinck, & Delvaux, 2004). The complexity of malt wort aroma and nutrient components offers opportunities for chemical or microbial generation of aroma compounds during lactic acid fermentation. Advances in brewing technology and suitable choice of cereal cultivars may result in malt and wort with defined aroma profile that will positively impact the resulting fermented beverage flavor.

5. Aroma-active compounds of cereal beverages produced by lactic acid fermentation

Aroma compound formation in lactic acid fermentations has extensively been reviewed (Hugenholtz, 1993; Mallia, Escher, & Schlichtherle-Cerny, 2008; Quintans, Blancato, Repizo, Magni, & Lopez, 2008; Smid & Kleerebezem, 2014; G.; Smit, Smit, & Engels, 2005). Several reactions from extracellular precursors are involved. Proteins, amino acids, carbohydrates, fatty acids are the main precursors in lactic acid fermentation. Lactic acid fermentation of cereal substrates may deliver different aroma composition. Alcohols, aldehydes, ketones, esters, furans, phenolic compounds, organic acids and fatty acids are reported. Table 4 summarizes seventy-four aroma compounds detected in cereal beverages produced by lactic acid fermentation of barley, oat, wheat, and emmer flour, and barley and emmer malt. In malt based fermented beverages produced from lactic acid fermentation, aroma profile may be constituted from: i) malt wort derived aroma compounds that may have resisted the fermentation process, ii) conversion of malt wort derived precursors by LAB enzymes and changes in environmental conditions. The main differences include: phenolic compounds (phenylethanol, 4-vinylguiacol, guiacol, 4-vinylphenol and vanillin), furans (furfural, maltol, furaneol and sotolon), higher alcohols (2/3-methylbutanol, 2-propanol and 2-methylpropanol), terpenoids (linalool), sulfur compounds (methional, dimethyldisulfide and dimethyltrisulfide) and norisoprenoid (β-damascenone). It is evident that cereal type affects the aroma composition of the resulted beverages. However, it is difficult to point out unambiguously the impact of the starter culture.

To the best of our knowledge, there exists not yet literature data about key aroma compounds of cereal beverages based on lactic acid fermentation. Yogurt key aroma compounds acetaldehyde, diacetyl, acetoin, acetone, 2-butanone, and lactic acid were also detected in cereal beverages (Cheng, 2010; Routray & Mishra, 2011). Quantitation studies in cereal beverages considered acetaldehyde, acetone, ethanol, acetoin, lactic acid, and acetic acid. Their concentrations were 0.11–1.36 mg/l, 0.73–1.30 mg/l, 0.001–0.050% abv., 0.07–0.11 g/l, 0.2–5.7 g/l, and 0.02–0.62 g/l, respectively depending on the starter culture and cereal type (Charalampopoulos et al., 2002; Coda et al., 2011; Kedia et al., 2007; Rathore et al., 2012; Rozada-Sánchez et al., 2008; Salmerón et al., 2015). Except acetone and ethanol, their concentrations are greater than their odor detection thresholds of 0.025 mg/l, 200 mg/l, 0.09%abv., 0.05 g/l, 0.18 g/l, and 0.09 g/l, respectively as reported in water (Czerny et al., 2008; Shallenberger, 2012; Tan & Siebert, 2004). Therefore, they may contribute to the overall aroma provided the flavor threshold is reached. However, there is not yet an evidence of their contribution as key aroma compounds of cereal

Table 3
Aroma active compounds detected in malt and unhopped wort, and their odor qualities.

Group of compounds	Odor quality	Unhopped wort/malt	References
Alcohols			
3-methylbutanol	whiskey, malt, burnt	wort/malt	a,b
1-pentanol	sweet, balsamic	wort/malt	a,b,d
1-penten-3-ol	Butter, pungent	wort/malt	a,b
1-hexanol	resin, flower, green	wort/malt	a,b
2-ethylhexanol	rose, green	wort	a,c
1-octen-3-ol	mushroom	wort/malt	a,b
1-nonanol	fat, green	wort/malt	a,c
phenylethanol	honey, rose	wort/malt	a,b,d
furfuryl alcohol	burnt	wort/malt	a,e,f
tyrosol	beewax, honey-like	wort	g
Aldehydes			
propanal	malty	wort/malt	a,b,i
2-methylpropanal[a]	solvent, malty	wort	a,h
butanal	malty	wort/malt	a,b,d,e i
3-methylbutanal[a]	malty	wort/malt	a,b,d,i
2-methylbutanal[a]	malty, cacao	wort/malt	b
pentanal	almond, malt, purgent	wort/malt	a,b,d,e
hexanal	grass tallow, fat	wort/malt	a,b
heptanal	fat, citrus, rancid	wort/malt	i
2-furfural	almond, toasted	wort/malt	a,d,f,j
5-methyl-2-furfural	almond, sweet	wort/malt	a,d,f,h
5-hydroxymethyl-2-furfural	nf	wort	a,f,j
benzaldehyde	harsh, green, honey	wort/malt	a,b,d
4-hydroxybenzaldehyde	almond, burnt sugar	wort	b,f
phenylacetaldehyde	honey-like	wort	a,i
nonanal	fat, citrus, green	wort/malt	a,b
2,4-decadienal[a]	fatty, deep-fried	wort	a,i
4-methyl-2-phenyl-2-pentenal	nf	wort	a
5-methyl-2-phenyl-2-hexenal	nf	wort/malt	d,a,
2-phenyl-2-butenal	nf	wort	a
Ketones			
acetone	ether, grape	wort	a,h
2-butanone	sharp, sweet	wort	a,h
2,3-butanedione (diacetyl)[a]	butter	wort/malt	a,b,e,i
2-pentanone	fruity	wort	a,h
2,3-pentanedione[a]	buttery	wort/malt	a,d,e,i
2-octanone	soapy, fruity	wort	a,h
3-octen-2-one	earthy	wort	a,h
2-nonanone	fruity, soapy	wort	a,k
methyl isobutyl ketone	nf	wort	a
4-cyclopentene-1,3-dione	nf	wort	a
6-methyl-5-hepten-2-one	herbal, oily, pungent	wort/malt	a,b
acetophenone	nf	wort	a
4-hydroxyacetophenone	nf	wort	f
3,5-octadien-2-one	nf	wort	a
4-hydroxy-2,5-dimethyl-3(2H)-furanone (furaneol)[a]	caramel-like	wort/malt	i,l,m
4-hydroxy-5-methyl-3(2H)-furanone[a]	caramel-like	wort/malt	i,l,m
3-Hydroxy-4,5-dimethyl-2(5H)-furanone (sotolon)	seasoning-like	malt	i,l,m
5-ethyl-3-hydroxy-4-methyl-2(5H)-furanone[a]	seasoning-like	wort/malt	i,l,m
β-damascenone	baked apple-like	wort	n
3-hydroxy-damascone	nf	wort	a
Esters			
vinyl acetate	nf	wort	a
Nitrogenous compounds			
2,5-dimethylpyrazine	nutty	wort/malt	a,d,o
2,6-dimethylpyrazine	nutty	wort/malt	d, f,o
trimethylpyrazine	roasty	wort/malt	d,f,o
3-ethyl-2,5/2,6-dimethylpyrazine	roasty, earthy	wort	a,o
2-ethyl-3-methylpyrazine	nutty	wort/malt	d,f,o
3-ethyl-5-methylpyrazine	nf	wort	f
2-ethyl-3,5-dimethylpyrazine	roasty, earthy	wort	f,o
2-ethyl-3,6-dimethylpyrazine	earthy	wort	f,h
indole	faecal, mothball-like	wort	n,f
Phenolic compounds			
4-vinylphenol	rice and wine	wort	f,p
guaiacol	smoky, sweet	wort	f
4-methylguaiacol	smoky	wort	f,q
4-vinylguaiacol	clove-like	wort	a,e,f
4-vinyl-2,6-dimethoxyphenol	smoked, burnt	wort	f

(*continued on next page*)

Table 3 (*continued*)

Group of compounds	Odor quality	Unhopped wort/malt	References
acetovanillone	faintly vanilla-like	wort	g,f
Other heterocyclic compounds			
2-methylfuran	chocolate	wort	a,h
2-ethylfuran	nf	wort	a
2-vinylfuran	nf	wort	a
2-pentylfuran	green bean, butter	wort/malt	a,b,d,f
2-acetylfuran	sweet, cabbage, balsamic	wort/malt	a,d,f
5-methyl-2-acetylfuran	nf	wort	f
3-hydroxy-2-methyl-4(H)-pyran-4-one[a]	caramel-like	wort/malt	d,i,f,j
5-hydroxymaltol	nf	wort	f
5-hydroxy-5,6-dihydromaltol[a]	caramel like	wort/malt	i
γ-butyrolactone	creamy type	wort	a, h
1-methylpyrrole	woody	wort	f,h
2-acetylpyrrole	roasted, biscut	wort	f,h
5-methylpyrrolaldehyde	nf	wort	f
2-acetylpyridine	biscuit, cracker-like	wort	f,h
ethylpyridine	nf	wort	f
Sulfur compounds			
methanethiol	sulfury	wort	a,k
dimethyl sulfide[a]	cabbage, sulfurous	wort/malt	a,b,i,r,f
thiophene	benzene-like	wort	a
dimethyl disulfide	cabbage-like	wort	a
dimethyl trisulfide	sulfurous	wort/malt	a,d,i,k
3-methylthio-propionaldehyde[a]	potato-like	wort/malt	a,b,d,i
Terpenoids			
linalool	citrus-like	wort	a,n
isoeugenol	spicy, honey-like	wort	f
Organic acids			
acetic acid	vinegar	wort	e
lactic acid	sour	wort/malt	b,f
isovaleric acid	sweaty	wort	a,h
palmitic acid	waxy	wort	h,j

[(a)](De Schutter et al., 2008a).
[(b)](Dong et al., 2013).
[(c)](Acree & Arn, 2015).
[(d)](Beal & Mottram, 1994).
[(e)](Coghe et al., 2004).
[(f)](Krüger & Anger, 1990).
[(g)](Jackson, 2014).
[(h)](Pico, Bernal, & Gómez, 2015).
[(i)](Fickert & Schieberle, 1998).
[(j)] (Salmeron, Fuciños, Charalampopoulos, & Pandiella, 2009).
[(k)](Rychlik, Schieberle, & Grosch, 1998).
[(l)](Mackie & Slaughter, 2000).
[(m)](Mackie & Slaughter, 2002).
[(n)](Czerny et al., 2008).
[(o)](Wagner, Czerny, Bielohradsky, & Grosch, 1999).
[(p)](Czerny, Romy Brueckner, Eva Kirchhoff, Schmitt, & Buettner, 2011).
[(q)](Nollet et al., 2008).
[(r)](Hartough, 2009).
[a] Compounds identified as most odor active in caramalt (Fickert & Schieberle, 1998); nf: not found, wort/malt: detected in wort and malt; wort: detected only in wort.

beverages. Comparative studies revealed higher concentrations when malted cereals, and *Lactobacillus plantarum* strain were considered. Acetaldehyde is produced in lactic fermentation from pyruvate in the glycolysis or from the conversion of threonine by the enzyme threonine aldolase. Acetone, diacetyl, acetoin are generated from citrate metabolism instead (Gänzle et al., 2007; Hugenholtz, 1993).

Fusel alcohols were mostly found in barley malt beverages whereas fatty esters were reported in oat based beverages. It may be in relation with higher amino acid availability in malted barley substrates and higher lipid content in oat substrates, respectively (Muñoz-Insa, Gastl, Zarnkow, & Becker, 2011). Fusel alcohols and esters are formed from the breakdown of branched chain amino acids in the Ehrlich mechanism. Ethanol is the sole alcohol formed in the glycolysis (Gänzle et al., 2007). Even so, the major mechanism of esters biosynthesis in LAB is the transfer of a fatty acyl group from acylglycerol and acyl-CoA to an alcohol (S. Q. Liu et al., 2004). The low alcohol content of these beverages (<0.5% v/v) (Zannini et al., 2013) may not be in favor of esterification. Esters are positively regarded because they impart fruity flavor. Fatty acids are sensory not desirable because they impart a soapy-like odor but they may serve as precursors for ester formation. Lactic acid and acetic acid, the major organic acids of lactic acid fermentation, can be involved in ester formation such as ethyl lactate and ethyl acetate, respectively.

3-Hydroxy-damascone, found in wort, is one of the glyco-conjugate carotenoid precursors of β-damascenone (Puglisi, Elsey, Prager, Skouroumounis, & Sefton, 2001). β-Damascenone with its apple juice fruity flavor and very low detection threshold in water (0.013 μg/l) (Czerny et al., 2008) could positively affect cereal

Table 4
Aroma active compounds and their odor qualities detected in beverages produced by lactic acid fermentation of various cereal substrates. BMB: barley malt based beverage; BB: barley substrate based beverage; EMB: emmer malt based beverage; EB: emmer substrate based beverage; OB: oat substrate based beverage; WB: wheat substrate based beverage.

	BMB[i,j,k]	BB[j,l]	OB[j,l]	WB[j]	EMB[m]	EB[m]	Odor quality (references)
Alcohols							
ethanol	+	+	+	–	+	+	sweet, alcohol (a)
1,2-ethanediol	+	–	–	–	–	–	nf
2-propanol	–	–	–	–	–	+	fruity, alcoholic (b)
1-butanol	–	–	–	–	+	–	malty, solvent (b)
2-butanol	–	+	–	–	+	+	nf
2-methylpropanol	+	+	–	–	–	–	malty, wine-like (b)
2-ethoxyethanol	+	–	+	–	–	–	sweet, ether-like (b)
3-methylbutanol	+	–	–	–	+	+	malt, burnt (a)
1-hexanol	–	–	–	–	+	+	resin, flower, green (a)
2-butoxyethanol	+	–	–	–	–	–	nf
1-pentanol	–	–	–	–	+	+	sweet, balsamic (a)
2-ethylhexan-1-ol	–	–	–	–	–	+	green, vegetable (b)
2-dodecanol	–	+	–	–	–	–	waxy type (b)
Aldehydes							
acetaldehyde	+	+	+	–	–	+	fresh, green (c)
hexanal	–	–	–	–	–	+	grass tallow, fat (a)
benzaldehyde	–	–	–	–	–	+	harsh, green, honey (a)
2,4-decadienal	–	–	–	–	+	+	fatty, deep-fried (d)
9,17-octadecadienal	–	–	–	+	–	–	nf
Ketones							
acetone	+	+	+	–	+	+	ether, grape (b)
acetoin	+	+	–	+	–	–	butter, cream (e)
2-butanone	–	–	–	–	–	+	etheric (e)
2,3 butanedione (diacethyl)	+	–	–	–	+	+	butter (a, d)
2-pentanone	+	–	–	–	+	–	pungent, fish like (e)
2-pentanone, 4-hydroxy, 4-methyl	+	–	–	–	–	–	truffle, herb, nut (f)
1-penten-3-one	–	–	–	–	–	+	sewer-like, fruity (g)
Esters							
ethyl acetate	+	+	+	–	+	+	solvent-like, fruity (e)
2-ethoxyethyl acetate	+	–	+	–	–	–	nf
isoamyl salicylate	+	+	–	–	–	–	peppermint (f)
methyl furoate	+	–	–	–	–	–	berry-like fruity (h)
methyl dodecanoate	–	+	–	–	–	–	wine (f)
methyl palmitate	–	–	+	–	–	–	nf
ethyl palmitate	–	–	–	+	–	–	waxy (b)
methyl oleate	–	–	+	+	–	–	nf
Phenolic compounds							
2-phenylethanol	+	–	–	–	–	–	honey, rose (a)
3,5-dimethoxyphenol	+	–	–	–	–	–	nf
2-methoxyethylbenzene	–	+	–	–	–	–	nf
1,4-dihydroxy-2-methoxybenzene	–	+	–	+	–	–	nf
vanillin	–	+	+	–	–	–	vanilla-like (d)
Heterocyclic compounds							
3-furanmethanol	+	–	–	–	–	–	nf
2-ethylfuran	–	–	–	–	–	+	nf
2-pentylfuran	–	–	–	–	+	+	green bean, butter (a)
2-acetyllfuran	+	–	–	–	–	–	sweet, balsamic (e)
furfural	+	–	–	–	–	–	bread, almond (b)
5-hydroxyl-methylfurfural	+	–	–	–	–	–	almond, caramel, burnt (b)
3-hydroxyl-2-methyl-4-pyrone (maltol)	+	–	–	–	–	–	caramel-like (d)
5,6 dihydro-5-hydroxymaltol	+	+	–	–	–	–	nf
Organic acids							
formic acid	+	–	–	–	–	–	pungent (b)
acetic acid	+	+	+	+	+	–	sour (a, d)
propionic acid	+	+	–	–	–	–	fruity, pungent (b)
lactic acid	+	+	+	+	+	+	sour (a)
butanoic acid	+	+	+	+	+	+	sweaty, rancid (e)
2-methylpropanoic acid	–	+	–	–	–	–	sweaty (e)
pentanoic acid	–	+	–	–	–	–	sweaty (e)
hexanoic acid	–	+	+	+	+	+	goat-like (e)
heptanoic acid	–	+	–	+	–	–	sweaty (e)
octanoic acid	–	–	+	–	–	+	sweaty (e)
nonanoic acid	–	+	+	–	–	–	sweaty (e)
myristic acid	–	+	+	–	–	–	nf
palmitic acid	+	+	+	+	–	–	waxy (b)

-, not present; +, present; nf, not found.
[a] (Dong et al., 2013).
[b] (Pico et al., 2015).

[c] (Czerny et al., 2008).
[d] (Fickert & Schieberle, 1998).
[e] (Rychlik et al., 1998).
[f] (Acree & Arn, 2015).
[g] (Carrapiso, Ventanas, & García, 2002).
[h] (Flament & Bessière-Thomas, 2002).
[i] *L. plantarum* NCIMB 8826 (Salmeron et al., 2009; Salmerón et al., 2015).
[j] *Bifidobacterium breve* NCIMB 702257 (Salmerón, Rozada et al., 2014).
[k] *L. acidophilus*, *L. reuteri;* concerns acetaldehyde, acetone, ethanol and ethylacetate (Salmerón et al., 2015).
[l] *L. plantarum* LP09 (Nionelli et al., 2014).
[m] *L. plantarum* 6E (Coda et al., 2011).

beverage flavor, if present at adequate concentration. Linalool is not reported in beverages although it was detected in wort. It may be due to the extraction method used by the authors when considering the complexity of essential oils extraction (Bicchi et al., 2008).

2-Phenylethanol, and 3,5-dimethoxyphenol are the phenolic derived aroma compounds identified in cereal fermented beverages. Phenolic compounds apart from been related to food flavor, astringency and color, are also precursors of aroma compounds. In the same way, vanillin, 4-vinylphenol, 4-ethylphenol, 4-vinylguaicol, 4-ethylguaiacol are generated from phenolic acids via LAB phenolic acid decarboxylases and reductases (Baljinder Kaur, Debkumar Chakraborty, & Kumar, 2013; Bloem, Bertrand, Lonvaud-Funel, & De Revel, 2007; Rodríguez et al., 2009). Vanillin for example has a pleasant vanilla-like flavor (Czerny et al., 2008) which may have a positive impact on cereal beverage flavor. Wort content in phenolic acids, led to suggest that phenolic aroma compounds can be formed in malt wort beverages provided the suitable starter culture is used.

Neither the knowledge of the aroma composition nor the relative abundance suffices to apprehend beverage flavor. Reported results push to the necessity for further studies on the characterization of key odorants in order to describe contributing aroma compounds of the typical flavor of cereal beverages. Further studies on the synergistic effect of key odorants and the interaction with non-volatile matrix composition on flavor perception are also of necessity.

Odor active values (OAV) and flavor dilution factors (FD) are proposed for the determination of the effect of individual aroma compounds to the final product flavor. Furthermore, aroma reconstitution and omission tests are usually performed to evaluate the additive effects of aroma compounds in a mixture (Fickert & Schieberle, 1998; Grosch & Schieberle, 1997; Grosch, 1994). On the other hand, volatile isolation methods considered so far are liquid to liquid solvent extraction, head space isolation and solid phase extraction followed. Each isolation method offers advantages and drawbacks on the aroma composition yield of cereals (Zhou, Robards, Glennie-Holmes, & Helliwell, 1999). Therefore, isolation methods, extraction solvent, and sample volume should be carefully chosen.

6. Sensory profile and impact of acidic odorants

Food sensory profile determines its acceptance and attraction to consumers. Therefore, its identity and unique character relies on the flavor quality. Sensory attributes of cereal beverages produced by lactic acid fermentation are reported similar, whatever the raw material or starter culture. Flavor attributes are sour, sweetish, wort-like, malty, cereal-like, roasted, and vegetable-like whereas taste and trigeminal attributes are sour, sweet, bitter, and astringent (Coda et al., 2011; Gebremariam et al., 2015; Nionelli et al., 2014; Salmerón, Rozada, et al., 2014). Due to the diversity of consumer acceptance of sour products, and the lack of more attractive attributes like fruitiness, they are not always accepted by consumers. Flavor profile improvement was demanded as the most important characteristic by consumers (Yu & Bogue, 2013). Furthermore, better acceptance was reported for beverages produced from barley and malt and *Lactobacillus plantarum* as starter culture (Salmerón et al., 2015).

On the other hand, organic acid production in lactic acid fermentation improves food palatability. Citric acid, lactic acid, acetic acid, and malic acid are already used in beverage industries as acidulants and flavor enhancers (Ashurst, 2005). Formic acid and acetic acid are not desirable because of the potential pungent sensory properties. Malic acid can provide pleasant taste to the beverage. Succinic acid should be regarded as negative because of the bitter aftertaste as described in emmer based beverages (Coda et al., 2011).

Customization of organic acid concentrations through the ratio lactic acid/acetic acid and sugar/acidity could be an asset for acceptance improvement. A pH value of 4–4.5 was proposed as sensorial acceptable (Angelov, Gotcheva, Hristozova, & Gargova, 2005) while a sugar/acidity ratio of 1.5 is recommended for lemon juice, for example (Ashurst, 2005).

A boost of sensory acceptance relies consequently on the aroma, organic acid composition and sugar content. A sensory study for consumer's preferences in terms of acidity, ratio sugar/acidity, sweetness, palate-fullness, and flavors could be an important input to drive the experimental design for flavor improvement. In regard to wort content in aroma compound precursors, there are several possible routes for the generation of targeted aroma compounds from LAB.

7. Potential routes of aroma generation in lactic acid fermentation of malt substrates

We summarized briefly in Fig. 1 the potential metabolic routes and aroma compounds that can be generated from malt and wort precursors through lactic acid fermentation. Pyruvate is an important intermediate for short chain aroma compounds; end metabolites depend on the presence or absence of electron acceptors such as oxygen, fructose and nitrate. Further potential attractive aroma compounds of malt based beverages produced from lactic acid fermentation could be β-damascenone, acetaldehyde, alcohols, esters, δ- and γ-lactones, phenolic aroma compounds and some terpenoids (linalool, geraniol). Most of them are not yet identified in cereal fermentation; although the precursors are available. The bottleneck may be the absence of optimum environmental conditions or enzyme availability related to starter culture.

8. Proposed research prospects for flavor improvement of malt wort beverages

8.1. Importance of starter culture and substrate selection

Selection of suitable starter culture for fermented food production has always proved to be an effective approach in the production of target compounds. The potential of LAB as functional

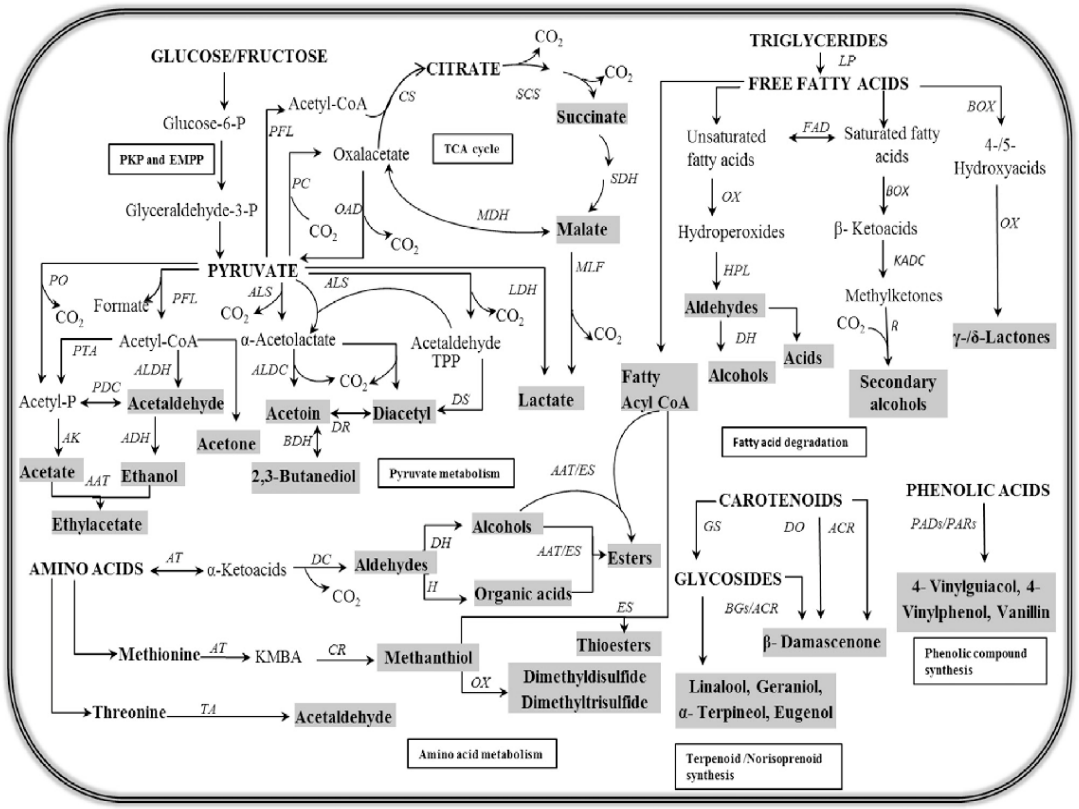

Fig. 1. Proposed schematic representation of potential aroma compounds and organic acids that can be generated in lactic acid fermentation of malt wort from existing precursors. In bold are major aroma compounds ; PKP, phosphoketolase pathways; EMPP, Embden-Meyerhof-Parnas pathways; TCA, tricarboxylic acid cycle; PC, pyruvate carboxylase; CS, citrate synthase; SCS, succinyl-CoA synthase; SDH, succinate dehydrogenase; FA, fumarase; MDH, malate dehydrogenase; OAD, oxaloacetate decarboxylase; PO, pyruvate oxidase; PFL, pyruvate formate lyase; ALS, acetolactate synthase; LDH, lactate dehydrogenase; PTA, phosphate acetyltransferase; ALDH, acetaldehyde dehydrogenase; ALDC, acetolactate decarboxylase; PDC, pyruvate decarboxylase; AK, acetate kinase; AAT, alcohol acyltransferase; DR, diacetyl reductase; DS, diacetyl synthase; BDH, 2,3-butanediol dehydrogenase; MLF, malolactic fermentation; AT, aminotransferases; DC, decarboxylase; DH; dehydrogenase; H, hydrogenase; TA, threonine aldolase; OX, oxidation; KMBA, 2-oxo-4-methylthiobutyric acid; CR, chemical reaction; LP, lipase; FAD, fatty acid desaturase; BOX, β-oxidation; HPL, hydroperoxide lyase; KADC, β-ketoacyldecarboxylase; R, reductase; ES, esterase; GS, glycosylation; DO, dioxygenase; BGs, β-glucosidase; ACR, acidic chemical reaction; PADs, phenolic acid decarboxylases; PARs, phenolic acid reductases. This figure is adapted from (McSweeney & Sousa, 2000; Mendes-Pinto, 2009; Skouroumounis & Sefton, 2000; Smid & Kleerebezem, 2014; Smit et al., 2005).

starter cultures for a more appealing product in terms of aroma, flavour and texture in food fermentation industry was described (Leroy & De Vuyst, 2004). Selection criteria of LAB should be driven by the targeted flavour with focus on the relevant metabolic activity, strain potential and technology (Buckenhüskes, 1993). Fig. 2 outlines important traits in the selection of LAB strain to be used for cereal based beverages production. Three starter cultures of *Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Lactobacillus reuteri* yielded different concentrations of acetaldehyde, acetone and ethanol in three cereal substrates of barley, barley malt, and oat. It seemed to be related to the metabolic affiliation. Facultative heterofermentative *Lactobacillus plantarum* likely produced more acetaldehyde in the three substrates, while obligate homofermentative *Lactobacillus acidophilus* produced more ethanol and acetone in barley and oat substrates, respectively. Obligate heterofermentative *Lactobacillus reuteri* produced more ethanol in barley substrate while acetone was produced only in oat substrate (Salmerón, Thomas, & Pandiella, 2014; Salmerón et al., 2015).

Combination of starter cultures possessing different enzymatic profile was considered for flavor enhancement. Tailor-made culture combination of high proteolytic activity and high decarboxylation activity dairy strains of *Lactococcus lactis* subsp. *cremoris* increased 3-methylbutanal yield (Ayad, Verheul, Engels, Wouters, & Smit, 2001). Mesophilic non-starter lactobacilli culture *Lactobacillus plantarum* is commonly used as adjunct starter for intensification and acceleration of flavor formation (Schaffner & Beuchat, 1986; Settanni & Moschetti, 2010).

Facultative heterofermentative LAB, *Lactobacillus plantarum* for instance, may be advantageous since they can switch from the Embden-Meyerhof-Parnas to the phosphoketolase pathways in sugar metabolism according to the environmental conditions leading to more diverse metabolites. Besides, acetaldehyde (apple fruitiness) is the end product of anaerobic conversion of pyruvate as compared to obligate homofermentative and heterofermentative which produces ethanol as the end product. Fermentation pathway affiliation, enzyme availability, considerations of starter culture potential and consociation, and fermentation technology can be exploited as selection criteria.

Metabolic engineering is described as a powerful tool for the exploitation of LAB as cell factories for aroma compounds (Chaves et al., 2002; de Vos & Hugenholtz, 2004). Nonetheless, the general skeptic belief in genetically modified foods does not favor its uses in food production (Sybesma, Hugenholtz, Vos, & Smid, 2006). With the plethora of LAB strain's origins and diversity, thorough strain selection can successfully replace the need of metabolic engineering. High-throughput screening method of LAB for a specific aroma formation was proposed (Smit et al., 2004). This approach is

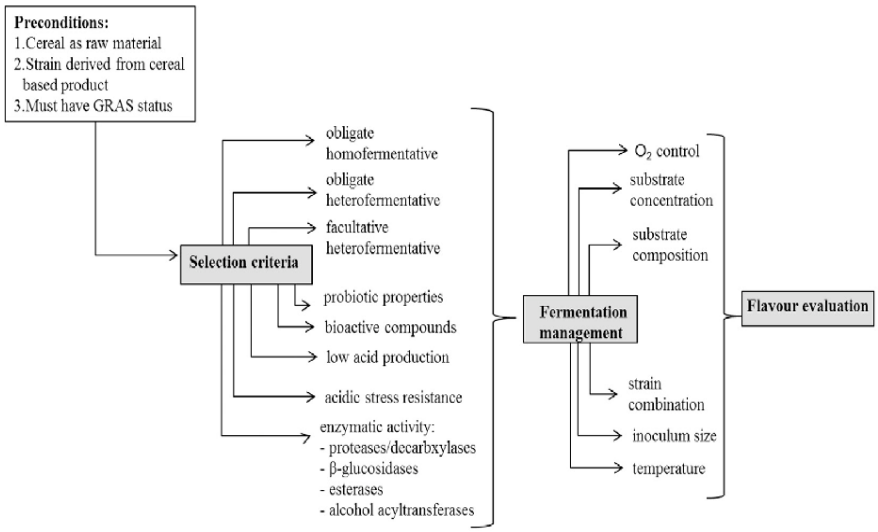

Fig. 2. Proposed schematic representation of the main traits necessary in LAB strain selection.

promising since several strains can be screened by very short time at micro-scale level, but the flavor of a product is not only dependent of a specific aroma compound but to the total contribution of key odorants. Screening at enzyme level is also an often used approach; however, fermentation environmental conditions and substrate composition does not only affect starter culture activity and subsequently metabolites yield but also the expression of genes coding for important enzymes.

Preference should be given to strains that deliver an overall pleasant aroma to the end-product with no off-flavors or less unwanted acids such as acetic acid, succinic acid or tartaric acid. Strain safeness in regard to the GRAS status, consideration of cereal natural environments or those that mimic cereal substrates, like wheat flour hydrolysate, at the expenses of synthetic medium (Buckenhüskes, 1993; Hayek & Ibrahim, 2013) are other aspects that should be considered in the selection.

8.2. Fermentation process management as tool for flavor improvement

The inoculation rate, dissolved oxygen, substrate availability, substrate type, buffering capacity, pH and temperature are fermentation process conditions that may affect and control LAB metabolic responses leading to metabolite generation. Variations in these conditions during fermentation become stressful to bacteria cells resulting to the rerouting of metabolic responses.

8.2.1. Impact of inoculum state and size

Cell reproduction efficiency, vitality and membrane integrity are the prerequisite that determine fermentation performance. Acidification power test was proposed as suitable for fermentation activity assessment of LAB in combination with flow cytometry or standard colony count methods (Bunthof & Abee, 2002; Sigler, 2013). Inoculation level may be used to modulate the generation of aroma compounds in lactic acid fermentation of cereal beverages. In wine for example, inoculation timing and size, significantly affect wine final aroma composition. Higher inoculum size resulted to the increase in higher alcohols while esters were reduced (Erten, Tanguler, Cabaroglu, & Canbas, 2006; Knoll et al., 2012). Research on lactic acid fermented cereal beverages individually considered varying inoculation size often given as inoculation percent volumes that are not always defined. Inoculation size ranges are 1–5% wt/wt (or %v/v), and 0.1–96 $\times$ 10^6 cells/ml. Nonetheless, the inoculation size, and fermentation time was found as main factors that influence the sugar to acid ratio in teff fermented beverages (Angelov et al., 2005; Gebremariam, Zarnkow, & Becker, 2014; Rozada-Sánchez et al., 2008; Salmeron et al., 2009; Zannini et al., 2013). On the other hand, LAB cells respond to high cell density in a concerted manner through production of quorum sensing signaling molecules, auto inducers, which regulate the expression of genes involved in specific metabolite production (Kuipers, de Ruyter, Kleerebezem, & de Vos, 1998). Some typical examples are: i) the accumulation of plantaricin A (PlnA) at a certain *Lactobacillus plantarum* DC400 cell density (Di Cagno et al., 2010), ii) the accumulation of bacteriocin when the 30mer quorum sensing induction peptide was added to the culture of *Streptococcus thermophilus* (Somkuti & Renye, 2014), iii) production of flavor enzymes through the NICE (nisin-controlled expression) system based on quorum sensing, iv) induction of *Lactobacillus lactis* lysis for release of intracellular enzymes involved in flavor formation. High cell density seems promising for flavor compound formation but additional environmental conditions such as low pH value may affect LAB quorum sensing (Kuipers et al., 1998).

8.2.2. Impact of substrate composition and pH value

As mentioned above, metabolite formation is also a function of cell viability and vitality. Use of damaged cells or rapid cell death is doubtless unfavorable to aroma compound formation. Fermentation duration of lactic acid fermentation of cereal substrates ranged from 6 to 72 h. The rapid decrease in the pH value due to the accumulation of organic acids, and the accumulation of undissociated form of lactic acid are strong inhibitors of LAB growth (Adamberg, Kask, Laht, & Paalme, 2003; Pieterse, Leer, Schuren, & Werf, 2005). Thus, fermentation with defined pH-control was recommended (Yoo, Ik-Keun Chang, Lee, Chang, & Moon, 1996). However, in pH-controlled fermentation, accumulation of organic acids caused growth decrease or cessation (Garro, Aguirre, & Savoy de Giori, 2006). The decrease of the pH value is lowered in the presence of a medium with high buffering capacity.

The natural buffering capacity of amphoteric compounds contained in malt wort such as amino acids, proteins and peptones (Yuldasheva, Zhidomirov, Leszczynski, & Ilin, 2013) can be managed to extend fermentation duration. Prolongation of fermentation duration may increase the yield of aroma compound in cereal

beverage production. Likewise, volatile production from LAB increased by three in sourdough fermentation when the leavening time was prolonged by 4 h (Lund, Hansen, & Lewis, 1989). High gravity fermentation could be a promising approach for delivery of increased concentration of relevant aroma compounds and fermentation extension. High gravity substrate provides higher buffering capacity from high concentration of amphoteric compounds and sufficient substrate for high enzymatic activity.

Malt worts prepared from different cereal types and process techniques differ in composition. Table 4 clearly demonstrates the difference in aroma composition when different cereal types are used for fermentation. Enrichment in lactose, glucose, threonine and citrate influenced the level of lactic acid, diacetyl, acetoin and ethanol in yogurt samples. Acetaldehyde concentration significantly raised at higher threonine concentration. Higher lactose concentration augmented the level of diacetyl and acetoin whereas higher glucose concentration significantly increased ethanol concentration (Baranowska, 2006). Sucrose and starch addition in wheat flour caused an increase in fatty acid concentration and a decrease in phenylethanol after fermentation with *Lactobacillus sanfranciscencis* (Vernocchi et al., 2008). Moreover, glucose content negatively affected the esterase activity from *Lactobacillus brevis* whereas sucrose and $CaCl_2$ content had a positive impact (Kim et al., 2013). Enzyme activity rate are strongly related to substrate concentration as demonstrated by Michaelis-Menten equation where the maximum enzyme reaction rate is achieved at saturating substrate concentration.

Therefore, rich nutrient medium may stimulate LAB growth but the concentration of individual components may affect the metabolic activity and aroma compound yield in different manner. Determination of individual component concentrations in wort prepared from different cereal type and processes may serve as basis for cereal choice and process technology.

8.2.3. Controversial benefits of oxygen

Although LAB are generally considered as facultative anaerobic organisms, some of them are equipped with respiration metabolism related to the presence of *cyd*ABCD genes encoding for cytochrome oxidases. They can switch from fermentation to aerobic respiration metabolism. Lactobacilli require exogenous heme and menaquinone for respiration in the presence of an electron acceptor (oxygen, nitrate, or fumarate). However, strains of *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactobacillus iners*, and *Lactobacillus sakei* cannot respire because they lack the *cyd*ABCD genes (Pedersen, Gaudu, Lechardeur, Petit, & Gruss, 2012).

Extended bacterial growth period with long-term survival of cells was attributed to LAB respiration when compared to anaerobic fermentation (Cesselin et al., 2010). Furthermore, respiration removes oxygen from the environment to form water with proton H^+ preventing cell from oxidative stress. Metabolic impact of respiration include: i) reduced lactate production with higher end pH value resulting from lower lactate dehydrogenase activity. In contrast, lactate dehydrogenase did not control lactate production in *Lactobacillus lactis* (Andersen, Pedersen, Hammer, & Jensen, 2001); ii) pyruvate rerouting to acetate, acetoin, and diacetyl formation with less ethanol production (Duwat et al., 2001; Pedersen et al., 2012). However, acetate, diacetyl, and acetoin may be unwanted in cereal beverages. Furthermore, oxygen strongly inhibits the activity of pyruvate formate lyase (PFL) which converts pyruvate to acetyl-CoA and formate under anaeorobic or substrate limited conditions. Acetyl-CoA is subsequently converted to ethanol via acetaldehyde catalysed by alcohol dehydrogenase and acetaldehyde dehydrogenase, respectively. Acetaldehyde, the most important aroma compound in yoghurt, is the major end product of anaerobic conversion of pyruvate by PFL in LAB that lack the enzyme alcohol dehydrogenase (Holzapfel & Wood, 2014).

Respiration can be exploited as a strategy to improve cell growth and survivability to stress, to reduce oxidative and acid stress in favor to extended fermentation that may positively affect aroma compound yield. But on the other hand, it reroutes pyruvate metabolism to diacetyl, acetone and acetoin formation and inhibit the activity of PFL responsible of ethanol or acetaldehyde formation. These observations underline the controversial impact of oxygen on the aroma yield. Consequently, it can be advantageously exploited to modulate the aroma profile of lactic acid fermented malt beverages.

8.2.4. Environmental stresses as tool for aroma generation

Exposure to stress conditions provokes broad transcriptional responses such as proteins overexpression, changes of metabolic pathways that are reflected in metabolite composition (Serrazanetti et al., 2009). Acid, oxidative, or osmotic stress responses are exploited in lactic acid fermentation for aroma compound generation (Serrazanetti et al., 2009). Acid stress is one of the most encountered challenges in lactic acid fermentation. Cereal biotransformations are surely not an exception.

Some LAB produced 2(5H)-furanones and medium-chain fatty acids as response to exposure to oxidative, osmotic, chemical, and heat stresses in whey fermentation (Ndagijimana et al., 2006; Vannini et al., 2007). Cells of *Lactobacillus sanfranciscensis* LSCE1 overproduced γ-decalactone, 2(5H)-furanones and aldehydes at oxidative stress exposure, whereas organic acids and phenylethanol were more accumulated at acid stress during wheat flour fermentation (Guerzoni, Vernocchi, Ndagijimana, Gianotti, & Lanciotti, 2007). Metabolites from branched chain amino acid catabolism increased by seven-fold under acid stress conditions in wheat flour fermentation by *Lactobacillus sanfranciscencis*; genes *glu*AT coding for glutamine amino transferase, *bc*At and *hid* coding for branched-chain aminotransferase and hydroxyisocaproate dehydrogenase, respectively, were overexpressed (Serrazanetti et al., 2011). Furthermore, acid stress induced the metabolic shift toward the overproduction of 3-methylbutanoic acid and 2-methylbutanoic in sourdough fermentation.

Cold stress exposure of sourdough LAB strains at reduced temperature of 15 °C resulted to an increase in the lag phase (from 2 to 5 h), generation time (from 10 to 18 h) and gene expression (M. Gobbetti, De Angelis, Corsetti, & Di Cagno, 2005). Cold stress can consequently be exploited as a promising tool for fermentation duration extension. Nutrient starvation stress applied to *Lactococcus lactis* revealed an overproduction of acetic acid and propionic acid. Similarly, production of 2-methylbutyric acid, α-ketoisocaproate, α-ketoisovalerate, glutamate and citrate from leucine was observed at carbohydrate starvation (Ganesan, Dobrowolski, & Weimer, 2006).

It is evident that application of stresses can be exploited to modulate the concentration of individual or groups of compounds for flavor improvement. Hence, investigation on the impact of individual stress to the yield of targeted aroma compounds in lactic acid fermentation of malt wort will be of great importance.

8.2.5. Contribution of temperature and other potential processes

The choice of fermentation temperature depends on starter culture requirements but it also determines the end product flavor. Fermentation temperature affects the aroma compound formation in wine production. Increase in temperature significantly lowered threonine aldolase activity in *Streptococcus thermophilus*, the enzyme responsible for threonine conversion to acetaldehyde (Routray & Mishra, 2011). Similarly, temperature increase adjusted Shiraz wine flavor by enhancing black currant flavor while

simultaneously reducing herbaceous flavor. Low fermentation temperature at 15 °C improved wine flavor by yielding higher concentrations of fruity aroma compounds as compared to 28 °C (Reynolds, Cliff, Girard, & Kopp, 2001). On the other hand, some off-flavors may be developed at low temperature (Cheng, 2010). Enzymatic activities are also temperature dependent; very low temperatures reduce the reaction rate while very high temperatures may cause enzyme denaturation.

Differentiation between growth associated aroma compounds and non-growth associated aroma compounds is worth to be done. This knowledge is necessary for the consideration of after fermentation processes that contribute to the generation of targeted aroma compounds. Post-fermentation treatments are commonly used for flavor enhancement of many beverages. However, in lactic acid fermentation, post fermentation may lead to further acidification as a result of further cell growth. The concept of attenuated adjunct cultures is established for ripening acceleration and flavor enhancement. Attenuated cultures are treated in order to eliminate or delay acidification capacity so that they become unable to grow and to produce significant level of lactic acid but still can deliver enzymes that are involved in aroma generation. Heating, freezing-thawing and sonication of cells are the often used methods (Gobbetti, De Angelis, Di Cagno, Mancini, & Fox, 2015). Careful modulation and further optimization of fermentation temperature could be favorable for the aroma profile of malt based beverages. Considerations of post-fermentation processes for aroma compound generation without further acidification using attenuated cells can thus be a promising approach for malt based beverages.

9. Conclusion

This review presented current research status on the flavor of lactic acid fermented cereal beverages and opportunities that can be exploited for flavor improvement. While some research have brought a contribution to the aroma composition and sensory characteristics, still much has to be investigated on specific aroma compounds contributing to the flavor of cereal beverages, their sensory impact alone and in synergy on the overall flavor. Immediate further studies for flavor improvement would be directed through: 1) characterization of key aroma compounds of malt wort beverages, 2) consumer sensory studies for sensory requirement definition, in terms of flavors, sugar to acid ratio, palate-fullness, 3) the impact of starter culture and cereal type on these aroma compounds for starter culture definition and for strain consociation possibilities, 4) investigations on fermentation process parameters as tool for generation and modulation of targeted aroma compounds; pitching rate, medium composition adjustment, dissolved oxygen content, environmental stresses and temperature are of prime importance, 5) last but not the least, optimization of the influencing parameters. This may open doors to fine-tune flavor quality during fermentation for the delivery of malt based beverages with attractable and distinguishable flavor and fully acceptable to meet society quest for new tastes and functional ingredients.

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2.3. Key volatile aroma compounds of lactic acid fermented malt based beverages – impact of lactic acid bacteria strains

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Key volatile aroma compounds of lactic acid fermented malt based beverages – impact of lactic acid bacteria strains

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ABSTRACT

This study aims to define the aroma composition and key aroma compounds of barley malt wort beverages produced from fermentation using six lactic acid bacteria (LAB) strains. Gas chromatography mass spectrometry–olfactometry and flame ionization detection was employed; key aroma compounds were determined by means of aroma extract dilution analysis. Fifty-six detected volatile compounds were similar among beverages. However, significant differences were observed in the concentration of individual compounds. Key aroma compounds (flavor dilution (FD) factors ≥16) were β-damascenone, furaneol, phenylacetic acid, 2-phenylethanol, 4-vinylguaiacol, sotolon, methional, vanillin, acetic acid, norfuraneol, guaiacol and ethyl 2-methylbutanoate. Furthermore, acetaldehyde had the greatest odor activity value of up to 4266. Sensory analyses revealed large differences in the flavor profile. Beverage from *L. plantarum* Lp. 758 showed the highest FD factors in key aroma compounds and was correlated to fruity flavors. Therefore, we suggest that suitable LAB strain selection may improve the flavor of malt based beverages.

1. Introduction

Due to increasing consumer awareness to the importance of healthy nutrition, the market for functional, natural and non-alcoholic beverages is steadily increasing all over the world (Fortitech., 2011). Cereal based beverages produced from lactic acid fermentation (LAFCB) are gaining a lot of attention because of their high nutritional values and functional properties that meet diverse and changing consumer nutrition demands such as lactose free, vegan, low cholesterol, non-alcoholic, gluten-free, natural and functional foods. However, their flavor was not accepted by consumers who requested flavor improvement as the most important aspect before nutritional properties (Yu & Bogue, 2013).

Cereals and cereal derived substrates are very rich in nutrients including proteins and bioactive compounds (Hassani, Zarnkow, & Becker, 2013). Barley is the cereal of choice with respect to its enzymatic activity, processability, nutrient content and sensory profile (Charalampopoulos, Pandiella, & Webb, 2003; Steiner, Gastl, & Becker, 2011). Malting and mashing, performed in breweries for malt wort production, and lactic acid fermentation further increase the nutrient availability and reduce anti-nutrient compounds such as phytates and oxalic acids occurring in cereals (Hassani, Procopio, & Becker, 2016; Kanauchi, Milet, & Bamforth, 2009; Singh, Rehal, Kaur, & Jyot, 2013). Hence, optimized malting and mashing conditions of diverse cereals were proposed for the production of lactic acid based beverages (Hassani et al., 2013; Zarnkow, Keßler, Back, Arendt, & Gastl, 2010). Furthermore, malt-

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ing and mashing improve the sensory quality and volatile composition of the resulted substrates which may significantly contribute to the final beverage flavor. Malt wort is therefore a complex nutrient rich substrate suited for LAFCB production than unmalted cereal substrates.

The volatile composition of LAFCB is affected by the type of cereal and processing treatment. Diverse types of cereal and substrates have been considered so far for LAFCB production. The volatile composition and sensory profile of LAFCBs produced from different cereal types were different even when same lactic acid bacteria (LAB) strain was used for fermentation. As such, a considerable amount of new volatiles was generated in barley malt than in non-malted cereal substrates during lactic acid fermentation (Coda, Rizzello, Trani, & Gobbetti, 2011; Salmerón, Fuciños, Charalampopoulos, & Pandiella, 2009; Salmerón, Thomas, & Pandiella, 2015).

However, LAB strains also influence the sensory and aroma profile of LAFCB. Significant differences in the acetaldehyde content and sensory acceptance were reported in malt based beverages formulated with *L. plantarum* and those with *L. acidophilus* and *L. reuteri*. Whether for acetaldehyde content or sensory acceptance, very large differences were observed in the values (Salmerón et al., 2015). Significant differences among LAB strains were described in the specific activity of some relevant enzymes involved in flavor formation during lactic acid fermentation (Smit, Smit, & Engels, 2005). Furthermore, beverage produced from *L. plantarum* NCIMB 8826 fermentation of barley malt had a better sensory acceptance than its counterparts produced with other LAB strains. It was tentatively attributed to differences in acetaldehyde content (Salmerón et al., 2015). Consequently, LAB strain selection was proposed as an approach for flavor improvement of LAFCB (Nsogning Dongmo, Procopio, Sacher, & Becker, 2016).

A prerequisite for flavor improvement is the thorough elucidation of the aroma composition and key aroma compounds in order to outline existing shortcomings. The aroma profile of LAFCB was not given an important focus while it is the main character which determines the acceptance. The most studied aroma compounds were acetoin, acetone, ethanol and acetaldehyde.

To the best of our knowledge, key aroma compounds that determine the flavor of LAFCB are not yet reported. The aim of this study is to define the aroma composition and key aroma compounds of barley malt wort beverages produced from lactic acid fermentation. Ultimately, the impact of six LAB strains on the aroma profile was investigated. This is an important basic knowledge for future attempts to the flavor improvement of cereal based beverages.

2. Materials and methods

2.1. Wort preparation and fermentation

Wort at 14% concentration was prepared from 72% standardized unhopped Bavarian pilsner barley malt extract from Weyermann® (Bamberg, Germany) using distilled water and autoclaved at 110 °C for 10 min. Hot break materials were separated after cooling. Six selected and previously identified strains of *L. plantarum* Lp. 758, Lp. 765 and Lp. 725, *L. brevis* Lb. 986 and *L. amylolyticus* La. TL3 and La. TL5 were obtained from the strain collection of the Institute of Brewing and Beverage Technology, Technische Universität München (München, Germany). These strains were preselected in preliminary screening experiments based on their current use in breweries (*L. amylolyticus, L. plantarum*) or their natural occurrence in malt wort (*L. brevis*). Cultures were stored in MRS broth containing 60% (v/v) glycerol at −80 °C. They were propagated twice in MRS broth (Sigma Aldrich, Germany) for 24–36 h and precultured in wort for 12 h at 28 °C (*L. brevis* and *L. plantarum)* and 48 °C (*L. amylolyticus)* before use for the experiments. Cells were washed thrice with sterile quarter strength Ringer's solution at 4000 rpm (rpm), 4 °C for 10 min. Fermentation was carried out in triplicate for each LAB strain at laboratory scale in 500 mL wort volume in static conditions for 72 h. The inoculation rate was $5.8 \pm 1.1 \times 10^6$ CFU (colony forming unit)/mL. Fermented beverages were immediately stored at 2 °C for sensory evaluation within 24 h and at −20 °C for aroma compounds analysis.

2.2. Volatile compounds extraction

2.2.1. Head-space-solid-phase-micro-extraction (HS-SPME)

An amount of 5 g fermented cold sample was weighed into a 20 mL glass headspace vial, tightly sealed with silicon septum and flanged cap and pre-equilibrated for 10 min at 36 °C. The septum was pierced with the SPME needle, and subsequently, the fiber was manually exposed to the sample headspace for an adsorption at 36 °C for 30 min for better estimation of aroma profile as perceived by the human nose (Sagratini et al., 2012). The SPME fiber material was 50/30 μm Divinylbenzol/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) (Supelco Inc., Bellefonte, USA). After adsorption, the fiber was directly transferred to the Gas-Chromatography–Mass Spectrometry–Olfactometry (GC–MS–O) injection port. Volatiles were then desorbed at 250 °C for 30 s.

2.2.2. Steam distillation

Volatile fraction was isolated using a Büchi distillation unit (Büchi K-314, Büchi Labortechnik, Germany) as previously described (Krahl, Zarnkow, Stürmer, & Becker, 2009). To 100 mL of cold samples, 9 mL ethanol (purity ≥99.5) and 1 mL of internal standards (methyl heptanoate and benzyl alcohol) were added. Samples were distilled at 100 °C into an ice cooled 100 mL volumetric flask and stored at 4 °C. Subsequently, 80 mL distillates, 22.2 g sodium chloride and 0.5 mL dichloromethane were given in a centrifuge glass tube, shaken for 30 min (Turbula-Shaker, Willy A. Bachhofen, Switzerland) and centrifuged at 0 °C with 2400 rpm for 15 min (Heraeus Megafuge 1.0 R, Thermo Fischer Scientific Inc., Massachusetts, USA). The supernatant was removed and the dichloromethane pearl in the bottom was pipetted into a GC–vial with integrated glass insert and filled with distilled water using a Pasteur pipette due to dichloromethane high volatility and to allow water separation from the dichloromethane pearl.

2.2.3. Solvent assisted flavor evaporation (SAFE)

The extraction was based on the procedure proposed by Schieberle (1996). A volume of 100 mL fermented sample was separated three times in a separatory funnel with diethyl ether (3×150 mL) after addition of 0.5 mL internal standard (methyl heptanoate and benzyl alcohol) The extracts were collected and washed twice with saturated NaCl solution (2×225 mL). The washed extract was dried by the addition of Na_2SO_4 and filtered through filter paper. The extract was subsequently concentrated to 30 mL using a Vigreux column (40 °C). The volatile fraction of the concentrated extract was isolated by means of SAFE (Engel, Bahr, & Schieberle, 1999). Isolated volatile fraction was further concentrated to 1 mL using the Vigreux column and stored at −20 °C prior to GC–MS–O analyses.

2.3. Analysis of volatile compounds by GC–MS–O and aroma extract dilution analysis

Identification of volatiles from SAFE distillate was performed on a Thermo Scientific GC Trace 1300 directly coupled to an ISQ-mass spectrometer (Thermo Fischer Scientific Inc, Darmstadt, Germany) as previously described (Roth, Schuster, Kollmannsberger, Jekle, & Becker, 2016). The column effluent was split by a 2-way μ-flow-

splitter to the mass spectrometer and an ODP-3 olfactory detection port (Gerstel, Mühlheim an der Ruhr, Germany). The split ratio was 2:1. The sniffing port was heated to 250 °C and rinsed with humidified air to avoid dehydration of assessor nasal membranes. Samples were separated using a TG-5MS column (Thermo Fischer Scientific Inc, Darmstadt, Germany) (60 m × 0.25 mm i.d., 0.25 μm film thickness). Carrier gas was helium at a constant flow of 1.85 mL/min. 2 μL of sample was applied with a split ratio of 1:10. Injector and transfer line temperature was 250 °C; ion source temperature was 200 °C. The temperature program was: 60 °C hold for 4 min then a rise to 220 °C at a rate of 5 °C/min with 5 min hold, and subsequent rise to finally 250 °C at a rate of 10 °C/min with 2 min hold. MS detection was performed with an electron impact energy of 70 eV. The analyzed mass range was 35–350 amu. Volatile identification was achieved by comparison of odor description, linear retention indices (RI) and mass spectra (MS) with authentic reference compounds and literature data. Data were analyzed using Xcalibur Software. Linear retention indices (RI) were determined according to van den Dool and Kratz (1963) using a mixture of linear alkanes C4–C20 under the same chromatographic conditions described above.

Aroma extract dilution analysis (AEDA) was performed according to the procedure described by Schieberle (1996). Aroma extracts were diluted stepwise with diethyl ether in a series of 1:1 dilution. 2 μL of each dilution was analyzed by GC–MS–O and the perceived intensities at the sniffing port were rated on a three-point intensity scale (1 = weak, 2 = moderate, 3 = strong). The greatest dilution factor at which a compound could still be detected at the sniffing port was considered as the flavor dilution factor (FD factor). Values from two assessors were considered.

2.4. Analysis of volatile compounds by GC–FID

Aroma compounds isolated by steam distillation were quantified by Gas-Chromatography-Flame Ionization Detection (GC-FID) (Hewlett-Packard 5890 Series II Plus) with a Hewlett Packard 7673 A automatic sampler (HP Inc, Böblingen, Germany) based on the method previously reported (Krahl et al., 2009). Compounds were separated using two capillary columns with different polarities. Column I was a 60 m HP Innovax Polyethylene glycol and column II was a 60 m HP5 column both with 0.25 μm film thickness and 0.25 mm internal diameter. Carrier gas was hydrogen at a constant flow of 3.8 mL/min (split 1:10) and the injection volume was 3 μL at 250 °C. The following temperature program was applied: 60 °C for 4 min, increase at 5 °C/min to 220 °C, with 30 min hold. Concentrations were calculated from internal calibrations with commercial reference substances and the values are means of independent duplicates. Odor activity values (OAVs) of aroma compounds were calculated by dividing their concentrations to the corresponding odor thresholds in water available in the literature (Grosch, 1994).

2.5. Analysis of acetaldehyde, higher alcohols and ethanol by GC–FID

Ethanol, acetaldehyde, propan-1-ol, 2-methylpropanol, 3-methylbutanol and 2-methylbutanol were quantified by headspace–GC–FID (Hewlett-Packard 6890 gas chromatograph equipped with a HP 7694 headspace autosampler) (Hewlett-Packard, Waldbronn, Germany) as described earlier (Procopio, Sprung, & Becker, 2015). Cold fermented samples of 5 mL and 0.1 mL internal standard mixtures (butan-1-ol and methyl hexanoate in 5% ethanol solution) were added to a 20 mL headspace vial and sealed with flanged caps. For ethanol quantitation, butan-1-ol (30 g/L in water) was used as internal standard. Samples were heated for 20 min at 65 °C in the autosampler. A 50 m HP-Ultra 2 silica capillary column, with 0.52 μm film thickness and 0.32 mm internal diameter was used. The following conditions were applied: injection temperature 150 °C; oven temperature 80 °C for 1 min, increase at 10 °C/min to 120 °C, hold for 5 min, increase 20 °C/min to 220 °C; detector temperature 250 °C. The carrier gas was helium (flow rate 1.5 mL/min, split 1:10). Concentrations were calculated from external calibrations with commercial reference compounds. Agilent Chemstation Rev. A.10.01 software was used for chromatogram analyses. Chemicals were provided by Sigma Aldrich® (Taufkirchen, Germany).

2.6. Sensory evaluation

Attribute difference test (Meilgaard, Civille, & Carr, 2007) was performed with seventeen trained panelists (eight females and nine males) in the age ranging from 26 to 38 years from the Institute of Brewing and Beverage Technology. Panelists were asked to rate each aroma descriptors in the six fermented beverages on a nine-point intensity scale (1: not present, 9: very intensive). Aroma descriptors, determined in preliminary sensory experiments, were: gustatory (sour, sweet and fullness) and olfactory (roasted-like, vinegar-like, caramel-like, wort-like, malty, fruity (pear-like, pineapple-like, apple-like), honey-like and putrid). The test was performed in randomized order at room temperature in clear plastic cups with 30 mL of beverage.

2.7. Statistical analysis

A one-way analysis of variance (ANOVA) was applied to the data. Tukey's test was used for mean values comparison to identify significant differences. Principal component analysis of the mean intensities was performed for the evaluated attributes and to determine the difference among samples. OriginPro 2015G (OriginLab Cooperation, Northampton, USA) was used as statistical tool.

3. Results and discussion

3.1. Volatile compounds in lactic acid fermented malt wort beverages (LAFMB)

Since odor-active compounds of LAFMB are partially studied, multiple extraction techniques were used to avoid misapprehension of the results. Then, headspace techniques (HS–SPME and HS–autosampling) and solvent extraction techniques (SAFE, distillation) were considered. As illustrated in Table 1, a total of fifty-six volatile compounds from eleven classes were identified in LAFMB with the four applied extraction methods. Some compounds could only be identified with specific extraction technique. Six unknown compounds could not be identified after comparison of odor description, linear retention indices (RI) and MS with literature data. Some alcohols, ketones, carboxylic acids and esters were previously reported in the volatile composition of LAFCB (Coda et al., 2011; Salmerón et al., 2009).

Aroma compounds of interest such as β-damascenone, furaneol, phenylacetic acid, 4-vinylguaiacol, sotolon, methional, norfuraneol, guaiacol, ethyl 2-methylbutanoate, linalool and geraniol described in this study, are reported for the first time in cereal based beverages produced from lactic acid fermentation. Their possible formation routes in lactic acid fermentation of malt wort from existing precursors were proposed (Nsogning Dongmo, Procopio, Sacher, & Becker, 2016).

Linalool and geraniol have always been associated with hop essential oils although they were reported in unhopped wort (De Schutter et al., 2008). They may be formed during fermentation from glycosides enzymatic cleavage by bacterial glycosidase or thermal cleavage by heat treatment in acid conditions. Further-

Table 1
Volatile compounds detected in LAFMBs after extraction by SPME, SAFE (S), distillation (D) and headspace autosampling (HS).

Aroma compounds	Isolation	Identification	Lp. 758	Lp. 765	Lp. 725	La. TL5	La. TL3	Lb. 986
Esters								
Ethyl acetate	HS	MS, RI, odor, reference	+	+	+	+	+	+
Ethyl 2-methylbutanoate	S, HS, D	MS, RI, odor	+	+	+	+	+	+
Ethyl 3-methylbutanoate	S, HS, D	MS, RI, odor	–	+	–	–	–	–
Ethyl 4-methylpentanoate	S	MS, RI, odor	+	–	–	–	–	–
Alcohols								
Ethanol	HS	RI, reference, odor	+	+	+	+	+	+
Propan-1-ol	HS	RI, reference, odor	+	+	+	+	+	+
2-methylpropanol	HS	RI, reference, odor	+	+	+	+	+	+
3-methylbutanol	HS, D, S	MS, RI, reference, odor	+	+	+	+	+	+
2-methylbutanol	HS, D	MS, RI, reference	+	+	+	+	+	+
1-octen-3-ol	SPME	MS, RI, reference	–	–	+	–	+	–
Hexan-1-ol	D, SPME	MS, RI, odor, reference	+	+	+	+	+	+
Nonan-2-ol	D, SPME	MS, RI, odor, reference	+	+	+	+	+	+
Ketones								
Diacetyl	S	MS, RI, odor	+	+	+	–	–	+
β-damascenone	S	MS, RI, odor	+	+	+	+	+	+
Heptan-2-one	D	MS, RI, reference	+	+	+	+	+	+
Nonan-2-one	D	MS, RI, reference	+	+	+	+	+	–
2-methylheptan-3-one	D	MS, RI, reference	+	+	+	+	+	–
Aldehydes								
Acetaldehyde	HS	RI, reference	+	+	+	+	+	+
Nonanal	S, D	MS, RI, odor	+	+	+	+	+	+
Terpenes								
Linalool	D, S, SPME	MS, RI, odor, reference	–	+	+	+	–	–
Geraniol	D	MS, RI, odor, reference	–	–	+	–	–	–
Acids								
Acetic acid	S	MS, RI, odor	+	+	+	+	+	+
Propionic acid	S	MS, RI, odor	+	–	–	–	–	–
Butyric acid	S	MS, RI, odor	–	–	–	–	–	+
2-methylpropanoic acid	S, D	MS, RI, odor	+	–	–	–	–	–
3-methylbutyric acid	S	MS, RI, odor	+	+	+	+	+	+
2-methylbutyric acid	S	MS, RI, odor	+	–	+	–	–	–
2-methyl-2-butenoic acid	S	MS, RI, odor, reference	+	–	–	–	–	–
Hexanoic acid	D, S	MS, RI, odor, reference	+	+	+	+	+	+
Octanoic acid	D	MS, RI, reference	+	+	+	+	+	+
Decanoic acid	D	MS, RI, reference	+	+	+	+	+	+
Ether								
Prenyl ethyl ether	D	MS, RI, reference, odor	+	+	+	+	+	+
Sulfurous compounds								
3-methylthiopropionic acid	S	MS, RI, reference, odor	–	–	+	–	+	–
Methional	S, D, SPME	MS, RI, odor	+	+	+	+	+	+
Heterocyclic compounds								
nor-furaneol	S	MS, RI, odor	+	+	+	+	–	+
Furaneol	S, D	MS, RI, odor	+	+	+	+	+	+
2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone	S, SPME	MS, RI, odor	+	+	+	–	+	–
Methyl furoate	S	MS, RI, odor	+	+	+	+	+	+
Sotolon	S	MS, RI, odor, reference	+	+	+	+	+	+
2-hydroxy-3-methyl-2-cyclopenten-1-one	S	RI, MS, odor, reference	+	–	–	+	+	–
Maltol	S	MS, RI	+	–	+	–	–	–
Phenolic compounds								
Guaiacol	S, D	MS, RI, odor	+	+	+	+	+	+
2-phenylethanol	S, D, SPME	MS, RI, odor, reference	+	+	+	+	+	+
4-vinylguaiacol	S, SPME	MS, RI, odor	+	+	+	+	+	+
4-ethylphenol	D, SPME	MS, RI, reference, odor	+	+	+	+	+	+
Phenylacetic acid	S	MS, RI, odor	+	+	+	+	+	+
Phenylpropanoic acid	S	MS, RI, odor	–	–	+	–	–	–
Vanillin	S	MS, RI, odor	+	+	+	+	+	+
Lactones								
γ-nonalactone	D, S, SPME	MS, RI, odor, reference	+	+	+	+	+	+
γ-dodecalactone	D, S	MS, RI, odor, reference	+	+	+	+	+	+
Unknown								
n.i.	S	RI, odor	–	–	+	–	–	–
n.i.	S	RI, odor	+	+	+	+	+	+
n.i.	S	RI, odor	–	–	+	+	+	–

Table 1 (*continued*)

Aroma compounds	Isolation	Identification	Lp. 758	Lp. 765	Lp. 725	La. TL5	La. TL3	Lb. 986
n.i.	S	RI, odor	–	+	+	+	–	+
n.i.	S	RI, odor	+	+	+	+	+	+
n.i.	S	RI, odor	–	+	–	–	–	–

+Positive detection; –Not detected; n.i., not identified.
Lp, *L. plantarum*; La, *L. amylolyticus*; Lb, *L. brevis*.
S, SAFE; D, steam distillation; SPME, headspace-solid phase-microextraction, HS, headspace autosampling by GC; MS, mass spectrum; RI, retention index; reference, comparison with reference compound.

more, linalool and geraniol formation by glycoside degradation from LAB strain *Oenococcus oeni* glycosidase and arabinosidase was reported in wine (Michlmayr et al., 2012). Glycosides were reported in cereals (Maier, Peipp, Schmidt, Wray, & Strack, 1995) but there is no knowledge yet about their occurence in malt wort.

Vanillin with its pleasant vanilla aroma may be of great importance to the final flavor. It was reported to be formed from ferulic acid by some *Lactobacillus* sp. and *Oenococcus oeni* in wine fermentation (Kaur, Chakraborty, & Kumar, 2013). β-Damascenone and its precursor 3-hydroxy-damascone are reported in malt wort. The concentration of β-damascenone significantly increased when the pH value was lowered (De Schutter et al., 2008). pH decrease that occurs during lactic acid fermentation may therefore favor the formation of β-damascenone from 3-hydroxy-damascone in malt wort.

As per sotolon (3-hydroxy-4,5-dimethyl-2(5*H*)-furanone), diverse formation pathways from chemical reactions were proposed in wine, among them is the aldol condensation of α-keto-butyric acid with pyruvic acid or acetaldehyde (Pham, Guichard, Schlich, & Charpentier, 1995). Possible formation pathway is not yet proposed in lactic acid fermentation. However, the availability of α-keto-butyric acid, pyruvic acid and acetaldehyde during lactic acid fermentation may explain its occurrence. Lactones are formed from β-oxidation of free fatty acids to 4/5-hydroxyacids which are further oxidized to lactones as reported in cheese (Nsogning Dongmo et al., 2016).

In summary, a large spectrum of aroma compounds was found and similarities observed among beverages led to suggest that LAB strains have less impact on the aroma composition of malt wort based beverages. Moreover, their contribution to the final beverage flavor was further investigated.

3.2. Key aroma compounds in LAFMB

The FD factor is an indicator of the contribution of an aroma active compound to the overall flavor of a particular food product. Therefore, the higher the FD factor of a compound, the most it contributes to the final flavor. The overall flavor is determined by the individual contribution of each key aroma compounds. For this reason, flavor dilution factors (FD) of odor-active compounds in the extracts obtained by SAFE were determined by means of AEDA.

Contrary to the aroma composition, there were large differences in the FD factors of individual aroma compounds among beverages (Table 2). Main differences were observed for β-damascenone, phenylacetic acid, 4-vinylguaiacol and vanillin. FD factors of up to 1024 were obtained for β-damascenone, furaneol and phenylacetic acid, depending on the LAB strains used for fermentation. Key compounds with FD factors ≥16 in at least one of the beverages were: β-Damascenone, furaneol, phenylacetic acid, 2-phenylethanol, 4-vinylguaiacol, sotolon, methional, vanillin, acetic acid, nor-furaneol, guaiacol and ethyl 2-methylbutanoate. Among them, vanillin and 2-phenylethanol were already reported in LAFCB (Salmerón et al., 2009).

Methional and acetic acid with a flavor note of cooked potatoes and vinegar, respectively may contribute as off-flavors to the sensory characteristics of these beverages. Methional is formed enzymatically through the Ehrlich pathway from methionine in *L. lactis*. This activity was strain dependent (Amárita, Fernández-Esplá, Requena, & Pelaez, 2001). 4-Vinylguaiacol is formed in lactic acid fermentation from ferulic acid by ferulic acid decarboxylases (Rodríguez et al., 2009). Furthermore, ferulic acid concentration of 51.4 μmol/L was reported in barley wort (Szwajgier, 2009).

Fig. 1a depicts the distribution of key aroma compounds among six LAB strains. Beverages produced from *L. brevis* Lb. 986 fermentation were more characterized by furaneol, nor-furaneol and acetic acid whereas beverages from *L. amylolyticus* La. TL3 and La. TL5 were more characterized by methional and furaneol. *L. brevis* is an obligate heterolactic which produces more acetic acid than other LAB strains. On the contrary, beverages produced from *L. plantarum* Lp. 758 showed highest FD factors of aforementioned key aroma compounds, except for acetic acid and nor-furaneol.

Table 3 shows the concentrations of volatile compounds extracted by steam distillation in fermented beverages. Compound concentrations were significantly different among beverages produced from different LAB strains. Furthermore, concentrations were lower than the detection thresholds in most compounds. The odor activity values (OAV) of compounds were then calculated and those with values greater than 1 in at least one of the beverages were selected and gathered in Table 4. Due to the lack of knowledge about the odor detection thresholds in lactic acid fermented malt based beverages and the large threshold ranges in the literature, minimal and maximal OAVs were considered. Selected compounds were acetaldehyde, propan-1-ol, γ-dodecalactone, ethanol, geraniol, linalool and 2-methylpropanol. Acetaldehyde was the most important aroma compound followed by propan-1-ol and γ-dodecalactone. Beverages produced from *L. plantarum* strains fermentation had very high OAVs for acetaldehyde as compared to others (Fig. 1b).

Propan-1-ol with its pungent and alcoholic odor quality may constitute an off-flavor to the overall flavor. The formation of propan-1-ol from propan-1,2-diol, an intermediate of lactic acid fermentation, by *L. diolivorans* was evidenced in maize silage fermentation (Krooneman et al., 2002).

Ethanol concentrations were significantly different in beverages to a maximum of 0.1% abv. which is quite lesser than 0.5% abv. recommended by the Food & Drug Administration for non-alcoholic beverages. Ethanol is necessary for ester synthesis with fatty acyl-CoA, the major ester synthesis pathways in lactic acid fermentation. Esters are positively regarded because of their fruity flavor which may enhance the sensory profile. Therefore, increased ethanol production may favor ester formation in lactic acid fermented malt based beverages. Obligate homolactics and heterolactics La. TL5, La. TL3 and Lb. 986 produced more ethanol. However, low FD factors for esters (Table 2) were recorded in the corresponding beverages. As such, esterification is not only dependent on the substrate availability but also on its concentration, the esterase occurrence and activity level.

Table 2
Flavor dilution factors (FD) of volatile aroma compounds in LAFMBs extracted by SAFE.

RI_{DB5}[1]	Aroma compounds	Odor quality[2]	Lp. 758	Lp. 765	Lp. 725	La. TL5	La. TL3	Lb. 986
Esters								
849	Ethyl 2-methylbutanoate	Sweet, strawberry	16	2	4	2	2	8
852	Ethyl 3-methylbutanoate	Fruity, honey	–	1	–	–	–	–
969	Ethyl 4-methylpentanoate	Fruity	1	–	–	–	–	–
Alcohols								
729	3-methylbutanol	Malty, musty	1	n.a.	n.a.	n.a.	n.a.	n.a.
982	1-octen-3-ol	Solvent-like	–	–	1	–	1	–
Ketones								
587	Diacetyl	Butter	2	4	1	–	–	–
1399	β-damascenone	Apple juice	1024	4	1	1	2	1
Acids								
621	Acetic acid	Vinegar	2	32	64	n.a.	n.a.	32
673	Propionic acid	Fruity, apple	1	–	–	–	–	–
739	2-methylpropanoic acid	Cheesy	1	–	–	–	–	–
775	Butyric acid	Rancid	–	–	–	–	–	1
831	3-methylbutyric acid	Cheesy, musty	2	4	2	1	n.a.	1
847	2-methylbutyric acid	Musty, sweet	1	–	1	–	–	–
921	2-methyl-2-butenoic acid	Bonbon	1	–	–	–	–	–
982	Hexanoic acid	Sweet	n.a.	n.a.	1	n.a.	n.a.	n.a.
Terpenes								
1103	Linalool	Flowery, solvent-like	–	1	1	1	–	–
1259	Geraniol	Fruity	–	–	1	–	–	–
Sulfurous compounds								
908	Methional	Cooked potatoes	128	32	32	16	32	8
1096	3-methylthiopropionic acid	Smoky	–	–	2	–	1	–
Heterocyclic compounds								
917	2-hydroxy-3-methyl-2-Cyclopenten-1-one	Spicy, maggi	2	–	–	1	1	–
983	2,4-dihydroxy-2,5-dimethyl-3(2 H)-furanone	Caramel	2	4	4	–	2	–
1047	nor-furaneol	Fruity, caramel	4	1	n.a.	–	–	32
1065	Furaneol	Fruity, strawberry, caramel	1024	64	256	128	128	32
1090	Methyl furoate	Musty, papier	8	1	2	4	8	8
1108	Sotolon	Seasoning-like	512	4	16	4	4	4
Aromatic compounds								
1096	Guaiacol	Smoky	32	2	1	n.a.	1	4
1121	2-phenylethanol	Citrus-like, rose-like	512	2	16	n.a.	n.a.	4
1251	Phenylacetic acid	Flowery, old paper	1024	4	8	1	8	4
1288	Phenylpropanoic acid	Strawberry, apple	–	–	1	–	–	–
1325	4-vinylguaiacol	Smoky, musty	512	1	n.a.	n.a.	n.a.	4
1413	Vanillin	Vanilla, sweet	64	2	2	2	1	–
Lactones								
1373	γ-nonalactone	Fruity, coconut	1	1	1	1	1	1
1510	γ-dodecalactone	Coconut, milk	1	1	1	1	1	1
Unknown								
720	n.i.	Apple	–	–	1	–	–	–
868	n.i.	Peanut	1	2	16	2	2	n.a.
1039	n.i.	Caramel, musty	–	–	1	1	1	–
1130	n.i.	Green, cucumber	–	2	n.a.	n.a.	–	n.a.
1149	n.i.	Fruity, apple	32	2	4	1	4	n.a.
1196	n.i.	Apple	–	1	–	–	–	–

n.a., not applicable (compounds were detected on GC–MS but the concentration in the non-diluted aroma extract was too low to be detected at the sniffing port.)
- compounds were not detected; n.i. not identified.
Lp, *L. plantarum*; La, *L. amylolyticus*; Lb, *L. brevis*.
[1] RIs on a DB-5 column.
[2] Odor quality perceived at the sniffing port of the GC–MS–O.

During glycolysis, ethanol can be formed from acetyl-CoA via acetaldehyde by the alcohol dehydrogenase. High activity of alcohol dehydrogenase may therefore result in the accumulation of ethanol at the expenses of acetaldehyde and vice versa. Per se, acetaldehyde concentration was low in the beverages where ethanol content was high. Acetaldehyde metabolism to ethanol was tested positive for obligate homolactic *L. delbrueckii* subsp. *lactis* and obligate heterolactic *L. mesenteroides* in wine fermentation (Osborne, Mira de Orduña, Pilone, & Liu, 2000).

Acetaldehyde with its pleasant fruity apple note at low concentration is of interest to the flavor of the resulted beverages. Large concentration ranges of 1.6–64 mg/L were recorded in the six beverages with significant differences (Table 3). These concentrations are greater than those of a previous study (Salmerón et al., 2015), probably due to different fermentation times and LAB strains considered. Furthermore, a concentration range of 0.1–77.5 mg/L was reported in yogurt (Routray & Mishra, 2011). Monitoring the changes during fermentation (Fig. S1) revealed a continuous production of acetaldehyde during fermentation. The differences observed in the final concentrations were related to low or no production by obligate homolactics La. TL5, La. TL3 and obligate heterolactics Lb. 986 strains. Besides, acetaldehyde is also formed from the conversion of threonine by threonine aldolase, the major source of acetaldehyde in yogurt. However, threonine aldolase

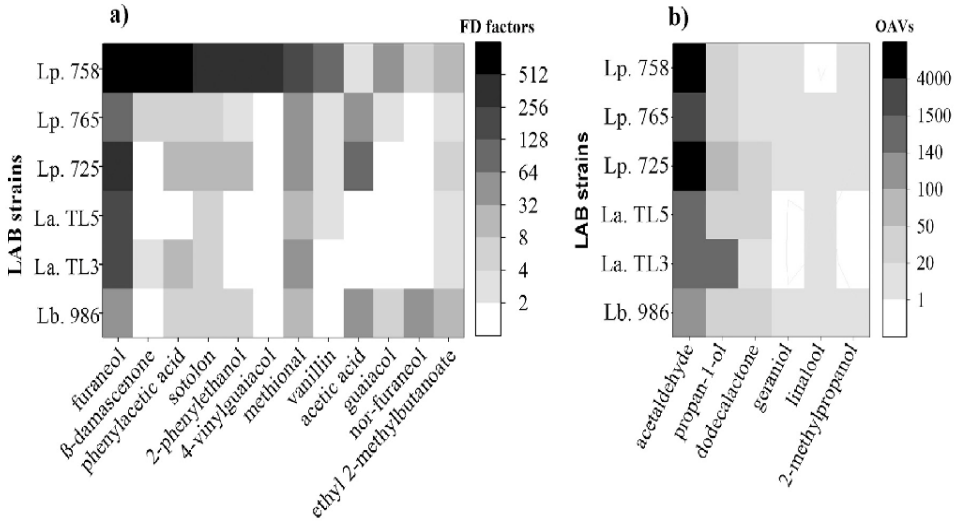

Fig. 1. Heatmap representations illustrating the distribution of FD factors (left) and OAVs (right) of selected aroma compounds among LAFMBs fermented using six LAB strains. Considered compounds are those with FD ≥ 16 and OAVs ≥ 1 in at least one of the beverages. Lp, *L. plantarum*, La, *L. amylolyticus*, Lb, *L. brevis*.

Table 3
Concentrations (μg/L) of volatile compounds extracted by steam distillation and headspace in LAFMBs.

Aroma compounds	Odor quality	Concentrations (mean ± standard deviation)*					
		Lp. 758	Lp. 765	Lp. 725	La. TL5	La. TL3	Lb. 986
Alcohols							
Ethanol[ε,·]	Ethanol-like[ii]	$363^{a} \pm 73$	$150^{b} \pm 25$	$87.6^{c} \pm 61$	$1171^{d} \pm 67$	$931.5^{e} \pm 67$	$897^{e} \pm 58$
Propan-1-ol	Alcohol, pungent[i]	$1531^{a} \pm 471$	$1245^{a} \pm 141$	$2314^{a} \pm 299$	$1450^{a} \pm 201$	$9066^{b} \pm 2039$	$1412^{a} \pm 290$
2-methylpropanol	Malty[ii]	$883^{a} \pm 43$	$724^{b} \pm 68$	$502^{c} \pm 38$	$241^{d} \pm 41$	$390^{d} \pm 49$	$658^{b} \pm 35$
3-methylbutanol	Malty, musty[iii]	$94^{a} \pm 15$	$138^{a} \pm 23$	$108^{a} \pm 0$	$3.3^{b} \pm 0.4$	$4.1^{b} \pm 0.4$	$121^{a} \pm 0$
2-methylbutanol	Malty[iii]	$82^{ab} \pm 15$	$101^{b} \pm 7$	$48^{ac} \pm 0$	$19\ c^{d} \pm 4$	$32^{d} \pm 11$	$66^{abd} \pm 0$
Hexan-1-ol	Flower, green[i]	$7.7^{ab} \pm 0.7$	$8.8^{a} \pm 1.9$	$3.6^{bc} \pm 1.3$	$2.2^{c} \pm 0.7$	$2.7^{c} \pm 0.3$	$6.4^{abc} \pm 0.7$
Nonan-2-ol	Fat, green[i]	$6.5^{a} \pm 0.4$	$3.4^{b} \pm 0.9$	$4.5^{ab} \pm 0$	$9.7^{c} \pm 1.3$	$11^{c} \pm 0.1$	$4.4^{ab} \pm 0.0$
Ketones							
Heptan-2-one	Sweet, cheese[iv]	$4.9^{a} \pm 1.2$	$7.6^{a} \pm 1.2$	$4.9^{a} \pm 0.3$	$4.8^{a} \pm 0.1$	$5.5^{a} \pm 0.4$	$1.4^{b} \pm 0.7$
Nonan-2-one	Musty, tea-like[iv]	$3.9^{a} \pm 0.03$	$3.4^{a} \pm 0.9$	$4.1^{a} \pm 0.0$	$1.3^{b} \pm 0.4$	$1.8^{b} \pm 0.02$	$0.9^{b} \pm 0.1$
2-methylheptan-3-one	Fruity[i]	$1.4^{a} \pm 0.2$	$1.9^{a} \pm 0.1$	$1.1^{a} \pm 0.2$	$0.5^{a} \pm 0.0$	$1.4^{a} \pm 0.1$	$2.1^{a} \pm 1.2$
Aldehydes							
Acetaldehyde[ε],	Green apple[ii]	$64^{a} \pm 2$	$23^{b} \pm 1$	$61^{a} \pm 4$	$2.2^{c} \pm 0.3$	$2.7^{c} \pm 0.4$	$1.6^{c} \pm 0.2$
Acids							
Hexanoic acid	Sweet[iii]	$50^{ac} \pm 3$	$57^{a} \pm 11$	$56^{a} \pm 9$	$9^{b} \pm 1$	$36^{c} \pm 3$	$60^{a} \pm 0$
Octanoic acid	Sweet[iv]	$10^{a} \pm 2$	$18^{b} \pm 3$	$11^{ab} \pm 3$	$8^{a} \pm 0.8$	$7^{a} \pm 0.5$	$44^{c} \pm 2$
Decanoic acid	Soapy, waxy[iv]	$4.6^{a} \pm 0.1$	$6.8^{a} \pm 1.5$	$5.5^{a} \pm 0.0$	$4.0^{b} \pm 0.1$	$4.9^{b} \pm 0.6$	$10^{c} \pm 1$
Terpenoids							
Linalool	Flowery[iii]	$0.8^{a} \pm 0.2$	$1.1^{a} \pm 0.2$	$1.0^{a} \pm 0.2$	$1.1^{a} \pm 0.1$	$1.3^{a} \pm 0.2$	$0.9^{a} \pm 0.3$
Geraniol	Fruity, citrus-like[iii]	$2.4^{ab} \pm 0.2$	$2.7^{a} \pm 0.4$	$2.5^{ab} \pm 0.0$	$0.4^{c} \pm 0.02$	$0.6^{c} \pm 0.1$	$1.9^{b} \pm 0.0$
Ethers							
Prenyl ethyl ether	Nussy, alcohol-like[iii]	$1.8^{a} \pm 0.4$	$1.9^{a} \pm 0.4$	$1.4^{a} \pm 0.3$	$1.7^{a} \pm 0.2$	$1.8^{a} \pm 0.6$	$0.9^{a} \pm 0.4$
Aromatic compounds							
2-phenylethanol	Grassy, citrus-like[iii]	$59^{a} \pm 3$	$75^{b} \pm 3$	$56^{a} \pm 5$	$7.2^{c} \pm 0.5$	$6.6^{c} \pm 0.4$	$94.9^{d} \pm 2.1$
4-ethylphenol	Green gras, cheesy[iii]	$0.7^{a} \pm 0.1$	$5.03^{b} \pm 1.1$	$0.6^{a} \pm 0.0$	$0.5^{a} \pm 0.1$	$0.5^{a} \pm 0.3$	$0.5^{a} \pm 0.05$
Lactones							
γ-nonalactone	Fruity, coconut[iii]	$5.4^{a} \pm 0.4$	$10^{ab} \pm 2$	$11^{ab} \pm 0.0$	$13.8^{ab} \pm 1.7$	$16^{b} \pm 4.8$	$5.1^{a} \pm 0.9$
γ-dodecalactone	Coconut, milk[iii]	$2.7^{a} \pm 0.4$	$1.4^{a} \pm 0.0$	$9.8^{bc} \pm 0.0$	$9.0^{bc} \pm 2.2$	$5.5^{ab} \pm 1.4$	$16.9^{c} \pm 2.7$

* Mean values in the same row with different lower-case letters indicate that they are significantly different at $p < 0.05$.
ε Concentration in mg/L.
Compounds extracted by headspace autosampling Lp, *L. plantarum*, La, *L. amylolyticus*, Lb, *L. brevis*.
i Rychlik, Schieberle, and Grosch (1998).
ii Czerny et al. (2008).
iii Odor quality perceived at the sniffing port of the GC–MS–O.
iv Qian and Burbank (2007).

activity is also strain dependent in LAB (Wilkins, Schmidt, & Kennedy, 1986). Threonine availability in malt wort (39–109 mg/L) (Schönberger, 2004) may contribute to high acetaldehyde concentrations. Furthermore, very high OAVs of up to 4266 was obtained for acetaldehyde (Table 4). This suggests that acetaldehyde may greatly contribute to the flavor of these beverages. It

Table 4
Odor activity values (OAV) ranges of selected compounds with values greater than 1 in LAFMBs.

Aroma compounds	Odor activity values*						
	Lp. 758	Lp. 765	Lp. 725	La. TL5	La. TL3	Lb. 986	Threshold in water (μg/L)
Acetaldehyde	1561–4266	561–1533	1487–4066	53–146	65–180	39–106	15–41[i,ii]
Propan-1-ol	13–41	10–33	20–63	12–39	79–245	12–38	37–115[i]
γ-dodecalactone	0.4–6.3	0.2–3.3	1.4–22.8	1.3–21	0.8–12.8	2.4–39	0.43–7[i,ii]
Ethanol	0.4–15	0.2–6	0.1–3	1.2–47	0.9–37	0.9–36	24900–990,000[i,ii]
Geraniol	0–2.2	0–2.5	0.0–2.3	<1	<1	0.0–1.7	1.1–75[i,ii]
Linalool	<1	0.2–1.3	0.2–1.2	0.2–1.3	0.2–1.5	0.2–1.0	0.87–6[i,ii]
2-methylpropanol	1.6	1.3	<1	<1	<1	1.2	550[ii]

* OAVs were calculated for the minimal and maximal threshold value reported in the literature. Lp, *L. plantarum*, La, *L. amylolyticus*, Lb, *L. brevis*.
[i] Rychlik, Schieberle, and Grosch (1998).
[ii] Czerny et al. (2008).

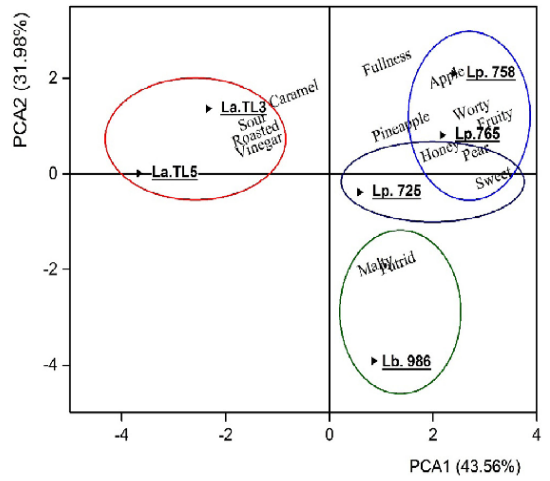

Fig. 2. Principal component plot illustrating the relationship between the sensory descriptors and the beverages fermented with corresponding LAB strain. Lp, *L. plantarum*, La, *L. amylolyticus*, Lb, *L. brevis*. Aroma descriptors, determined in preliminary sensory experiments, were: gustatory (sour, sweet and fullness) and olfactory (roasted-like, vinegar-like, caramel-like, wort-like, malty, fruity (pear-like, pineapple-like, apple-like), honey-like and putrid).

should therefore be considered as a key compound for the flavor of LAFMB.

The significant impact of LAB strains on the key aroma composition of LAFMB is clearly demonstrated in Fig. 1a and b. Differences in genetic background may be the explanation as it is with dairy LAB strains (Smit et al., 2005).

Proposed key aroma compounds of LAFMB with FD factors ≥16 are therefore: β-damascenone, furaneol, phenylacetic acid, 2-phenylethanol, 4-vinylguaiacol, sotolon, methional, vanillin, acetic acid, nor-furaneol, guaiacol and ethyl 2-methylbutanoate. Acetaldehyde is also of great importance. Furthermore, omission tests and aroma recombination studies will help to determine the contribution of each of the reported key aroma compounds to the final flavor. Studies on their synergistic effect will also be necessary.

3.3. Sensory characteristics of LAFMBs

Principal component analysis plot of Fig. 2 presents the correlation between each beverage produced from fermentation with the corresponding LAB strains and the sensory descriptors. The plot reveals 75% of the variation in the sample set. Four sample groups and characteristic flavor attributes could be identified. Small variations were observed for samples fermented with strains of the same metabolic pathways while very large variations are observed among strains of different metabolic pathways. Beverages fermented with *L. amylolyticus* and *L. brevis* were completely different from each other and from those produced from *L. plantarum*.

Those fermented with *L. amylolyticus* and *L. brevis* had relatively less attractive flavor notes such as vinegar, sour, roasted and malty, putrid, respectively. It is consistent with the fact that key aroma compounds in these beverages were methional, acetic acid and nor-furaneol with aroma notes of cooked potatoes, vinegar and caramel-like, respectively.

Samples fermented with *L. plantarum* Lp. 758 strain were clustered with positive flavor attributes such as fruitiness, apple-like, pear-like, honey-like and pineapple-like. This observation is in agreement with the high FD factors obtained for key aroma compounds. Among them are fruity flavor compounds such as β-damascenone, furaneol, 2-phenylethanol and ethyl 2-methylbutanoate with flavor like apple juice, strawberry, caramel and citrus as recorded during GC–O analysis.

4. Conclusions

The aroma profile of barley malt wort beverages and the impact of six selected LAB strains were described in this study. Thereafter, we proposed twelve key aroma compounds that contribute to the flavor profile of malt wort beverages produced from lactic acid fermentation. To the best of our knowledge, β-damascenone, furaneol, phenylacetic acid, 4-vinylguaiacol, sotolon, methional, nor-furaneol, guaiacol, ethyl 2-methylbutanoate, linalool and geraniol are reported for the first time in LAFCB. Our findings provide evidence of the significant impact of LAB strains on the key aroma composition and the sensory profile of LAFMB. However, LAB strains had less impact on the aroma composition itself. Based on these results, suitable selection of LAB strain may lead to a more appealing beverage with high acceptance; compounds such as β-damascenone, acetaldehyde, furaneol, phenylacetic acid and ethyl 2-methylbutanoate are of interest for the flavor of LAFMB and can therefore be considered in LAB strain selection or further studies aiming to improve the flavor of LAFCB. Therefore, we suggest that existing shortcoming in the flavor of LAFCB could be related to the low concentration of the reported key aroma compounds and by extension to the LAB strain. The strain of *L. plantarum* Lp. 758 which delivers high concentrations of key aroma compounds and consequently, a beverage with fruity flavor, was proposed for LAFMB production. Sensory acceptance studies are necessary to validate this strain. Furthermore, investigation into the fermentation behavior of these LAB strains in malt wort may bring insights into the significant differences observed in this study.

Conflict of interest statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.02.091.

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2.4. Investigating on the fermentation behavior of six lactic acid bacteria strains in barley malt wort reveals limitation in key amino acids and buffer capacity

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Investigating on the fermentation behavior of six lactic acid bacteria strains in barley malt wort reveals limitation in key amino acids and buffer capacity

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ABSTRACT

Understanding lactic acid bacteria (LAB) fermentation behavior in malt wort is a milestone towards flavor improvement of lactic acid fermented malt beverages. Therefore, this study aims to outline deficiencies that may exist in malt wort fermentation. First, based on six LAB strains, cell viability and vitality were evaluated. Second, sugars, organic acids, amino acids, pH value and buffering capacity (BC) were monitored. Finally, the implication of key amino acids, fructose and wort BC on LAB growth was determined. Short growth phase coupled with prompt cell death and a decrease in metabolic activity was observed. Low wort BC caused rapid pH drop with lactic acid accumulation, which conversely increased the BC leading to less pH change at late-stage fermentation. Lactic acid content (≤3.9 g/L) was higher than the reported inhibitory concentration (1.8 g/L). Furthermore, sugars were still available but fructose and key amino acids lysine, arginine and glutamic acid were considerably exhausted (≤98%). Wort supplementations improved cell growth and viability leading to conclude that key amino acid depletion coupled with low BC limits LAB growth in malt wort. Then, a further increase in organic acid reduces LAB viability. This knowledge opens doors for LAB fermentation process optimization in malt wort.

1. Introduction

Although their tremendous reported health benefits and possibilities for technological innovation, lactic acid fermented cereal-based beverages are scarce on the market. Low consumer's acceptance and limitations in the flavor profile are the shortcomings (Nsogning Dongmo et al., 2016; Yu and Bogue, 2013). It is now known that limitations in the flavor profile of lactic acid fermented malt-based beverages (LAFMB) are not due to the aroma composition but to an insufficient concentration of important aroma compounds. The fermenting LAB strain has a significant impact on the concentration of key aroma compounds, the flavor, and acceptance of the resulting beverages (Nsogning Dongmo et al., 2017; Salmerón et al., 2015). However, the question of what causes insufficient aroma compound formation by LAB during malt wort fermentation is fundamental for further attempts aiming to improve the flavor profile of LAFMB.

The central issue that could be addressed is an adequate fermentation performance. For that, good cell physiological conditions and nutrient availability are key determinants. Nutrient deficiency or low viable and vital cell could impair fermentation performance resulting undoubtedly to short growth and low aroma yield. The basics on how is the fermentation performance of LAB in malt wort remained partially elucidated. Studies have described the growth behavior of LAB, change in fermentable sugars, free amino nitrogen, and lactic acid and acetic acid accumulation in cereal-based substrates fermentation. LAB growth phase in cereal-based substrates is generally very short ranging from 6 to 48 h depending on the substrate type, fermentation temperature, inoculation rate and LAB strain (Charalampopoulos et al., 2002, 2003; Peyer et al., 2015; Salmerón et al., 2014). Furthermore, studies on the evaluation of limitations that may exist in lactic acid fermentation of malt wort do not exist yet.

LAB are strictly fermentative but require fermentable sugars, amino acids, fatty acids, vitamins and purines for their development. As such, they are auxotrophic to several amino acids and unable to grow in a simple media containing carbon sources and minerals (Hebert et al., 2000). Any lack of nutrients could,

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therefore, be a real challenge for LAB growth.

Besides, medium composition changes such as pH drop, consequent to organic acid accumulation occurring in lactic acid fermentation, cause acidic stress that may also affect LAB viability and metabolic activity. The toxic effect of the undissociated lactic acid on LAB cells has been described (Adamberg et al., 2003; Pieterse et al., 2005). To overcome the acidic stress they are subject to in their fermentation environment, LAB cells exploit four main systems: the glutamate decarboxylase (GAD), lysine decarboxylase, arginine deiminase (ADI) and F1Fo ATPase multisubunit (Azcarate-Peril and Klaenhammer, 2010). However, whether to increase the intracellular pH or to intensify protons extrusion from the cytoplasm, they all rely on proteins or amino acids. Furthermore, amino acids are known to be strongly involved in enzymatic functions and as aroma compound precursors. Lack of some amino acids could, therefore, result to low acidic resistance, cell death, and impaired metabolic activity leading too low aroma formation.

Thus, nutrient composition of malt wort is of major importance to LAB cell fermentation performance and by extension to aroma compound yield. Barley malt substrate is a complex rich nutrient medium that was described to support LAB growth and aroma compound formation (Charalampopoulos et al., 2003; Nsogning Dongmo et al., 2016; Peyer et al., 2015) than other considered cereals like wheat and oat. But whether malt wort is an optimum medium to meet LAB nutritional requirement for efficient and long-lasting LAB growth is still to be determined.

To understand the limitations occurring in lactic acid fermentation of malt wort, this study aims at investigating on the viability, vitality and death rate of six LAB strains, medium physical and compositional changes regarding residual nutrients, organic acids, buffering capacity and pH value change in barley malt wort fermentation. In the end, the implication of key amino acids, fructose, and wort buffering capacity in LAB growth was evaluated. This knowledge will orientate in the optimization of malt wort composition and fermentation process for improved LAB growth and will bring a light to the understanding of the reported low aroma yield of lactic acid fermented cereal-based beverages as well.

2. Material and methods

2.1. Wort preparation, strains, and fermentation

Wort at 14% concentration was prepared from 72% standardized unhopped Bavarian pilsner barley malt extract from Weyermann (Bamberg, Germany) using distilled water and autoclaved at 110 °C for 10 min. Hot break materials were separated after cooling. Six selected and previously identified strains of *Lactobacillus plantarum* Lp.758, Lp.765 and Lp.725, *Lactobacillus brevis* Lb.986, and *Lactobacillus amylolyticus* La.TL3 and La.TL5 were obtained from the strain collection of the chair for Brewing and Beverage Technology (Technical University of Munich, Germany). Cultures were propagated twice in MRS broth (Sigma Aldrich, Germany) for 24–36 h and pre-cultured in wort for 12 h at 28 °C for *L. brevis* and *L. plantarum* and at 48 °C for *L. amylolyticus* before use in the experiments. Cells were washed thrice with sterile quarter strength Ringer's solution (Sigma Aldrich) at 4000 rpm, 4 °C for 10 min. Fermentation was carried out in triplicate at laboratory scale in 500 mL wort volume under static conditions for 72 h. The inoculation rate was $5.8 \pm 1.1 \times 10^6$ CFU (colony forming unit)/mL. Fermented samples were immediately used for viability, vitality and staining procedures whereas those for pH, buffering capacity and analytical measurements were stored at 4 °C and −20 °C, respectively until analysis.

2.2. Viability and death kinetics

Cell viability was evaluated using plate counting method whereas fluorescence microscopic counting after cell labeling with carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) fluorescent probes was considered for death rate determination.

2.2.1. Plate count

The viability was measured by colony forming unit counting method. Serial decimal dilutions were prepared in sterile quarter strength Ringer's solution and 0.1 mL diluted samples were plated on MRS agar in triplicate using pour-plate procedure and incubated at 28 °C and 48 °C for *L. plantarum*/*L. brevis* and *L. amylolyticus*, respectively for 48 h. Viable colonies were counted and recorded as the number of CFU/mL.

2.2.2. Cell fluorescence labeling with CFDA and PI

A solution of CFDA (10 mM) was prepared by dissolving 4.6 mg CFDA in 1 mL dimethyl sulfoxide (DMSO) and stored in aliquots of 1 mL at −20 °C in the dark and was diluted to 1 mmol using DMSO before use. PI was supplied as a 1 mg/mL solution and was stored in darkness in the refrigerator. Cells were washed thrice with Ringer's solution and resuspended into an OD_{600nm} of 1.2 prior to staining. 30 μL of CFDA was added to 940 μL bacteria suspension and kept in the darkness at 30 °C for 10 min. Thereafter, 30 μL of PI was added and kept in the darkness at 30 °C for 10 min (Bunthof et al., 1999). Cells were washed out twice to remove excess probes and resuspended in Ringer's solution; aliquots were then stored on ice until analysis within 1 h. Red-labelled and green-labeled cells of 10 visual fields were counted at 40x objective using a Zeiss Axioskop epifluorescence microscope equipped with three filter sets for DAPI/PI/CFDA and a camera (Carl Zeiss, Germany). Each sample was counted in triplicate. The death percentage was calculated as the ratio of the red-labeled cells to the total of red- and green-labeled cells.

2.3. Vitality assessment – acidification power (AP) test

Vitality was assessed by means of acidification power test, which measures the glycolytic activity through the ability of cell to lower the pH value of 0.1% glucose solution (Riis et al., 1995; Sigler, 2013). The applied method was adapted from previously reported methodology (Bunthof et al., 1999). Cells were washed thrice (4000 rpm, 4 °C, 10 min) and re-suspended in quarter strength Ringer's solution at pH 6.5 to a final OD_{600nm} of 1.0 ± 0.1 corresponding to $8 \pm 1 \times 10^7$ cells/mL. After equilibration at 25 °C, 200 μL glucose was added to 20 mL cell suspension to a final concentration of 6 mM. Medium acidification was assessed after 10 min by pH value measurement. The acidification power was calculated as the pH difference between the initial pH value (6.5) and the pH value after 10 min. The test was done in triplicate.

2.4. Analytical determinations

2.4.1. Sugar analysis

Extracellular sugars were analyzed by high-performance liquid chromatography (HPLC) using a pulsed amperometric detector (PAD) and a Dionex Carbopac PA10 carbohydrate column (Thermo Scientific, Germany). External commercial reference compounds were used for the calibration.

2.4.2. Organic acid analysis

Specific enzymatic kits (Megazyme, Ireland) were used to determine the concentrations of extracellular L-lactic acid (kit k-late 07/14), acetic acid (kit k-acetrm 07/12), L-malic acid (kit k-lmal-58A

0914) and succinic acid (kit k-succ 01/14) as per manufacturer instructions. Prior to the analysis, samples were heated at 80 °C for 5 min, centrifuged and the supernatant was used for the analysis.

2.4.3. Amino acids and free amino nitrogen

Free amino acid quantitation was performed by HPLC and free amino nitrogen (FAN) was determined by a ninhydrin-based method according to the approved methods of MEBAK (Jakob, 2012).

2.4.4. Buffering capacity change

The buffering capacity of beverages was determined by titration of 100 mL sample with 1 M HCl (Charalampopoulos et al., 2002; Li et al., 2016). Buffering capacity expressing the amount of HCl (mM) required to drop 1 pH unit per liter samples was calculated using the formula:

$$BC\left(mmol\ HCl\ pH^{-1}L^{-1}\right) = \frac{\Delta V_{HCl} \times C_{HCl}}{\Delta pH \times V_{sample}} \times 1000 \quad (1)$$

BC: buffering capacity; ΔV_{HCl}: required volume of HCl (L); C_{HCl}: concentration of HCl (M); V_{sample}; sample volume (L); ΔpH: change in the pH value.

2.5. Implication of lysine, arginine, glutamic acid or fructose and wort buffering capacity on LAB cell growth

14% barley malt wort was inoculated with the corresponding LAB strains and 10 mL of each was individually spiked with amino acids, fructose and buffer agent (20 g/L KH_2PO_4 and 0.18 g/L NaOH) at concentrations of 0.6 g/L, 2.5 g/L and 10.4 mmol HCl $pH^{-1}L^{-1}$. Fermentation was carried out on microplate with 250 μL volume using the same conditions as in section 2.1. Cell development was monitored by measuring the OD_{600nm} until the stationary phase was reached using microplate reader Cytation™ 5 (BioTek® Instruments, Inc.); the viable cell count at that period was assessed by colony forming unit counting method.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) was applied to the experimental data. Tukey's test was used for mean values comparison to identify significant differences. Results were considered significantly different at $p < 0.05$. OriginPro 2015G (OriginLab Corporation, Northampton, USA) was used as statistical tool. Results are presented as mean values ± standard deviations of independent replicates.

3. Results and discussion

3.1. LAB cell viability, death rate, and vitality

Cell viability and vitality are important parameters for cell physiological activity and product quality. As shown in Fig. 1a, short growth phase, and prompt death phase initiation were observed for the six LAB strains in malt wort fermentation. The growth phase itself took around 24–48 h depending on the strain. This is consistent with previous studies, which reported a growth phase of 10–40 h in cereal substrates and malt wort (Charalampopoulos et al., 2002; Peyer et al., 2015; Rathore et al., 2012; Rozada-Sánchez et al., 2008). A rapid decrease in the viable count was observed in *L. amylolyticus* and *L. plantarum* Lp.765 strains while *L. plantarum* Lp.758, Lp.725, and *L. brevis* Lb.986 delivered the highest viable count and longer growth time.

Whether the decrease of the viable count was due to cell death was determined by cell labeling with fluorescent probes and monitoring of cell death percentage during fermentation (Fig. 1b). A prompt increase in the death rate was observed from the exponential growth phase. At the death phase, up to 80% cells were dead depending on LAB strains. Lactic acid produced by LAB is known to be a strong inhibitor for its growth and viability (Pieterse et al., 2005). One of the inhibitory effects of lactic acid accumulation on LAB strains is the dissipation of the membrane potential. A complete inhibition of *L. plantarum* Lp.758 growth was observed at 1.7 g/L lactic acid concentration (data not shown). Similarly, growth inhibition of *L. lactis* was reported from a lactic acid concentration of 1.8 g/L, which resulted in a significant degradation of the membrane potential (Magni et al., 1999).

Only damaged membrane cells, including death and viable but not culturable cells (VNC), can absorb PI probe. VNC can, however, recover under favorable conditions as observed with PI-red labeled cells of *L. plantarum* in liquid culture at pH 6.5 (Ingham et al., 2008). Also, very rapid recovery of VNC was observed in wine LAB upon oxygen availability (Millet and Lonvaud-Funel, 2000). Furthermore, damaged and VNC LAB are reported to still undergo enzymatic activities as observed with spoilage capability of VNC *L. lindneri* in beer (Suzuki et al., 2006) and lactate dehydrogenase activity by *L. plantarum* in cheese (Gobbetti et al., 2015). Therefore, it was suggested that VNC could still hydrolyse fluorescent esters to be counted as viable with fluorescence technique although they are unable to form colonies on MRS agar (Millet and Lonvaud-Funel, 2000). Similarly, VNC of *L. lindneri* and *L. paracollinoides* were not detected on MRS agar whereas up to 460 viable cells were counted by fluorescence technique in beer (Suzuki et al., 2006). Consequently, it causes inconsistencies in LAB counts by epifluorescence and CFU as reported in wine (Millet and Lonvaud-Funel, 2000). Therefore, the discrepancy in the results of CFU and death rate observed in this study is explained.

Furthermore, as the dead rate is expressed in percentage, it considers and evaluates the number of death cells against the total cell count at each time in the medium whereas the CFU does measure only the remaining viable cell count at that same period. The continuous increase of the death rate leads, therefore, to suggest that during lactic acid fermentation of malt wort, as viable cells were multiplying, a proportion of that (non-resistant cells) were continuously dying and been accumulated whereby reducing the real number of viable cells at each time but increasing the number of dead cells. This further explains the observed discrepancy but also the exponential increase in the death rate.

Cell metabolic activity evaluated through vitality test represents the ability of cells to metabolize glucose and to actively transport hydrogen ions through its intact membrane into the medium. Only viable cells with intact membrane and metabolically active can achieve that (Bunthof et al., 1999). Fig. 1c shows that cell vitality decreased drastically throughout fermentation to reach very low values. Strains of *L. plantarum* Lp.758 and Lp.725 with the higher end viable count and lowest death rate had also higher vitality values at 72 h of fermentation. The low acidification power (AP) value of *L. brevis* Lb.986 does not absolutely reflect low metabolic activity since it is an obligate heterolactic which yield less acid than its homolactic counterparts. Furthermore, the AP test measures the metabolic activity with respect to the total cell count. That means the observed high dead cell percentage have therefore contributed to decreasing the total metabolic activity.

When referring to previous studies mentioning the low aroma yield in lactic acid fermented malt-based beverages, it is crucial to have living and metabolically active cells for high metabolite formation. However, we found short growth phase and rapid death phase initiation, continuous increase of cell death with a significant reduction in metabolic activity of LAB cells in malt wort. This

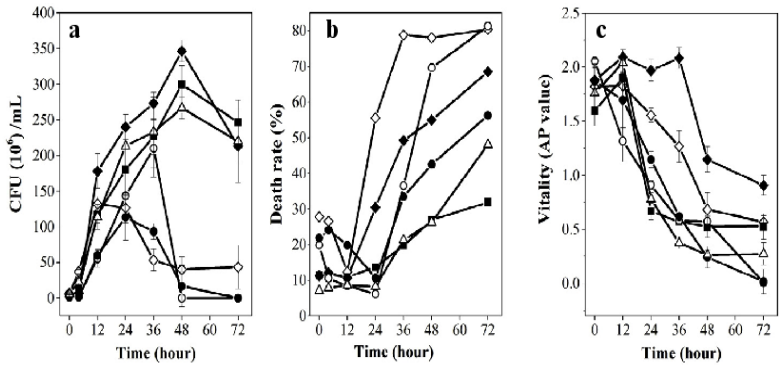

Fig. 1. Kinetics of cell growth (a), death rate (b) and vitality change (c) of six LAB during malt wort fermentation. *L. plantarum* Lp.758 (◆), Lp.765 (◊), Lp.725 (■); *L. amylolyticus* La.TL5 (○), La.TL3 (●); and *L. brevis* Lb.986 (Δ). CFU, colony forming units; AP, acidification power value. Vertical lines represent standard deviations from independent triplicates.

constitutes important limitations that may explain the low yield in aroma compounds reported in malt-based beverages (Nsogning Dongmo et al., 2017).

3.2. Medium composition changes

3.2.1. Sugar fermentation

Hereinafter, it was important to determine whether sugars may have been limited during fermentation to cause cell death. The main fermentable sugars in malt wort were glucose, fructose, sucrose, maltose, and maltotriose. The 14% barley malt wort used in this study had 11.9 g/L, 1.8 g/L, 5.2 g/L, 63.2 g/L and 15.9 g/L glucose, fructose, sucrose, maltose, and maltotriose, respectively. Glucose, fructose, and sucrose were the major utilized sugars; they were simultaneously and continuously degraded during fermentation with the extent depending on the fermenting LAB strain (Fig. 2).

52–83% of glucose remained in the medium corresponding to 6.2–9.9 g/L (Fig. 2a). To the best of our knowledge, there is no data about minimal glucose concentration required by LAB to growth but its concentration in the medium may influence its uptake and glycolytic activity (Papagianni et al., 2007). Moreover, LAB growth in 6% barley malt wort (5 g/L glucose content) was observed in our unpublished work. Therefore, glucose was not limiting in the medium and more likely have not been the cause of cell death.

Fructose was rapidly depleted in all cases except for *L. amylolyticus* strains (Fig. 2b). It is worth to mention that fructose concentration in malt wort is lesser than glucose at a molar ratio of 1:6. However, there is no report yet on fructose as an essential sugar for LAB growth although it is speculated that its metabolism increases LAB metabolic activity and growth rate (Zaunmüller et al., 2006). The utilization of fructose as electron acceptor provide to LAB cells an extra ATP gain with no ATP expenditure through the conversion of acetyl-P to acetate (Zaunmüller et al., 2006). In this case, limitation in fructose may affect LAB growth and viability.

Sucrose remained at 46–98% (Fig. 2c) in the fermentation medium. It does not seem to be an important fermentable sugar to the considered LAB strains. Sucrose metabolism by levansucrase or β-glucosidase to fructose and glucose was reported in both homolactics and heterolactics LAB (Bonestroo et al., 1992; Tung-Hai, 2003). The increased content of fructose at the late-stage fermentation may be related to the action of enzymes delivered from cytoplasm after cell death, which may have converted sucrose to fructose. It is further seeable that the content of sucrose significantly decreased at the same period (Fig. 2c), where a high proportion of cells were dead (Fig. 1a) for both *L. amylolyticus* La.T3/T5 strains.

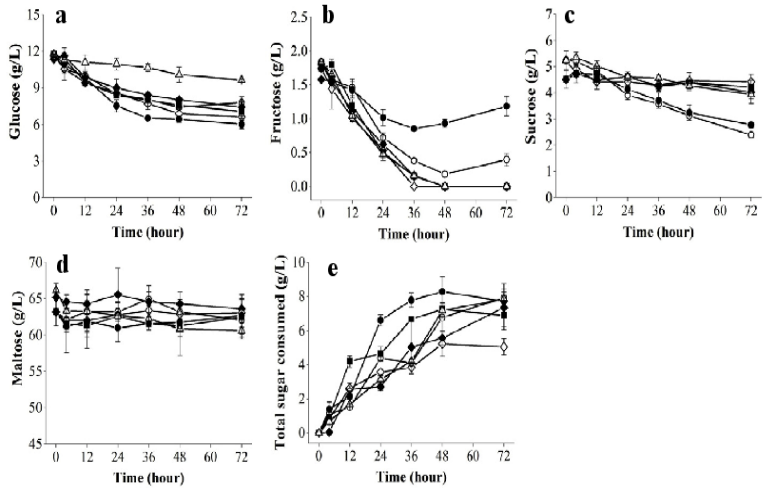

Fig. 2. Kinetics of malt wort fermentable sugar degradation and total sugar consumption during 72 h fermentation by six LAB strains. *L. plantarum* Lp.758 (◆), Lp.765 (◊), Lp.725 (■); *L. amylolyticus* La.TL5 (○), La.TL3 (●); and *L. brevis* Lb.986 (Δ). Glucose (a), fructose (b), sucrose (c), maltose (d), total degraded sugars (e). Vertical lines represent standard deviations from independent triplicates.

Maltose, the major sugar in malt wort remained in the medium at 96–99% (Fig. 2d) for a total consumption of 0.5–2.6 g/L. It is, therefore, the less consumed sugar during malt wort fermentation by LAB. Maltose is the preferred sugar of sourdough heterolactics LAB because the expenditure of 1 mol ATP necessary for the conversion of glucose to glucose-6-phosphate is saved. It is consistent with the higher percent of maltose utilization by heterolactic *L. brevis* observed in this study. Maltose metabolism in LAB occurs via intracellular maltose phosphorylase and 6-phospho-α-glucosidase cleavage for obligate heterolactics and homolactics, respectively (Ehrmann and Vogel, 1998; Gänzle et al., 2007). On the contrary, we could not find an explicit utilization of maltotriose by any of the six strains considered in this study (data not shown).

The total sugar utilization increased significantly at the steady growth phase to hibernate at the stationary growth phase although concentrations were different among strains (Fig. 2e). The continuous sugar consumption at late-stage fermentation, where cell began to die may be related to the metabolism of resistant living cells or to non-growth related glycolytic activity. Likewise, glycolytic activity was already reported in non-growing damaged *L. plantarum* cells (Gobbetti et al., 2015). The increase in lactic acid production at the same period supports this assertion. In total, only 5.3–8.2% sugar were consumed (Table 1). There was still enough sugar in the fermentation medium to support continuous growth of LAB. However, the possible indirect impact of fructose will be further investigated in this study.

3.2.2. Change in organic acid concentration during malt wort fermentation

Because lactic acid, acetic acid, and succinic acid were reported to inhibit LAB growth (Li et al., 2010; Magni et al., 1999), we became interested in the concentration of organic acids during malt wort fermentation as a possible cause of rapid LAB cells death.

Same patterns in the accumulation of lactic, acetic and succinic acid were observed but the concentrations were largely dependent on LAB strains (Fig. 3). There, the fermentative pathway of LAB strains is determinant because homolactics produce 2 mol lactic acid per mol hexose consumed whereas heterolactics produce 1 mol lactic acid (Pessione, 2012). An accumulation of up to 3.9 ± 0.4 g/L lactic acid (Fig. 3a) with a yield of 0.11–0.49 g/g sugar consumed (Table 1) was observed. Concentrations were much higher than the reported initial lactic acid concentration of 1.8 g/L, which inhibited *Lactococcus lactis* growth (Magni et al., 1999). Similarly, growth inhibition of LAB strains considered in this study, in malt wort at an initial lactic acid concentration of 1.7 g/L was observed in our unpublished work. The sensitivity to lactic acid may vary between strains and the inhibitory concentration in an ongoing fermentation may be higher than the one at shock stress conditions. Lactic acid accumulation may have therefore contributed to LAB cell death during fermentation.

Table 1
Fermentation rate of total sugars in malt wort and yield of lactic acid and acetic acid per total sugar consumed among six LAB strains.

	F_{ts}	$Y_{LA/ts}$	$Y_{AA/ts}$
Lp.758	7.5 ± 3.2	0.32 ± 0.04	0.029 ± 0.006
Lp.765	5.3 ± 3.4	0.34 ± 0.02	0.027 ± 0.010
Lp.725	7.1 ± 5.5	0.17 ± 0.01	0.015 ± 0.003
La.TL5	8.2 ± 1.2	0.49 ± 0.09	0.013 ± 0.001
La.TL3	8.1 ± 3.8	0.31 ± 0.01	0.009 ± 0.001
Lb.986	8.0 ± 2.7	0.11 ± 0.01	0.024 ± 0.005

F_{ts}: fermentability of total sugars (the ratio of total consumed sugars to the initial sugar concentration).
$Y_{LA/ts}$: yield of lactic acid produced per total sugar consumed (g/g).
$Y_{AA/ts}$: yield of acetic acid produced per total sugar consumed (g/g) (succinic acid was not considered since it is not a direct product of sugar fermentation); the yields were calculated by dividing the concentration of lactic acid or acetic acid by the concentration of total sugars consumed.

Acetic acid concentrations of 0.7–0.2 g/L (Fig. 3b) with a yield of 0.009–0.029 g/g sugar (Table 1) obtained were quite lower than that of 1.2 g/L and 3.6 g/L, which reduced respectively the growth rate and biomass yield of *Lactococcus lactis* (Magni et al., 1999). Acetic acid alone has unlikely hindered LAB growth but in combination with lactic acid, an inhibition may take place. Higher concentrations at late-stage fermentation may be explained by the mechanism of resistant living cells to overcome lactic acid stress by the overexpression of pyruvate metabolic enzymes to reroute pyruvate conversion to acetyl-CoA and acetyl-P for acetic acid production at the expenses of lactic acid (Gobbetti et al., 2015; Pedersen et al., 2012).

All the six LAB strains generated succinic acid during malt wort fermentation to up to 0.8 g/L (Fig. 3c). Therefore, succinic acid prevailed than acetic acid (0.2 g/L). Citrate metabolism to succinic acid or other acids was described to be used by *Lactococcus lactis* to overcome the inhibitory effect of lactate (Magni et al., 1999). However, succinic acid is unlikely to have affected LAB viability although an effect in combination with lactic acid may not be neglected. Therefore, succinic acid should be given more attention as an important acid in lactic acid fermentation of malt wort.

Malic acid was found in malt wort at a very low concentration of 0.4 g/L (Fig. 3d) as compared to must (6 g/L) (Knoll et al., 2012). It is formed during malting process by endogenous microorganisms (Li and Fang Liu, 2015). In this study, the degradation of malic acid was noticed in all the considered LAB strains, where the extent was higher by *L. plantarum* and *L. brevis*. Malolactic activities were already reported in several *L. plantarum* and *L. brevis* strains (Bravo-Ferrada et al., 2013; Iorizzo et al., 2016; Zhang and Lovitt, 2006). As such, malolactic fermentation is already reported in juice and cider fermentation (Costantini et al., 2008; Sánchez et al., 2014). It may have encountered in malt wort fermentation as well.

To sum up, lactic acid prevailed at concentrations much higher than the reported inhibitory concentration and may, therefore, have contributed to LAB cell death during fermentation. Further investigations on the combined inhibitory effects of organic acids on LAB growth and death in malt wort are pending. Whether it was the sole cause of cell death was further investigated in this study.

3.2.3. Change of malt wort pH value and buffering capacity during fermentation

The buffering capacity (BC) is of major importance in lactic acid fermentation because of the pH decrease that occurs as a consequence of acid accumulation and its negative impact on LAB growth and viability. High BC will delay rapid pH drop to values below the optimum for LAB growth and viability. The rapid decrease in the pH values was consistent with organic acid production (Fig. 4a). Total pH change was from 1.4 to 1.9 for end pH values of 3.6 and 3.1, respectively. However, at a pH value of round 3.5, the decrease was slow although organic acids were still significantly produced in the medium. To understand the low change of pH value at that fermentation stage, we next evaluated the capacity of malt wort to resist the pH change during lactic acid fermentation (Fig. 4b).

A typical nonlinear increase of malt wort BC with pH drop was obtained during fermentation. The initial value of 4.5 mmol HCl $pH^{-1}L^{-1}$ was low, which caused rapid pH drop in early-stage fermentation. Thereafter, it increased slowly to reach exponential increase at pH value of circa 3.5. At that phase, the pH value hardly changed although organic acids were continuously accumulated. Higher values (up to 31.3 mmol HCl $pH^{-1}L^{-1}$) were obtained at late-stage fermentation and the increase was proportional to acid concentrations. BC value of 8.5 mmol HCl $pH^{-1}L^{-1}$ was reported in

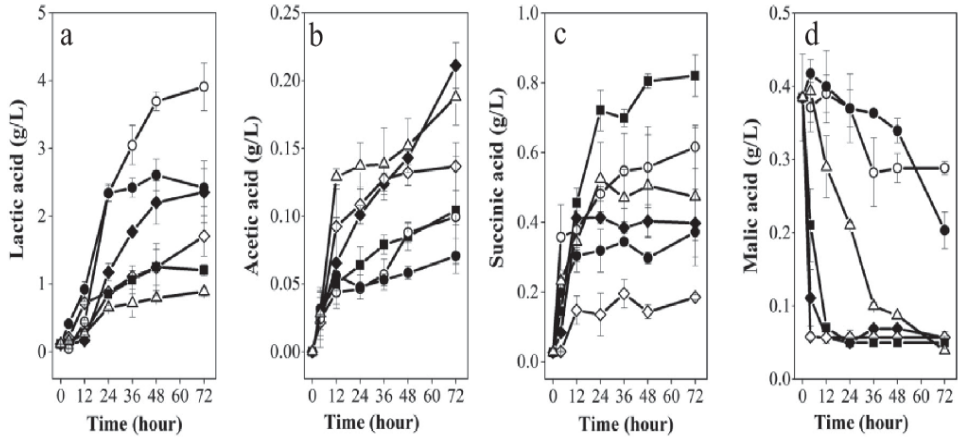

Fig. 3. Kinetics of major organic acid accumulation during malt wort fermentation by six LAB strains. *L. plantarum* Lp.758 (◆), Lp.765 (◊), Lp.725 (■); *L. amylolyticus* La.TL5 (○), La.TL3 (●); and *L. brevis* Lb.986 (Δ). Lactic acid (a), acetic acid (b), succinic acid (c) and malic acid (d). Vertical lines represent standard deviations from independent triplicates.

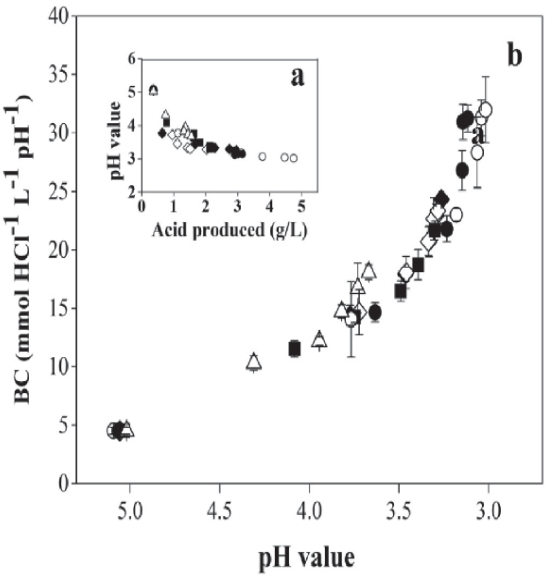

Fig. 4. Change in the pH value as a function of organic acid accumulation (a) and change in the buffering capacity (BC) with the change in the medium pH value (b) during barley malt wort fermentation by six LAB strains. *L. plantarum* Lp.758 (◆), Lp.765 (◊), Lp.725 (■); *L. amylolyticus* La.TL5 (○), La.TL3 (●); and *L. brevis* Lb.986 (Δ). Vertical lines represent standard deviations from independent triplicates.

malt medium (Charalampopoulos et al., 2002). The difference with our results may be due to different preparation process.

Malt wort buffering capacity was attributed to amphoteric substances such as amino acids, proteins, peptones and inorganic ions (Lewis and Bamforth, 2007; Yuldasheva et al., 2013). The buffering effect of lactic acid addition to wort was described in a previous study (Li et al., 2016). Lactic acid ($CH_3CH(OH)CO_2H$), with a pKa value of 3.85, is in equilibrium with its ionized form ($CH_3CH(OH)CO_2$-) when the medium pH is equal to its pKa. When a significant amount of both are present, the buffer is created and the capacity is proportional to the concentration of both agents. As such, BC values were higher in the pH range near lactic acid pKa value (3.86 ± 0.5). Then, we suggested that lactic acid and its conjugate base are the main buffer agents causing a buffering increase at late-stage fermentation of malt wort. In short, lactic acid accumulation decreased rapidly the pH value at early-stage fermentation, which conversely favors the formation of lactic acid/lactate buffer leading to further increase in the BC at late-stage fermentation. Therefore, constant pH value at late-stage fermentation does not necessarily mean the absence of acid production. Further, the ionization of lactic acids to create the buffer may reduce the availability of non-ionized lactic acid which is toxic to LAB cells. Consequently, low wort BC may hinder LAB cell growth through consequent rapid pH drop, since low pH values are deleterious to LAB growth and viability.

3.2.4. Residual amino acids and free amino nitrogen

LAB are known to be auxotroph to a wide range of essential amino acids. It raised our interest in the content of amino acids in malt wort fermentation by LAB. According to Fig. 5, malt wort contained 0.58 ± 0.02 g/L and 4.60 ± 0.03 g/L free amino nitrogen and total amino acids, respectively. Lysine, glutamic acid, arginine, leucine, phenylalanine, and alanine were the prevailing amino acids and constituted 58.8% of the total amino acids. There was no significant difference in the residual amino acids among *L. plantarum* strains but significant differences with other strains were observed (data not shown).

Amino acids were important to LAB growth, up to 76% of total amino acids and 77% of total free amino nitrogen were utilized (Fig. 5). They were tentatively organized into 4 groups according to their extent of utilization, which was consistent with their prevalence in malt wort. Lysine, arginine and glutamic acid, constituting group A, were the most important with lysine being consumed until less than 2% remained. Similarly, lysine and arginine were significantly consumed by a *L. plantarum* strain, as compared to other amino acids, in MRS fermentation (Lee et al., 2014) whereas glutamic acid was found to be essential for dozens of LAB strains (Costello et al., 2015; Terrade and Mira de Orduña, 2009). Amino acids are not only necessary as nitrogen source for cell growth but also for responding to the acid stress through their decarboxylation. Lysine and glutamate decarboxylation facilitates the increase of intracellular pH. The formation of ATP in the arginine catabolism constitutes an alternative source of energy to LAB whereas ammonia, generated from deamination, increases the medium pH value (Azcarate-Peril and Klaenhammer, 2010; Pessione, 2012). Therefore, lack of amino acids may not only cause nutrient related growth inhibition but also reduce the acidic stress resistance. However, all measured amino acids showed a residual concentration at late-stage fermentation. It was reported that the expression of genes involved in amino acid uptake significantly decrease under

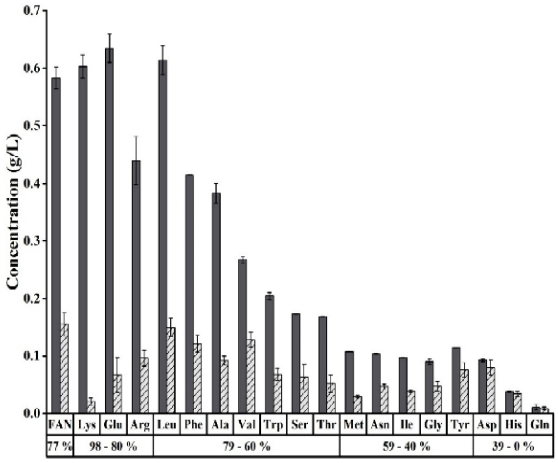

Fig. 5. Prevalence of free amino nitrogen (FAN) and amino acids in 14°P barley malt wort (black bars) and their residual concentrations after 72 h fermentation (striped bars). Amino acids were tentatively classified in increasing order of utilization by six LAB strains; percentage values are the percentage of utilization calculated from the initial values in malt wort; the residual amount represents the mean values from the six considered LAB strains. Vertical lines represent standard deviations from independent duplicates.

lactic acid stress (Pieterse et al., 2005) and the amino acid uptake by LAB is low (Vermeulen et al., 2006). Therefore, amino acid uptake potential of LAB became probably too low at late-stage fermentation. Depending on the LAB strain, amino acids could have been depleted between 24 and 48 h fermentation of malt wort. Furthermore, amino acid measurement at each fermentation stages will define the time of amino acid depletion in malt wort fermentation.

Our results reveal limitation in key amino acids during lactic acid fermentation of malt wort. This may have contributed to growth cessation and cell death. Therefore, it was of great importance to determine their implication to LAB growth behavior in malt wort.

3.2.5. *Implication of lysine, arginine, glutamic acid or fructose and wort buffering capacity on LAB growth behavior in malt wort*

The main limitations to LAB cell growth in malt wort outlined in this study are key amino acid, fructose, and BC, in addition to the speculated inhibitory effect of lactic acid. For that, further investigations were done to highlight their implications on LAB growth behavior. It was found that the addition of arginine and lysine to wort increased significantly cell growth (Fig. 6). Doubling their addition content led to further increase of the cell count (Fig. 7). Their importance to LAB growth was previously reported (Lee et al., 2014). They are basic amino acids and their addition to malt wort caused an increase in the initial pH value. For this reason, histidine, a basic amino acid also reported to be used by LAB to fight against acidic stress, was considered as a control although its content in malt wort was low. Comparable growth behavior with arginine and lysine was observed. Furthermore, the increase of the pH value due to their addition was not responsible for the greater cell development; a parallel experiment with wort at different pH values showed that high pH value increases cell growth but the effect was lesser than that with amino acid addition (data not shown). We could not draw a clear conclusion about the impact of glutamic acid because, as an acidic amino acid, its addition significantly decreased wort pH value. Then, it was concluded that the depletion of arginine and lysine in malt wort limited LAB cell growth.

The addition of fructose improved cell development when wort was fermented by *L. brevis* Lb.986 whereas it had no significant impact when *L. plantarum* Lp.758 and *L. amylolyticus* were used - the effect was lesser than that of amino acids. The contribution may be related to one ATP gain in the utilization of fructose as an electron acceptor (see section 3.2.1), which compensate low ATP gain by heterolactics in glucose metabolism (Gänzle et al., 2007). Therefore, the effect of fructose on LAB cell death was considered not substantial.

Wort buffering increased cell growth but lesser than amino acid addition, except for strain *L. amylolyticus*, where the effect was significantly higher than that of amino acid addition (Fig. 6). This strain, as an obligate homolactic, produced more lactic acid than its counterparts (see Fig. 3a). The buffering may have prevented rapid pH drop allowing more cell growth but the resulted increase in lactic acid content, and key amino acid limitation may have caused growth cessation. This result present not only the contribution of low wort buffering capacity but also that of amino acid depletion and lactic acid accumulation to LAB death in malt wort.

The effect of amino acids, fructose and wort buffering on the pH value change (Fig. 7) revealed that amino acid addition led to higher pH change during fermentation. Higher acid production as a result of higher cell development in combination to low wort buffering

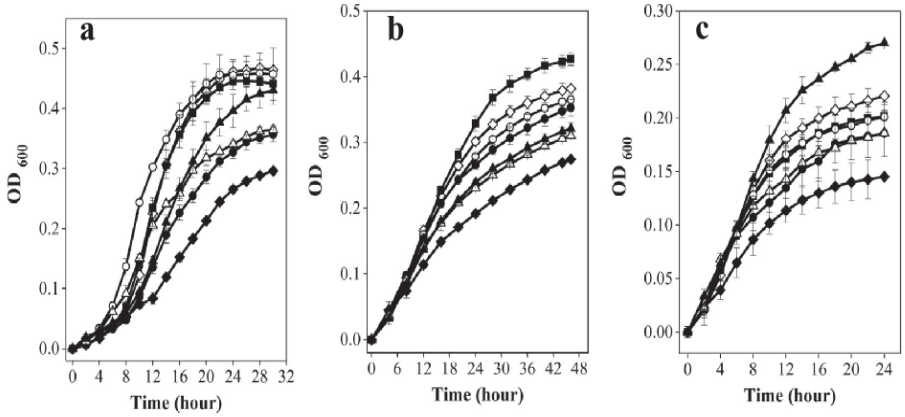

Fig. 6. Effect of arginine (■), lysine (◊), histidine (○), glutamic acid (◆) or fructose (●), and buffering (▲) on cell development of three LAB strains in 14°P malt wort as compared to wort without addition (Δ). (a), *L. plantarum* Lp.758; (b), *L. brevis* Lb.986; (c), *L. amylolyticus* La.TL5. Cell development was evaluated by monitoring the change in the OD_{600nm} value over time until the initiation of the stationary phase. Vertical lines represent standard deviations from independent triplicates.

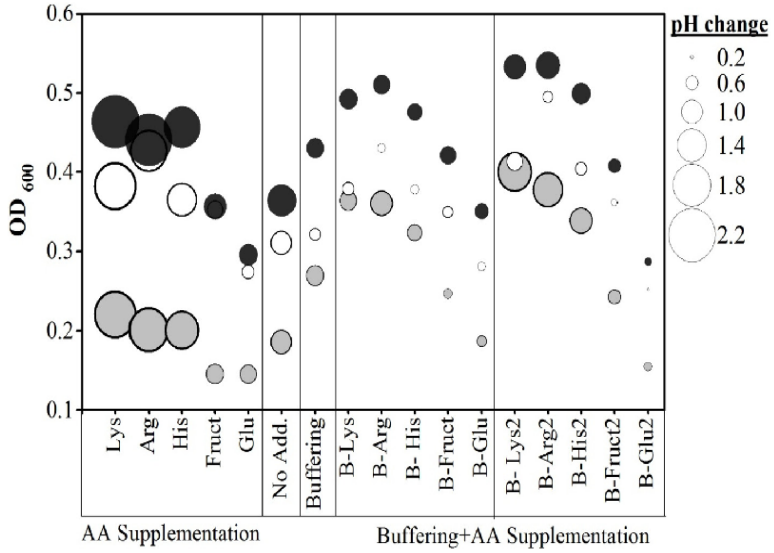

Fig. 7. Bubble plot showing differences in the total cell development (OD_{600nm}) in 14°P malt wort spiked with arginine (Arg), lysine (Lys), histidine (Hist), glutamic acid (Glu) or fructose (Fruct), buffer agents (B), the mixture of buffer agents and amino acid (B-Lys) and the mixture of buffer agents and double addition of amino acid ($B\text{-}Lys^2$) fermented by three LAB strains when compared to wort without any addition (No add.). The size of each bubble is proportional to the total pH value change during fermentation. Values are from independent triplicates. Black ovals represent samples fermented by *L. plantarum* Lp.758; open ovals represent those from *L. brevis* Lb.986; grey ovals represent those from *L. amylolyticus* La.TL5.

capacity may be the explanation. Wort buffering led to significant reduction in the total pH change. As such, combining amino acid and buffering further increased the cell count but the pH change remained less. However, doubling the amino acid supplementation led to further increase of the cell count and the pH change remained lesser than when no addition occurred.

As per LAB viability, Fig. 8 shows that amino acid addition significantly increased the total viable count at the stationary phase with arginine being the most important. However, wort buffering alone led to significant reduction of the viable count. When in combination with amino acid supplementation, the viable cell count was higher but still less than when only amino acid was supplemented. These results showed that malt wort content in these amino acids plays a great role in LAB viability during fermentation. They are used by LAB as an essential growth nutrient but also to resist and therefore survive to the acidic stress (Azcarate-Peril and Klaenhammer, 2010; Pessione, 2012). Wort buffering may have allowed more lactic acid to be produced causing further LAB cells death. This explains the low viable count even when in combination with amino acid, as compared to the high viable count when only amino acids were added. This further corroborates with the extreme low viability of strain *L. amylolyticus*, which produced the most lactic acid, even in increased amino acid conditions. These results showed that amino acid depletion, occurring in malt wort fermentation, significantly contributed to LAB prompt death as reported in section 3.1; the accumulation of organic acids being also of major contribution. Whether the improved LAB growth and viability consequent to amino acid supplementation, may increase the content in aroma compounds is worth to be investigated.

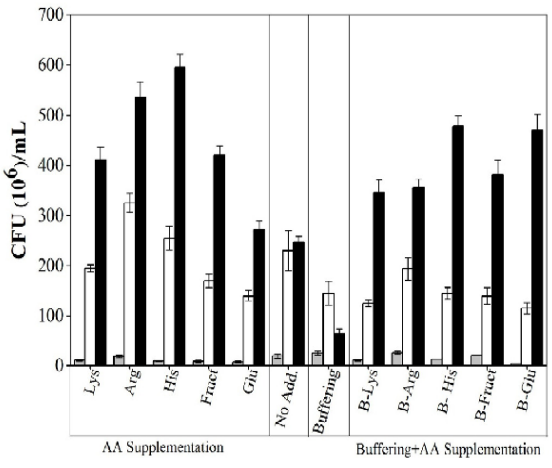

Fig. 8. Scatter plot showing differences in the total viable count in 14°P malt wort spiked with arginine (Arg), lysine (Lys), histidine (Hist), glutamic acid (Glu), fructose (Fruct) or buffer agents (B) and the mixture of buffer agents and amino acid (B-Lys) fermented by three LAB strains when compared to wort without addition (No add.). Values are from independent replicates. Black circles represent samples fermented by *L. plantarum* Lp.758; open circles represent those from *L. brevis* Lb.986; grey circles represent those from *L. amylolyticus* La.TL5.

4. Conclusions

The objective of this study was to elucidate the fermentation performance of LAB in barley malt wort fermentation to outline limitations that may exist. We found that LAB fermentation in malt wort is abridged with rapid attainment of death phase coupled to a rapid increase in cell death rate and rapid degradation of cell metabolic activity. Lactic acid accumulation, low wort buffering capacity, fructose depletion and key amino acids lysine, arginine and glutamic acid exhaustion were suggested as the main limiting factors.

The supplementation of malt wort with amino acids and buffer agents led to conclude that the depletion of key amino acids (arginine, lysine and glutamic acid) during fermentation caused LAB growth cessation and cell death during malt wort fermentation. Wort buffering was of contribution to LAB growth and viability only in combination with amino acid availability. More attention should, therefore, be given to these amino acid content in malt wort. Furthermore, we speculated on the significant contribution of

lactic acid accumulation to LAB cell death. However, good cell viability throughout fermentation, mainly at the stationary phase, where stress conditions are available, is of great advantage to metabolite formation. Further studies on the implication of improved LAB growth and viability, through amino acid and buffering capacity increase on the aroma yield, are worth to be done.

Moreover, wort supplementation did not improve the fermentation duration. It is therefore of great necessity to optimize malt wort composition. Mashing process management of protease activity, for instance, or considerations of pseudo-cereals or their mixture are valuable. Further, it is also important to develop a fermentation process that will attenuate the inhibitory effect of lactic acid to have extended viability for an improved flavor profile.

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2.5. Exploration of high-gravity fermentation to improve lactic acid bacteria performance and consumer's acceptance of malt wort-fermented beverages

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Exploration of high-gravity fermentation to improve lactic acid bacteria performance and consumer's acceptance of malt wort-fermented beverages

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Summary Lactic acid bacteria (LAB) fermentation performance is essential for aroma metabolites formation and product flavour quality. Hence, this study appraises high-gravity malt wort fermentation (HGF) by three LAB strains to improve the fermentation performance and consumer's acceptance of lactic acid-fermented malt-based beverages (LAFMB). HGF at 20% (w/w) provided higher amino acid content and buffering capacity that allowed greater cell development, viable cell count and sugar utilisation. Moreover, the pH change was lesser although marked lactic acid accumulation. It is noteworthy that HGF significantly incremented the content of higher alcohols (+0 – 161%), 2-phenylethanol (+11–147%), acetaldehyde (+27–44%) and β-damascenone (+25 – 66%) comparing to low-gravity malt wort at 12%. Thus, HG-fermented beverages were significantly preferred with greater hedonic scores (4.6 ± 2.1). Our results indicate that HGF is a valuable strategy for improving LAB fermentation performance in malt wort, which in turn increases key aroma compound content resulting in enhanced acceptance of LAFMB.

Keywords Aroma yield, buffering capacity, cereal-fermented beverages, flavour improvement, high-gravity wort, sensory acceptance.

Introduction

Flavour improvement remains the main challenge for the development of lactic acid-fermented beverages produced from cereal-based substrates. In fact, due to their high functional properties, lactic acid-fermented cereal-based beverages possess high market potential and are in demand to replace dairy-based products (Granato *et al.*, 2010). That is enforced by diversified consumer's choices and health conditions such as lactose intolerance, veganism, low cholesterol and low salt diet. Then, considerable research interest was dedicated to establish their potentials as functional beverages (Salmerón *et al.*, 2015; Hassani *et al.*, 2016; Enujiugha & Badejo, 2017). However, the development on the market failed or at least remains scarce as compared to dairy-based products (Corbo *et al.*, 2014), which might have reduced researchers and industrials interest in lactic acid fermented cereal-based beverages in the previous years. The reported low consumer's acceptance due to poor flavour profile coupled to understudied aroma profile and the quasi nonexisting process technology are the main limitations (Yu & Bogue, 2013; Nsogning Dongmo *et al.*, 2016). Recent studies revealed that the concentration of key aroma compounds in lactic acid-fermented malt wort beverages (LAFMB) was too low to contribute to the overall flavour (Nsogning Dongmo *et al.*, 2017).

The nowadays consumer's health consciousness and the quest for natural food give values to bioflavouring over artificial flavouring. In this regard, the acidic stress occurring in lactic acid fermentations were exploited to increase metabolites yield. A shift from sugar to amino acid catabolism under acidic stress was proven in *Lactobacillus sanfranciscencis* to promote aroma formation (Serrazanetti *et al.*, 2013). A typical example was the rise in 3-methylbutanoic acid yield from leucine catabolism at acidic stress (Serrazanetti *et al.*, 2011). Also, higher alcohols, aldehydes, γ-decalactone and 2(5)-furanones were accumulated under acidic stress exposure (Guerzoni *et al.*, 2007) and in combination with osmotic stress (Serrazanetti *et al.*, 2013). Osmotic stress may encounter in high-gravity fermentation (HGF) from high sugar concentration. However, sugar osmotic stress is less deleterious to lactic acid bacteria (LAB) because cells can balance the extra and intracellular concentration of sugars (Serrazanetti *et al.*, 2013). In fact, acidic stress conditions upregulate genes involved in amino acid and carbohydrates metabolism, and secondary metabolite

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biosynthesis and transport (Pieterse *et al.*, 2005). The prerequisite is therefore amino acid availability and LAB viability. Inversely, acidic stress is deleterious to LAB viability causing rapid cell death and, consequently, fermentation cessation (Pieterse *et al.*, 2005).

Abridged fermentation performance resulting from exponential cell death and degradation of metabolic activity was reported in the lactic acid fermentation of malt wort. Low buffering capacity (BC) and key amino acids depletion were suggested as key limitations (Nsogning Dongmo *et al.*, 2018). A possible solution to increase the aroma yield is therefore the improvement of LAB fermentation performance and medium composition. Fermentation performance depends on cell viability and vitality. It defines cell efficiency to metabolise substrate, develop new viable biomass or to form metabolites during fermentation. Hence, metabolites yield is closely related to the fermentation performance. In this regard, medium composition and environmental conditions are determinants (Serrazanetti *et al.*, 2011).

High-gravity wort (HGW), a concentrated brewer's malt wort, is already under consideration in industrial applications to improve the fermentation performance and product yields (Puligundla *et al.*, 2011). It would benefit LAB fermentation from (i) increased content in sugars, amino acids and essential nutrients; (ii) raised dextrin content that protects LAB from acidic stress (Charalampopoulos *et al.*, 2002); and (iii) high initial BC (Bamforth, 2001). To our knowledge, this is the first report on HGF to improve LAB fermentation performance in LAFMB.

Thus, the aim of this study was to increase malt wort amino acid content and BC through HGW for improved LAB fermentation performance and aroma concentration, and ultimately consumer's acceptance of the resulting beverage. This study provides a cost-efficient industrial application for the development of LAFMB.

Materials and methods

Malt wort preparation

Standardised unhopped Bavarian pilsner barley malt extract (72%) from Weyermann® (Bamberg, Germany) was diluted to the corresponding final wort gravities. Malt worts were then autoclaved at 110 °C for 10 min, and pellets were removed by sedimentation after cooling. Malt wort gravity was determined as the percentage by weight of sugars and soluble materials.

Bacteria strains and fermentation conditions

Strains of *Lactobacillus plantarum* Lp.758, *Lactobacillus brevis* Lb.986 and *Lactobacillus amylolyticus* La.TL5 were obtained from the strain collection of the Institute for Brewing and Beverage Technology, Technical University of Munich. Single colonies cultures were activated and propagated twice in MRS broth then precultured in 14% wort for 12 h at 28 °C (*L. brevis* and *L. plantarum)* and 48 °C (*L. amylolyticus)*. Cells were washed out thrice using sterile quarter strength Ringer's solution and inoculated at $6 \pm 1 \times 10^6$ CFU mL^{-1} into 500 mL presterilised wort contained in 500-mL shake flasks. Fermentation was carried out in triplicate for 48 h under static conditions.

Determination of cell growth and total viable cell count

Cell growth was monitored by measuring the optical density (OD) at 600 nm using Ultrospec™ 3100 pro UV/Visible spectrophotometer (Amersham Biosciences GmbH, Freiburg, Germany). The viable cell count was assessed by colony-forming unit (CFU) plate counting method as described previously (Peyer *et al.*, 2015).

Analytical methods

Residual sugars were measured by high-performance liquid chromatography using a pulsed amperometric detector (PAD) and a Dionex Carbopac PA10 carbohydrate column (Thermo Scientific, Darmstadt, Germany). Samples were filtered through a 0.25 µm membrane filter prior to the analysis. Extracellular lactic acid (kit k-late 07/14) and acetic acid (kit kacetrm 07/12) were determined using Megazyme enzymatic kits (Megazyme, Wicklow, Ireland). Samples were heated at 80 °C for 5 min and centrifuged, and the supernatant was used for analysis. The pH value was measured using WTW InoLab pH 720 digital pH Meter (WTW inoLab®, Weilheim, Germany). The BC was determined by titration based on previous methods (Charalampopoulos *et al.*, 2002; Nsogning Dongmo *et al.*, 2018).

Aroma isolation

Volatile fraction in the beverages was isolated by steam distillation using a Büchi K-314 distillation unit (Büchi Labortechnik, Essen, Germany) based on the previously described method (Krahl *et al.*, 2009). Higher alcohols (propan-1-ol, 2-methylpropanol, 3-methylbutanol and 2-methylbutanol) and acetaldehyde were isolated by headspace method using a HP 7694 GC-headspace autosampler (Hewlett-Packard, Waldbronn, Germany) as described previously (Nsogning Dongmo *et al.*, 2017).

GC-FID analysis

Higher alcohols and acetaldehyde

An Hewlett-Packard 6890 gas chromatograph equipped with a HP 7694 headspace autosampler and

a 50 m HP-Ultra 2 silica capillary column and with 0.52 μm film thickness and 0.32 mm internal diameter (Hewlett-Packard) was used for higher alcohols and acetaldehyde separation and quantification as described earlier (Nsogning Dongmo *et al*., 2017).

2-phenylethanol and β-damascenone

Isolated aroma volatiles from steam distillation were separated and quantified by GC-FID (Hewlett-Packard 5890 Series II Plus) with a Hewlett-Packard 7673 A automatic sampler (HP Inc, Böblingen, Germany) based on the method previously reported (Krahl *et al*., 2009). The mean values of independent duplicate experiments were considered.

Sensory analysis

Sensory tests for the evaluation of consumer's preference and acceptance of the beverages were performed using eighty nontrained consumers (ages: 20–30 years) according to the protocol described by Meilgaard *et al*. (2007).

Statistical analysis

Statistical significance was calculated by one-way analysis of variance (ANOVA) with Tukey's test ($n = 3$, $\alpha = 0.05$) on independent experiments. The correlation strength was estimated by Pearson bivariate correlation. These statistical analyses were performed using OriginPro 2017G (OriginLab Cooperation, Northampton, MA, USA).

Results and discussion

Effect of malt wort gravity on cell development and total viable cell count

Figure 1a–c shows that the increase in wort gravity led to more cell development, regardless of LAB strains. Although similar trends were observed, growth was significantly differentiable at the late-stage fermentation. The total viable cell count measured at that same period was significantly elevated as wort gravity was increased (Fig. 1d). That was worth 0.3-, 3.1- and 3.3-fold augmentation, respectively, for Lp.758, La.TL5 and Lb.986 corresponding to a gravity increment from 12% to 20%. LAB viability behaviour in malt wort fermentation was already reported in previous studies (Nsogning Dongmo *et al*., 2018). HGW brought along greater key and total amino acids content (Fig. 1e), which contributed to promote cell growth and to maintain cell viability at late-stage fermentation. Similarly, arginine, lysine, histidine and glutamic acid were reported to be used by LAB to resist and survive to acid stress conditions (Pessione, 2012).

The evaluation of ultra-HGF to up to 40% (Fig. S2) further augmented the viable cell count till 30%. However, the prolonged lag phase, and very low viability at 40%, was observed indicating ultra-HGF not relevant. Most LAB are osmosensitive; osmolality value of 0.914 OsM Kg^{-1} was obtained for 25% wort in this study, whereas a tolerated threshold of 0.933 OsM Kg^{-1} was reported (Kashket, 1987).

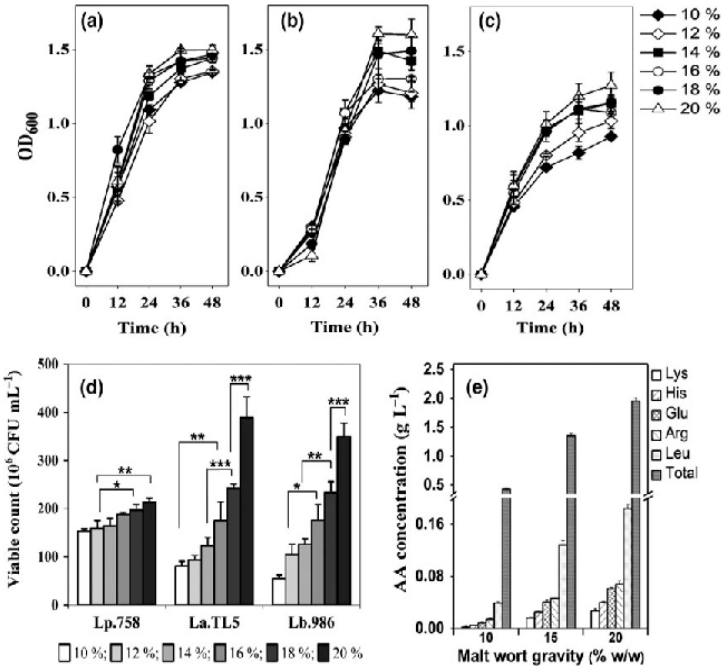

Figure 1 Comparison of cell development (a–c) and total viable count of three lactic acid bacteria strains after 48-h fermentation (d), malt wort amino acid content at increased gravity from 10% to 20% (w/w) (e). (a) *Lactobacillus plantarum* Lp.758, (b) *Lactobacillus amylolyticus* La.TL5, (c) *Lactobacillus brevis* Lb.986, CFU, total colony-forming unit; AA, amino acids. Vertical lines are SD; *Significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Table 1 Sugar consumption after 48-h fermentation by three lactic acid bacteria strains at increasing wort gravity (%w/w)

	Sugar (glucose + fructose) consumed (g L^{-1})		
	Lp.758	La.TL5	Lb.986
10%	2.4 ± 0.4	2.5 ± 0.5	1.7 ± 0.1
12%	2.9 ± 0.3	4.1 ± 0.5	2.0 ± 0.6
14%	4.0 ± 0.0	4.7 ± 0.2	2.5 ± 0.1
16%	4.7 ± 0.7	5.1 ± 0.4	2.6 ± 0.6
18%	5.2 ± 0.5	6.0 ± 0.4	2.7 ± 0.1
20%	6.3 ± 0.9	6.0 ± 0.1	3.9 ± 0.5

Lp, *Lactobacillus plantarum*, La, *Lactobacillus amylolyticus*, Lb, *Lactobacillus brevis*.
Data are presented as mean ± SD.

Sugar consumption

To further evaluate LAB fermentation performance in HGF, sugar consumption was evaluated. Focus was given in this study to glucose and fructose because they were previously reported the main fermentable sugars with up to 48% and 100% utilisation, respectively, in malt wort, whereas maltose and sucrose utilisation was not important (Peyer *et al.*, 2015; Nsogning Dongmo *et al.*, 2018). Sugars concentration in malt worts can be found in Table S1.

Table 1 indicates that sugar consumption was 0.5- to 1.2-fold higher at HGF (20%) than at low-gravity wort fermentation (LGF) (12%). Strong and high significant correlations were observed (Table S2). The elevated cell count at HGF might have led to increased sugar utilisation to gain energy. Figure S1 explains in detail the ATP gain from sugar metabolism by LAB. Moreover, no substantial change in wort gravity was observed after 48 h of fermentation (Table S4) because of a possible raised effect of organic acids on the measured value.

Effect of malt wort gravity on lactic acid and acetic acid content

Lactic and acetic acid were quantified to understand the implication of greater sugar consumption and viable count at HGF on product formation. Figure S1 explains the sugar fermentative behaviour of LAB to organic acids. HGF allowed significant conversion of sugars to lactic acid, regardless of LAB strains (Fig. 2a). When wort gravity was increased from 12% to 20%, lactic acid was 0.3- to 1.3-fold accumulated. Along with wort gravity, strong and significant correlation ($P < 0.05$) was observed (Table S2).

In contrast, there was neither a correlation nor a significant difference in the formation of acetic acid (Fig. 2b). Acetic acid formation in lactic acid fermentation is dependent on sugar metabolism in heterolactic fermentation, whereas in homolactic fermentation it is dependent on the availability of electron acceptors and acidic conditions LAB (Gänzle *et al.*, 2007) (Fig. S1).

Although lactic acid accretion is deleterious to LAB viability (Pieterse *et al.*, 2005), the viable count was still greater at HGF as compared to LGF. This strengthens the speculation on the contribution of higher amino acid content provided by HGW to LAB survivability in acidic stress.

Malt wort buffering capacity and pH value change at high-gravity fermentation

Usually, lactic acid production acidifies malt wort causing rapid pH drop during fermentation. pH inhibitory effect is greater at larger pH change (ΔpH) because lactic acid diffusion into cell is intensified (Pieterse *et al.*, 2005). It is unfavourable to cell growth and survival, and physiological activities (Hansen *et al.*, 2016). Thus, it is imperative to prevent significant and rapid pH change during malt wort

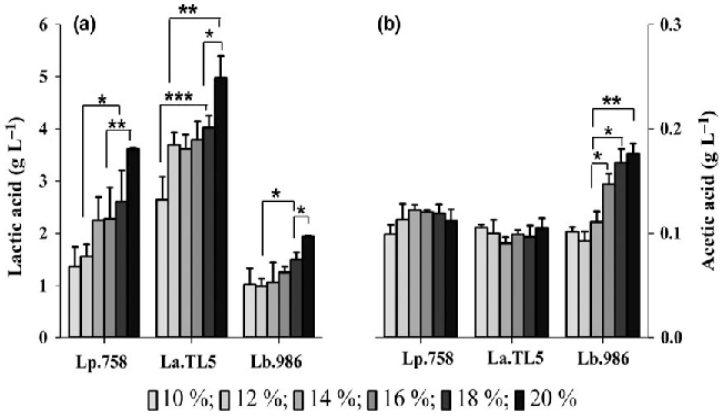

Figure 2 Lactic acid (a) and acetic acid (b) contents after 48-h malt wort fermentation at different wort gravity to up to 20% (w/w) by three lactic acid bacteria strains. Lp, *Lactobacillus plantarum*, La, *Lactobacillus amylolyticus*, Lb, *Lactobacillus brevis*. Vertical lines are SD. *Significant differences (*$P < 0.05$, **$P < 0.01$; ***$P < 0.001$).

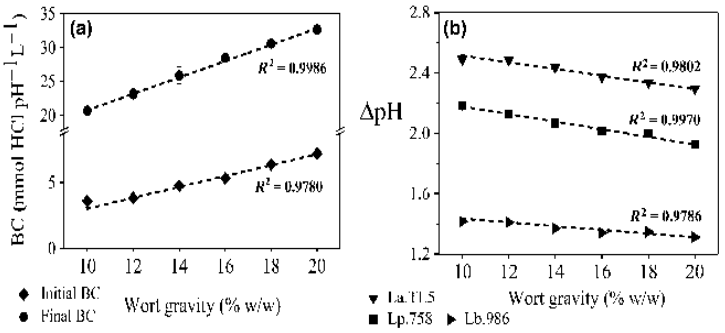

Figure 3 Effect of increasing malt wort gravity on (a) its buffering capacity (BC) and (b) the total pH change. R^2, Pearson's correlation coefficient; the values for final BC were considered for Lp.758.

fermentation for the sake of cell performance and metabolite formation. However, malt wort BC is low. Medium buffering with alkali may complex the fermentation process and lead to the loss of food naturalness. Also, malt wort pH buffering with buffer agents increased growth but the resulting high lactic acid concentration significantly reduced LAB viability (Nsogning Dongmo *et al.*, 2018).

The initial BC was twice elevated at HGW (7.6 mmol pH^{-1} L^{-1}) than at low-gravity wort (3.8 mmol pH^{-1} L^{-1}) when wort gravity was doubled from 12% to 20% (Fig. 3a); HGW might have provided more amphoteric substances such as proteins and phosphates in addition to amino acids that increased the BC. Furthermore, an increase in wort gravity by only 2% significantly ($P < 0.05$) lowered the total ΔpH for the three strains (Table S3). A strong negative linear correlation between wort gravity and the ΔpH in the fermentation end was observed (Fig. 3b). Indeed, greater ΔpH was expected at HGF instead as more lactic acid were produced. This indicates the tremendous contribution of high BC brought by HGW to reduce the pH drop. The time course of the pH drop during fermentation is presented in Fig. S3. The high BC in the fermentation end (Fig. 3a) is related to the substantial lactic acid content as described before (Nsogning Dongmo *et al.*, 2018).

Increase in the content of important aroma compounds at high-gravity fermentation

Whether the improved LAB fermentation performance at HGF and the greater amino acid content of HGW could contribute to increasing the aroma content was evaluated. Based on previous reports on key aroma compounds of LAFMB (Nsogning Dongmo *et al.*, 2017), acetaldehyde, β-damascenone, 2-phenylethanol and higher alcohols were considered.

Table 2 shows that increasing wort gravity significantly incremented the aroma concentrations ($P < 0.05$); strong positive correlations were observed (Table S2). An increase of up to 161%, 44%, 66% and 147% total higher alcohols, acetaldehyde, β-damascenone and 2-phenylethanol, respectively, was obtained at HGF (20%), depending on the fermenting strain, as compared to LGF (12%) (Table 2). Higher alcohols biosynthesis is directly associated with branched-chain amino acids catabolism via the Ehrlich pathway in lactic acid fermentation (Smid & Kleerebezem, 2014). Acetaldehyde is a product of pyruvate conversion under anaerobiosis by LAB in sugar metabolism pathway to gain energy for growth (Fig. S1).

Moreover, amino acid catabolism is also utlised by LAB to gain nitrogen in their growth metabolism. Therefore, it is assumed that higher alcohols, 2-phenylethanol and acetaldehyde are part of the growth-associated metabolism. Accordingly, differences in the aroma composition of nongrowing and growing LAB cells were reported (van de Bunt *et al.*, 2014). Amino acids utilisation by LAB in 14% (w/w) malt wort was already reported, where significant depletion to up to 98% was observed (Nsogning Dongmo *et al.*, 2018). This was

Table 2 Effect of increasing malt wort gravity (in %w/w) on the content of total higher alcohols (HA), acetaldehyde, 2-phenylethanol and β-damascenone after 48-h fermentation by three lactic acid bacteria strains

	Total HA (mg L^{-1})	Acetaldehyde (mg L^{-1})	2-phenylethanol (μg L^{-1})	β-damascenone (μg L^{-1})
Lp.758				
12%	1.2 ± 0.1^{a}	35.9 ± 3.1^{a}	40.9 ± 0.6^{a}	0.3 ± 0.0^{a}
16%	1.8 ± 0.1^{a}	41.0 ± 3.1^{ab}	53.2 ± 3.0^{ab}	0.4 ± 0.0^{ab}
20%	2.4 ± 0.2^{b}	46.5 ± 4.6^{b}	64.3 ± 7.6^{b}	0.5 ± 0.1^{b}
La.TL5				
12%	1.2 ± 0.2^{a}	1.1 ± 0.2^{a}	5.1 ± 0.9^{a}	0.5 ± 0.1^{a}
16%	1.3 ± 0.3^{a}	1.2 ± 0.0^{a}	6.5 ± 0.2^{a}	0.5 ± 0.1^{a}
20%	1.1 ± 0.1^{a}	1.4 ± 0.2^{a}	12.6 ± 0.1^{b}	0.7 ± 0.1^{b}
Lb.986				
12%	2.6 ± 0.2^{a}	1.6 ± 0.1^{a}	124.9 ± 5.3^{a}	0.4 ± 0.1^{a}
16%	4.8 ± 0.6^{b}	2.5 ± 0.2^{b}	126.6 ± 4.5^{a}	0.5 ± 0.0^{a}
20%	6.8 ± 0.6^{b}	2.3 ± 0.1^{b}	138.8 ± 14.0^{a}	0.4 ± 0.0^{a}

Lp, *Lactobacillus plantarum*, La, *Lactobacillus amylolyticus*, Lb, *Lactobacillus brevis*.

Data are means ± SD. Different letters in the same row indicate significant differences ($P < 0.05$).

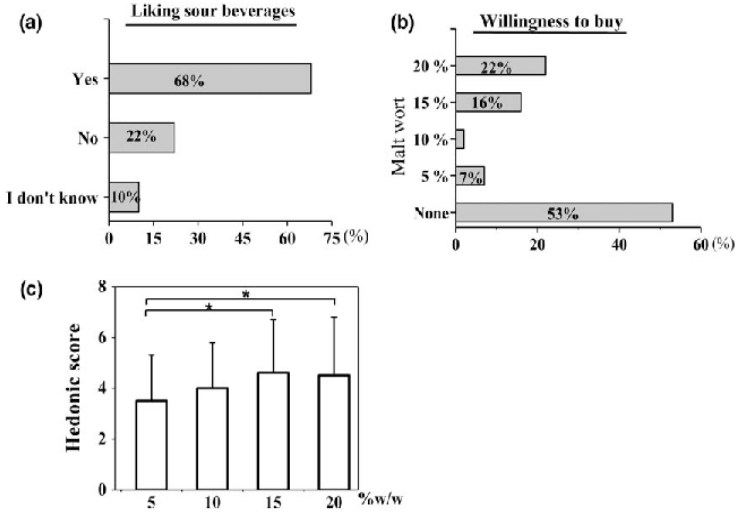

Figure 4 Sensory evaluation of the beverages produced from 5%, 10%, 15% and 20% (w/w) wort gravities for preferability (b), hedonic acceptability (c) and sour beverage liking (a). *Lactobacillus plantarum* Lp.758 was used for fermentation. Non-trained panellists performed the test (n = 80). Questions concerning the liking and the willingness to buy the beverage were as follows: (a) Do you like sour beverages? categories were 'yes', 'no' or 'I don't know', (b) which beverage (s) would you like to buy? panellists had also the possibility to choose none of the beverage, (c) nine-point hedonic scale ranging from 1 (dislike extremely) to 9 (like extremely) was used; *Are significant differences ($P < 0.05$).

solved in HGF by the greater content of amino acid (Fig. 1d). Acidic conditions of late-stage fermentation may have promoted the aroma formation. As per β-damascenone, its formation in malt wort from β-damascone is rather associated with low pH values (De Schutter *et al*., 2008).

The odour thresholds of 0.013, 25 and 140 μg L^{-1} in water, respectively, for β-damascenone, acetaldehyde and 2-phenylethanol (Czerny *et al*., 2008), were exceeded in HGF beverages. With odours of apple juice, green apple and rose-like, they might have positively contributed to the overall flavour individually or in combination.

High-gravity fermentation improves the sensory acceptance of lactic acid-fermented malt-based beverages

Food flavour is the first criteria for consumer choice (Azzurra & Paola, 2009). In acidic beverages, the sugar/acidity ratio is relevant for the harmony. Based on the greater aroma titre found in HGF beverages, we investigated on its contribution to consumer's acceptance and perception. In this regard, the fact that up to 68% of panellists (n = 80) liked sour beverages (Fig. 4a) validated their familiarity to the product and their frame reference for comparison. The beverage produced from 20% malt wort was the most preferred with 22% of panellists willing to buy it (Fig. 4b), in addition to the greater hedonic acceptability scores (4.6 ± 2.1) (Fig. 4c). So far, LAFMB was produced based on low concentrated cereal substrates of 6%, 12% or 14% malt wort.

Therefore, the greater aroma content at HGF may be of great contribution to the preeminent acceptance of HGF beverages. Furthermore, the greater sugar content of HGW has balanced the harmony through a masking effect on the elevated lactic acid content at HGF. That implies a subsequent dilution of the beverages would be ineffective on the sugar/acid ratio but further sensory tests will be supportive. Furthermore, 53% of panellists were not willing to buy any of the beverages (Fig. 4b) indicating the necessity to further enhance the acceptance of LAFMB.

Conclusion

Altogether, the mechanism of HGF to improve LAB performance in malt wort and consumer's acceptance is proposed. HGW provided greater amino acid content and BC; the combination with the consequent reduction in the pH change promoted LAB growth viability and, thus, the fermentation performance. Having greater viable cells in HGF mainly at the acid stress of late-stage fermentation was favourable to amino acid catabolism and pyruvate metabolism, which increased the content of aroma compounds associated with growth metabolism. Moreover, the greater sugar content of HGW masked the acidic taste of organic acids for a balanced harmony. The output was a better consumer's acceptance of HGF beverages. In this study, HGF successfully improved the consumer's acceptance of LAFMB, but further efforts to taking full advantage of HGF by LAB are still required.

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Conflict of interest

The authors declare that they have no competing interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Proposed malt wort sugar metabolism pathways in lactic acid bacteria showing the effect of anaerobic and aerobic conditions as well as electron acceptor on the product and energy yield.

Figure S2. Effect of ultra-high gravity fermentation on the viable cell count development of Lp.758.

Figure S3. pH drop during malt wort lactic acid fermentation at different gravities. a) *L. plantarum* Lp.758, b) *L. amylolyticus* La.TL5, c) *L. brevis* Lb.986.

Table S1. Malt wort fermentable sugars concentration (g L^{-1}) at different wort gravity (%w/w).

Table S2. Pearson correlation coefficient of fermentation parameters with wort gravity.

Table S3. Total pH value change after 48 h fermentation showing significant differences.

Table S4. Residual wort gravity after 48 h fermentation by three LAB strains at increasing wort gravity (%w/w). Lp, *L. plantarum*, La, *L. amylolyticus*, Lb, *L. brevis*. Data are presented as mean ± SD.

3. Discussion

In this dissertation, a process to design and improve the flavor of LAFMB was successfully developed based on brewing, fermentation and maturation processes. To reach that goal, six main strategies were appraised and presented partly in the previous embedded publications. Flavor, defined by the aroma composition, is the core aspect of product development. It was found in **Paper 1** that LAFMB flavor is poor, which causes poor consumer's acceptance and lack of market development (Nsogning Dongmo *et al,.* 2016). Furthermore, the organic acids content in LAFMB masks the flavor perception indicating the necessity of flavor enhancement. The nowadays general consumer quest for clean label and natural foods with less additives and preservatives make the use of flavorings not valuable. Here, the investigations for flavour improvement were based on natural processes. So far, little attention was given to the flavor of LAFMB, researches were focused on functional aspects instead. Neither an attempt for flavor improvement was done, nor has the aroma profile been extensively studied for an understanding of the poor aroma profile. The flavor of LAFMB was reviewed and perspectives for flavor improvement were proposed. Aroma characterisation, starter culture and substrate definition, and fermentation process optimization were found to be the main knowledge gap.

In that sense, this thesis opted to firstly investigate on the flavor profile of LAFMB prior to any attempt of flavor improvement. However, it was a priority to define the substrate and the fermenting strain to be used. In this regard, based on the literature data, barley malt wort was considered for the investigations of this study. Indeed, the high nutritional value and buffering capacity of pseudo-cereals may be of advantage to LAB growth, but according to the literature, malted barley was proven to support LAB growth and to deliver a more pleasant beverage as compared to wheat, emmer, and oat (Charalampopoulos *et al,.* 2003). Trials with oat, for instance, showed very less attractive sensory characteristics and poor processability as compared to barley (unpublished results). For these reasons, this study exclusively focussed on barley malt as a reference in order not to jeopardize in the investigations by considering other cereals.

In lactic acid fermentation of malt wort, LABs are the sole enzymes providers for aroma compound development comparing to other lactic acid fermentations, which consider multi-strain starter culture in addition to cereal enzymes. There, cereal naturally occurring enzymes are being denatured during brewing process step. LAB genetic and enzymatic background are complex and varying (van Kranenburg *et al,.* 2002). That is reflected not only in sugar metabolism enzymes but also on proteases, amino acid catabolic enzymes,

lipases and esterases, the core enzymes of aroma formation. The quality of fermenting LAB strain is therefore crucial for the aroma profile through the enzymatic package it brings along. For this reason, this thesis at first considered screening test to select strains of the investigations.

Microorganism screening is one of the most used techniques when it comes to strain selection for target compound production. Screening tests for aroma compound production is a difficult task due to the extensive steps involved in aroma analysis. Furthermore, the necessary use of high volume does not allow small-scale trials or the screening of large strains number at a time. By using Mass Spectrometry-based electronic nose and high throughput selection, a wide variety of LAB strains could be screened and differentiated according to their aroma-forming ability at small scale (Hugenschmidt *et al,.* 2010, Marilley *et al,.* 2004, Smit *et al,.* 2004). However, these studies were based on the identification of individual compounds whereby it did not provide substantial insights into the overall flavor, which is the final target. Though, it is the synergistic effect of aroma compounds, but not the individual effect, that confers the overall flavor. Furthermore, most of them considered synthetic medium, different to the original substrate, that may lead to result misinterpretations or difficult knowledge transfer.

The overall flavor of the finished product was considered during the screening test. Fruity, neutral or absence of off-flavors was considered as the selection criteria. A total of sixty LAB strains were screened based on their natural occurrence in cereal-based substrates: *L. plantarum* (10), *L. brevis* (22), *L. backii* (03), *L. corynifomis* (03), *L. casei* (03), *L. sanfrancicencis* (01), *P. pentosaceus* (02), *P. damnosus* (01), *L. collinoides* (01), *L. rossiae* (01), *L. delbrueckii* (02), *L. sakei* (01), *Leuconostoc oenos* (01), *P. acidilacti* (01), *L. parvus* (01), *L. pastorianus* (01), *L. paracasei* (01), *L. amylolyticus* (03), *L. sp*. (01), and *L. buchneri* (01). Based on the flavor, six strains subdivided into three groups of positive, neutral and negative control were selected for the investigations. The positive control strains of *L. plantarum* delivered the most pleasant flavor. Further, *L. brevis* was the typical negative control strains, which delivered a beverage with an unpleasant flavor. As per neutral control, *L. amylolyticus* delivered a neutral flavor instead.

Paper 2, describes the low concentration of key aroma compounds to be the main cause of the poor flavor of LAFMB (Nsogning Dongmo *et al,.* 2017). Four different methods including solvent assisted flavor evaporation (SAFE), solid phase-micro-extraction (SPME), distillation and direct headspace sampling was used for aroma compound extraction. The SAFE method allowed the extraction of temperature sensitive aroma

compounds because of the mild temperature of 36°C employed (Engel *et al,.* 1999). However, because this method is based on the evaporation of volatiles, high molecular compounds are not easily extracted. For this reason, distillation at 100°C was used for their extraction. Furthermore, direct head sampling was used for the extraction of highly volatile compounds such as acetaldehyde and higher alcohols. A total of fifty aroma compounds could be identified based on their spectral mass, retention index and odor quality by Gas Chromatography-Mass Spectrometry (GC-MS-O). Among them, several aroma compounds were identified for the first time in cereal-based beverages of LAF, particularly, furaneol, sotolon, 4-vinylguaiacol, linalool, geraniol and ethyl 4-methylpentanoate. Furthermore, it was found that LAB strain does not affect significantly the aroma composition.

Only two studies considered the aroma composition of LAFCB (Coda *et al,.* 2011, Salmeron *et al,.* 2009). However, attempt to define the key aroma compounds that defined the flavor of LAFCB was not done so far. Flavor dilution analysis on GC-MS-O and odor activity calculation based on internal calibration and analysis on Gas Chromatography coupled to a Flame Ionization Detection helped to define a total of thirteen key aroma compounds of LAFMB. Flavor dilution factors (FD) higher than 16 was considered (see Table 2, Paper 2). The higher the FD factor, the more the aroma compound contributes to the flavor. β-damascenone, furaneol, phenylacetic acid, 2-phenylethanol, vanillin, nor-furaneol, ethyl 2-methylbutanoate, and acetaldehyde were the positive contributors to the final flavor because of their high FD factors and pleasant odor qualities. However, the most considered aroma compounds of LAFCB so far were acetone, acetoin, diacetyl, and acetaldehyde, which are key aroma compounds of yogurt instead (Cheng 2010).

Not only aroma compound derived from amino acids, carbohydrate metabolism or fatty acids were found but also those from phenolic acids, carotenoids, and glycosides degradation. That involves other enzymatic and chemical reactions than well-known ones of LAF as previously reported in Fig. 1 (Paper 1). Furthermore, very few of these defined key aroma compounds are derived from amino acids catabolism. In this study, aroma compound concentration, but not the composition, was found to be strongly related to the starter culture. Limitations in aroma formation ability by LAB were reported in section 1.1: insufficiency of important enzymes aminotransferase and glutamate dehydrogenase, low amino acid uptake, low intracellular pool of precursors NAD/NADH ratio, α-ketoglutarate and pyridoxal-5-phosphate, lactic acid accumulation, and oxygen availability (Vermeulen *et al,.* 2006, Kunji *et al,.* 1996). Moreover, the small size of LAB cells (0.87-1.21 μm) may

not allow significant enzymatic conversion of aroma compounds as compared to bigger size cells such as yeast (3-4 μm) (Kokkinosa *et al,.* 1998). Likewise, cell size, volume and surface area were reported to affect the enzymatic activity (Weiss *et al,.* 1975).

Esters are important for the fruity odor they impart to the final flavor. They are formed from the esterification of ethanol and activated fatty acyl-CoA. Acyl-CoA and alcohol acyltransferases are the main esterase in LAB (Costello *et al,.* 2013). Ethanol addition (5% abv.) in MRS broth significantly favored ester formation although low pH value (3.5) was not favorable (Costello *et al,.* 2013). Ethanol is, therefore, one major precursor that is not present in malt wort and should be synthesized during fermentation. A maximum of 0.1 %abv. was found in LAFMB (see Table 3, Paper 2). Esters ethyl acetate, ethyl 2/3-methylbutanoate, and ethyl 4-methylpentanoate were found in LAFMB but at low concentration. Low ethanol content and low pH value of LAFMB might have therefore hindered ester formation.

Furthermore, based on the literature data, branched chain amino acids leucine, isoleucine, and valine are the amino acids implicated in key aroma compounds formation. Some authors reported leucine and isoleucine to be the most involved amino acid in esterification (Hazelwood *et al,.* 2008, Smit *et al,.* 2005). Except for leucine whose concentration lowered significantly (Paper 3, Fig. 5), branched chain amino acids were not limiting at the fermentation end. That would mean the low ester content would be less a limitation in amino acids only if the speculations about the combined contribution of amino acids to the aroma formation (Vermeulen *et al,.* 2006) find here a validation.

As per acetaldehyde formation, 90% in dairy fermentation are through pyruvate (Fig. 4) whereas very less are produced from threonine by threonine aldolase (Ott *et al,.* 2000). Furthermore, the formation of acetaldehyde via citrate metabolism from the conversion of acetaldehyde-TPP was speculated (Hugenholtz 1993, Le Bars and Yvon 2008, Beresford 2011). In this thesis, the inverse proportionality between acetaldehyde and ethanol content (Table 3, Paper 2) led to suggest that glycolysis is also the main acetaldehyde formation route in malt wort. However, it is to consider that this conversion is strongly inhibited by oxygen and dependent on medium pH (Lees and Jago 1976). These reports clearly explain then the significant increase in the acetaldehyde content at acid stress as seen in Fig. S1 (Paper 2). Contrary to cheese aroma profile, other aldehydes were not found in LAFMB, although many aldehydes are reported in malt wort. They seemed to have been reduced during fermentation to alcohols.

Unexpectedly, diacetyl, one of the major compounds of lactic acid fermentation was not of relevance in LAFMB because of very low FD factor. It was detected mainly when the facultative heterofermentative strains *L. plantarum* was applied for fermentation. Diacetyl formation by *L. plantarum* has been evidenced in beer whereas no production was evidenced in malt wort during biological acid production by *L. amylolyticus* (Vriesekoop *et al,.* 2012). The formation pathway of diacetyl is well described in Fig. 1 (Paper 1).

Terpenes, linalool and geraniol were found in this study although at low concentrations in LAFMB. So far, they have been only associated with hops and hop-derived products. In this study, an unhopped malt wort was used and both compounds were not detected in malt wort demonstrating their occurrence during LAF. Further, the formation of terpenes and vanillin from glycosides precursors by LAB was already evidenced in wine fermentation (Hernandez-Orte *et al,.* 2009).

The most pleasant beverage of this study was produced by *L. plantarum* Lp.758. It was more described with positive sensory attributes such as fruity, apple-like, pear-like, honey-like and sweet-like. These descriptors can be used as the basis for defining the sensory evaluation schema of LAFMB in the future. According to Fig. 1, *L. plantarum* has been the strain of choice so far in LAFCB production. Further, a comparison of various LAB strains in oat fermentation revealed a strain of *L. plantarum* to deliver a product with better sensory properties as compared to other strains (Luana *et al,.* 2014). Also, *L. plantarum* is widely used in lactic acid fermentation of cheese as adjunct culture for flavor development at the maturation phase (Gobbetti *et al,.* 2015).

Based on its ability to deliver a higher amount of key aroma compounds, which resulted in the fruity and more pleasant beverage, *L. plantarum* Lp.758 was suggested as the suitable starter culture for LAFCB production. Further studies on the functionalities of Lp.758 such as probiotic, bioactive compounds and bacteriocins delivery will fully complete the use of this strain as a starter culture for LAFMB. *L. plantarum* is used as preferred strain in the traditional production process of cereal-based beverages and malt production. It has not been reported so far to have pathogenic properties and can, therefore, be attributed the QPS status according to the EFSA requirements (EFSA *et al,.* 2017). Therefore, *L. plantarum* Lp.758 strain could be considered as basic starter culture of LAFMB without any previous assessment.

However, consociation with a functional strain for a multi-strain starter culture definition may help to bring together in the resulting beverage, the flavor, and functional asset. This approach is already under consideration in many fermentation systems. In wine, for

example, yeast is the major contributor of the aroma whereas LABs are employed for the primary role of deacidification although it contributes to the flavor formation as well. The same applies to sourdough fermentation. In this regard, a tailored made strain consociation based on their proteolytic and transaminase activity proved to strongly enhance the aroma formation in lactic acid fermentation (Ayad *et al,.* 2001).
Globally, the concentration of aroma compounds in LAFMB was low. Understanding the fermentation performance of LAB in malt wort may give insight into the explanation of low aroma concentration in LAFCB.

Paper 3 describes early and prompt cell death as well as the degradation of metabolic activity as one of the major problem of the low aroma yield in LAFMB (Nsogning Dongmo *et al,.* 2018b). As fermentation proceeded, the number of death cell increased. Dead cell proportion during fermentation was determined under the fluorescence microscope after labeling with Propidium Iodide (PI) and Carboxyfluorescein Diacetate (CFDA) probes. Only cells with damaged membrane fluidity could absorb the PI. Damaged membrane cells include dead cells and viable but not culturable cells (VNC).

VNC cells can, however, recover their viability and still undergo enzymatic activities as observed with LAB in wine, beer and cheese under favorable conditions such as high pH value or oxygen availability (Millet and Lonvaud-Funel, 2000, Suzuki et al., 2006, Gobbetti et al., 2015). In this regard, cells of *L. amylolyticus* La.TL5/TL3 from 72h fermentation that did not grow on MRS agar could grow in MRS broth. This indicates that VNC can still hydrolyse fluorescent esters to be counted as viable with fluorescence techniques causing a discrepancy between the fluorescence count, CFU and the death rate.

Further, as the death rate is expressed as a percentage, it considers and evaluates the number of dead cells against the total cell count at each time in the medium whereas the CFU does measure only the remaining viable cell count at that same period. Therefore, the continuous increase of the death rate even at the exponential growth phase leads to suggest that during fermentation, as viable cells were multiplying, a proportion (non-resistant cells) was continuously dying and been accumulated. The impact was the reduction of the real number of viable cells but the increase in the number of dead cells and total cell count at each time. This behavior is proposed and well described in Fig. 9. Indeed, it emerges that the observed attainment of the death phase in these results or the observed stationary phase in most LAB growth curves in the literature, does not necessarily mean growth or fermentation cessation but that cell growth and cell death are

occurring simultaneously, and the fate is dependent on the proportion of death cells. These explanations broadly give a sense to the continuous increase in the death rate and its disproportionality with the CFU. The observed lactic acid production at the death phase (Fig. 3a, Paper 3) demonstrates the ability of damaged VNC cells and resistant viable cells that still could undergo enzymatic activities. Similarly, low lactate dehydrogenase activity was observed in membrane damaged *L. plantarum* cell during cheese ripening (Gobbetti *et al,.* 2015).

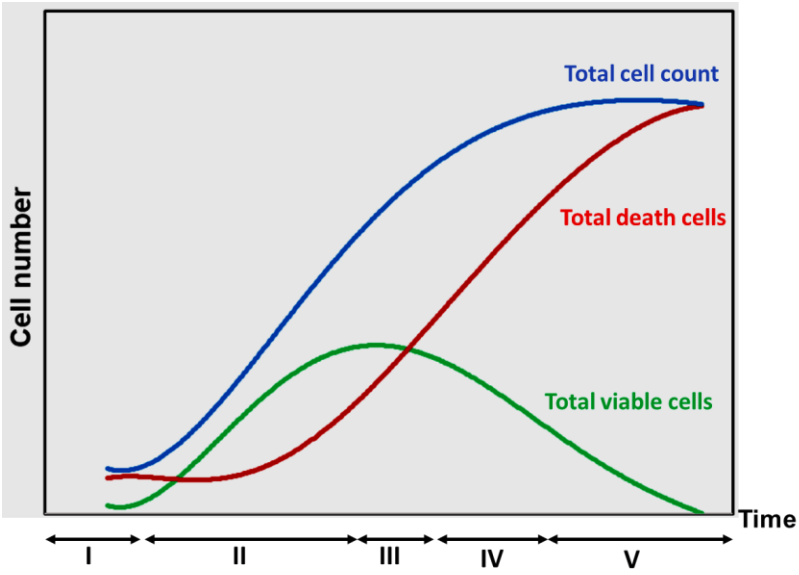

Figure 9. Proposed change behavior in viable, dead and total LAB cell count during barley malt wort fermentation based on the findings of this thesis. I: lag phase, II: log phase, III: deceleration phase, IV: accelerated death phase, V: decelerated death phase.

When cells were closely observed under the microscope, agglomeration-like flocculation of PI red-labeled and filamented cells were observed (Fig. 10) at late-stage fermentation (72h). That was peculiar to strains *L. amylolyticus* TL5/TL3 and *L. plantarum* Lp.765. These strains showed the higher and rapid dead cell percentage in addition to lowest end pH values. Elongated or filamented LAB cells were observed in *L. plantarum* under acidic conditions as well (Ingham *et al,.* 2008). However, this is the first report on agglomeration-like flocculation for LAB cells. Low pH value was reported to cause cell division failure, without obvious septation leading to cell filamentation. Those filamented cells had rougher cell surface but were still culturable (Ingham *et al,.* 2008). This agglomeration might have led to miscounting dead cells under the microscope creating the discrepancy in the death rate and the CFU value.

This thesis shows for the first time that LAB under acidic stress may also flocculate. The mechanism of formation of agglomerate-like flocculation still to be determined. Speculations can be the damaged and rougher cell surface that allows a binding of different cells surface components together to form an agglomeration of cells.

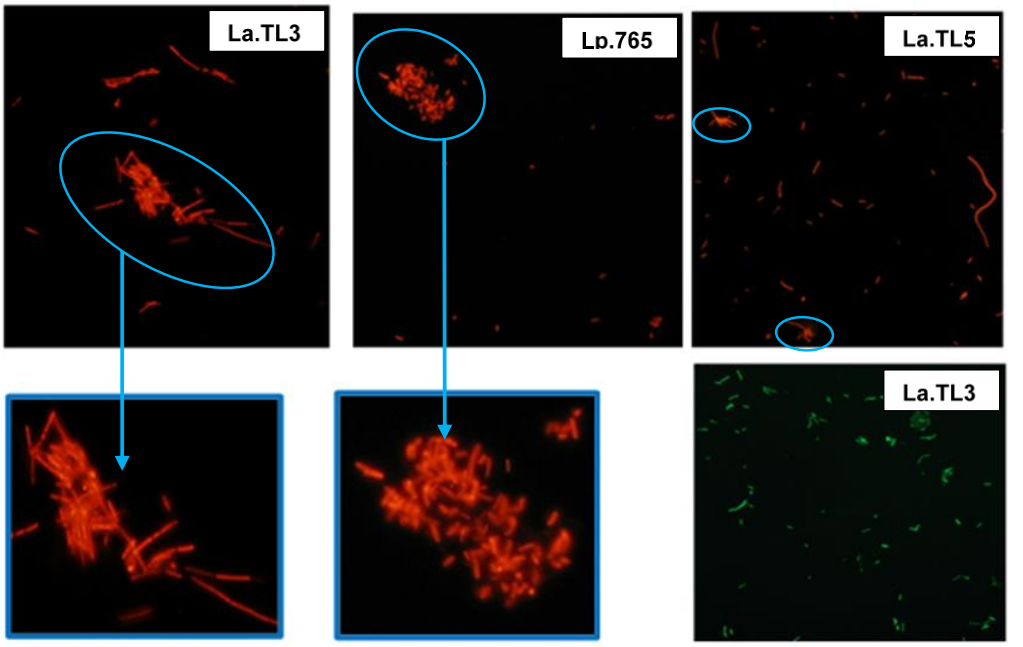

Figure 10. Fluorescence microscopy images showing flocculation of *L. amylolyticus* La.TL3/TL5 and *L. plantarum* Lp.765 at 72h fermentation after labeling with Propidium Iodide (red) as compared to *L. amylolyticus* La.TL3 at 24h fermentation after labeled with Carboxyfluorescein Diacetate (green).

The significant cell death was related to the depletion of key amino acids, low buffering capacity and the accumulation of lactic acid in malt wort fermentation (Nsogning Dongmo *et al,.* 2018b). Using malt wort instead of other cereal extracts has the prime advantage that the mashing process contributes to enriching the medium with amino acids through the activity of malt endopeptidases. However, high temperature that is employed destroys these enzymes afterward making them unavailable for further amino acid provision during fermentation. Also, mashing process reduces malt wort protein content through lautering and boiling step. Comparing with lactic acid fermentation of yogurt, milk is a very rich protein medium and LAB starter culture are naturally rich in casein proteases, which may explain why the problem of amino acid limitation was not questioned so far. Also, when comparing with other cereal fermentation such as sourdough fermentation, for instance, amino acid limitation was not questioned so far because yeast proteases and cereal endopeptidases increase the amino acids content, which in turn improves LAB

fermentation performance (Gänzle 2014). For example, yeasted dough had twice more free amino acids than non-yeasted dough (Collar *et al,.* 1992, El-Dash and Johnson 1970). The significant contribution of yeast was further proved in 5% barley malt, where yeast addition increased the FAN value during fermentation thereby improving *L. reuteri* growth as compared to non-yeasted malt (Kedia *et al,.* 2007). Therefore, consociation of yeast with LAB for fermentation in barley malt-based beverages is a viable approach to cope with the low amount of amino acids. This may have an additional advantage of providing more ethanol for ester formation, as mentioned earlier in this section.

Increasing the amino acid content should be considered as whole because of their combined contribution to the aroma formation. Peptide addition for example in fermentation medium increased the conversion of phenylalanine to phenylacetic by 2 - 4 fold more than single amino acid addition (Vermeulen *et al,.* 2006). Thus, increasing only the content of key amino acids may seem incomplete or not optimal when the aim is to improve the aroma yield in LAFMB.

Amino acids conversion to aroma compounds by LAB is regulated by the gene expression level. The gene expression in lactic acid fermentation varies depending on the fermentation stage. Under acid stress conditions, the level is increased; an up to 4-fold increase in the expression level of genes kivD (encoding an α-keto acid decarboxylase), araT (encoding aromatic amino acid) and bcaT (encoding aromatic and branched chain amino acid) at the late stationary phase was reported (Rijnen *et al,.* 2003). At the stationary phase, however, a significant cell death was found in this study, whereas leucine and branched chain amino acids isoleucine and valine were limited, explaining the low aroma yield.

Upon supplementation of key amino acids individually and in the mixture, improvement in cell yield and viability was observed (Fig. 6, Paper 3). This reveals the significance of amino acids to LAB growth in malt wort fermentation. Also, under acidic conditions, LABs utilise amino acids as an energy source in the presence of carbohydrates by switching from carbohydrates to amino acid metabolism (Jónsson *et al,.* 1983, Serrazanetti *et al,.* 2011). Further, amino acids are not only utilised as a nitrogen source by LAB to grow but also for acidic stress resistance (Zuljan *et al,.* 2016). Concerned amino acids are exactly those reported in this study to be limiting in LAFMB. Arginine seems the most important; it is metabolised through the ADI (arginine deiminase) pathway and is coupled to ATP and ammonium production, which could contribute to acidic tolerance by providing extra energy

and pH increase. Accordingly, VNC *Oe. oeni* cells were converted to culturable cells in medium supplemented with arginine (Tonon and Lonvaud-Funel 2000).

However, significant cell death and stationary phase attainment were still observed when amino acids were supplemented (Fig. 8, Paper 3) although significant residual high amino acid content (Fig. 11). This discards amino acid limitation to be the sole limiting factor and describes an additional unknown effect, here speculated to be lactic acid content and the consequent low pH value. Then, amino acid supplementation could reduce, but not repeal, lactic acid inhibition. Accordingly, evolutive growth adaptation of *L. plantarum* Lp.758 from 1.7 to 5.0 g/L lactic acid was successful but did not increase significantly LAB viability. This shows that amino acid limitation remains the main concern. Lactic acid in this study was much higher than the concentration of 1.7 g/L, which completely inhibited LAB growth in malt wort in a parallel study. From a concentration of 0.3 g/L, lactic acid exerted already a growth inhibition effect on *L. plantarum* (unpublished results). The accumulation of lactic acid might have therefore caused cell death in coordination with amino acids limitation during malt wort fermentation. Thus, it was relevant to consider lowering lactic acid production during malt wort fermentation. The hypothesis that the combination of amino acid increase with high buffering capacity and lactic acid reduction may bring significant improvement to LAB viability and aroma compound yield was then tested.

In Paper 4, high-gravity fermentation (HGF) provided more amino acids (Fig. 1e, Paper 4) and higher buffering capacity (Fig. 3a, Paper 4) (Nsogning Dongmo *et al,.* 2018a). These conditions improved LAB fermentation performance in malt wort (Fig. 1, Paper 4). The difference was particularly significant at the late-stage fermentation when compared to low gravity fermentation (LGF). The presence of more amino acid at HGF did not only allow cells to continue growing but also provided better resistance to acidic stress reducing cell death to increase the viable count. Lactic acid toxicity is more deleterious as its concentration increased. Higher lactic acid content was found at HGF but instead, higher viable cell count was obtained. Lactic acid dissociates at pH value near to its pKa value of 3.85 to its protonated form. The low pH change at HGF from high buffering capacity (Table S3, Paper 4) could have favored the conversion of the toxic undissociated lactic acid to its conjugated form to reduce lactic acid toxicity.

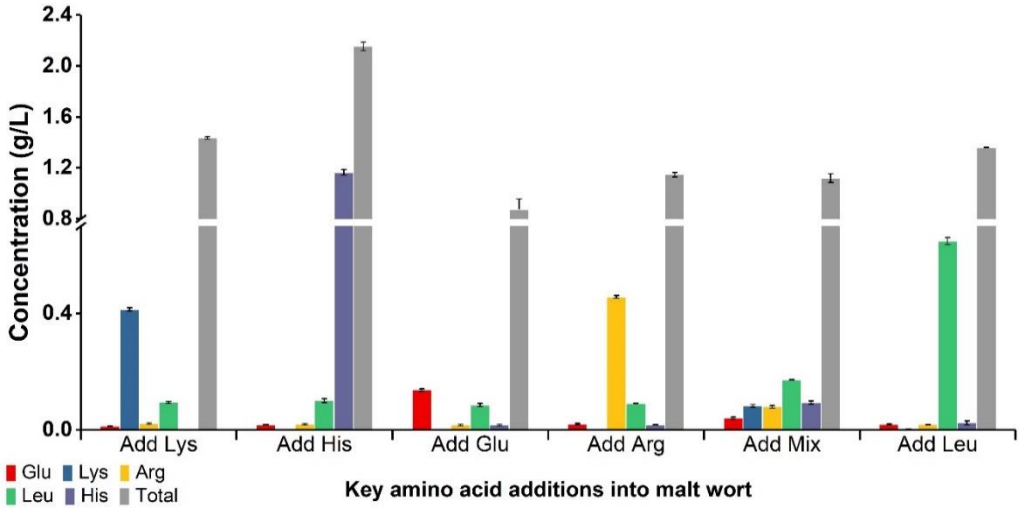

Figure 11. Residual amino acid content in barley malt worts spiked with the same concentration of key amino acids after 72h fermentation with *L. plantarum* Lp.758. Add: addition; Arg: arginine; Lys: lysine; Leu: leucine; His: histidine; Glu: glutamic acid

Furthermore, HGF significantly increased the aroma content (Table 2, Paper 4), which led to the improved acceptance of LAFMB in HGF (Fig. 4, Paper 4). This demonstrates that increasing the amino acids content significantly improves the aroma formation in LAFMB. It was found that although HGF brought significant improvement, the acceptance of LAFMB remained to be improved. In this regard, ultra-HGF was investigated but discarded because of the extended lag phase and the low viability observed (Fig. S2. Paper 4). Further, change in cell morphology was observed under the microscope (data not shown) from 25 %w/w, which was attributed to the high osmolality of ultra-high-gravity wort. It is well-known that LABs are osmosensitive (Kashket 1987).

Further, it was hypothesized that extending LAB viability in malt wort fermentation may significantly increase the aroma yield and thereby improve the flavor and the acceptance of LAFMB. In this regard, the approaches tested here were i) optimization of malt wort amino acid content, ii) slow and continuous provision of amino acid to LAB during fermentation, and iii) fermentation temperature reduction.

Previous studies optimized malt wort composition for the production of functional LAFCB (Muñoz-Insa *et al,.* 2011, Zarnkow *et al,.* 2010, Zarnkow *et al,.* 2007). However, they were based on yeast nutritional requirements and did not consider LAB nutritional requirements and the end-product quality. Here, based on the mashing regime described in Fig. 7, the extension of mashing protein rest from 30 min to 75 min at 52°C increased the amino acid content by 48.5 % (Fig. 12b). A sigmoidal increase was observed in the total free amino nitrogen (FAN) and amino acid content (Fig. 12). Jones and Budde reported the inhibition

of malt endopeptidases to reduce wort soluble protein content (Jones and Budde 2005). Then, it was assumed that the extension of their activity could favor amino acid liberation as observed in this study. That was achieved through the combination of thick mashing-in with protein rest time extension to promote malt protein solubilisation for optimum malt endopeptidase activity. Wort soluble proteins (Eq. 2) are hydrolysed to FAN and free amino acids through the action of malt endopeptidases.

However, enzyme activities decrease by half under optimum conditions after exposition at a determined time called half-life. Therefore, under extended exposition at these conditions, the activity continuously decreases until a complete deactivation. Further, as proteinases are also proteins, a possible cannibalism action by a self-reverse action of endopeptidases on themselves may have occurred at extended exposition explaining the low values at 105 min extension time. The increase in the total amino acids is rather positive for the aroma formation than an individual increase according to the previous speculations about the combined contribution of amino acids. However, excess amino acid content may cause off-flavors formation or bitter taste.

$$Total\ soluble\ protein = \sum(HM\ protein + MM\ protein + LM\ protein + FAN) \quad \text{Eq. 2}$$

HM: high molecular; MM: middle molecular, LM: Low molecular, FAN, free amino nitrogen.

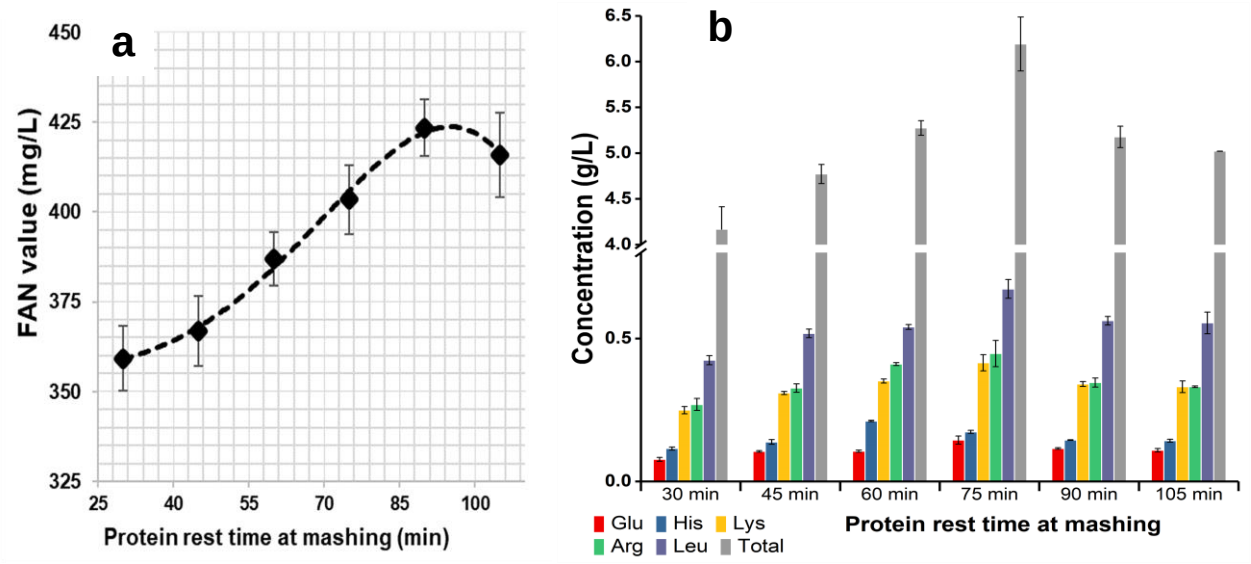

Figure 12. Effect of extending protein rest time from 30 to 105 min during mashing on the content of FAN (a), and key and total amino acids (b) in 20 %w/w barley malt wort.

Additionally, the buffering capacity being of concern in this study, extending the protein rest time also affects phosphatases, which optimum temperature is around 52°C. Phosphatase liberates phosphate ions from phytic acid. It is speculated that phosphate

ions increase the buffering capacity of malt wort although other authors do not agree with the contribution of phosphate to malt wort buffering capacity (Li *et al,.* 2016). The change in the phosphate content in malt worts obtained from different protein rest times showed proportionality with the FAN value (unpublished results). Then, the buffering capacity was increased with extended protein rest but remained proportional to the FAN value.

Further, the aim here was to gain as much as possible nitrogenous content. Boiling step of wort precipitates proteins reducing the final FAN value but the eventuality of skipping the boiling step to gain in proteins would not be of benefit for sterilisation purpose. In this thesis, the contamination of wort by *Bacillus* spore germination was of serious concern for fermentation process control and the stale flavor its impart to the beverage. *Bacillus* spp. are naturally occurring in cereals, they resist the boiling step in brewing to be found in malt wort in spore form, which requires very high temperature to be inactivated. However, because high temperature denatures proteins and complex amino acids to sugars, the approach of using high LAB inoculum to rapidly acidify wort thereby inhibiting the early germination of spores was considered instead. An inoculum of 100 Mio. cells/mL could inhibit successfully the development of spores by prompt and rapid medium acidification. Likewise, *L. plantarum* preparation was found effective against *Bacillus subtilis* in a previous study (Valerio *et al,.* 2008).

Furthermore, the imperative prompt acidification step is, however, not favorable to cell viability because of the toxicity of high lactic acid on LAB viability. Thus, focus on the process that may allow slow amino acids utilisation and slow production of lactic acid to maintain cell viability was achieved through a combination of fed-batch fermentation and fermentation temperature reduction from 28°C to 18°C.

That was based on the principle of the Q^{10} rule (van't Hoff-rule) on the temperature sensitivity of enzymatic reaction rate. It stipulates that for a Q^{10} value of 2.0, for most enzymes, a temperature rise by 10°C lead to a double in the reaction rate (Purich, 2010). For instance, more than twice higher lactate dehydrogenase activity was observed at 45°C as compared to 30°C fermentation that led to higher lactic acid production (Medina de Figueroa *et al,.* 2001). Inversely, that should be true for double reduction of reaction rate by 10°C temperature reduction. So, the fermentation rate should slow down at low temperature thereby reducing product yield.

It was found that medium feeding during fermentation increases significantly LAB viability under low pH value. Thus, a combination of fed-batch fermentation with temperature reduction achieved to maintain cells viability at extended fermentation duration from 3 to

up to ten days (Fig. 13a). The principle idea was a continuous amino acid provision for LAB growth through medium feeding to avoid substrate limitation combined with mild fermentation temperature to reduce reaction rate and lactic acid production. In standard fed-batch fermentation with variable volume as considered here, the biomass concentration and growth rate are constant (Stanbury *et al,.* 1995). In this study instead, a decrease in the viable count was observed probably due to the lactic acid toxicity.

It is noteworthy that fermentation temperature reduction to 18°C considerably decreased lactic acid production as observed in Fig. 13b as compared to 28°C fermentation. This would have reduced lactic acid toxicity and therefore cell death reduction to maintain high cell viability as compared to 28°C fermentation (Fig. 13a). Then, the issue of lactic acid toxicity as a limiting factor of LAB viability in malt wort was reduced. Low lactic acid production is not only favorable to LAB viability but also to balance the sensory harmony of the beverages. Furthermore, after microbiological investigations, the inhibition of naturally occurring *Bacillus* spores was still achieved at low-temperature fermentation as well although lactic acid production was lower.

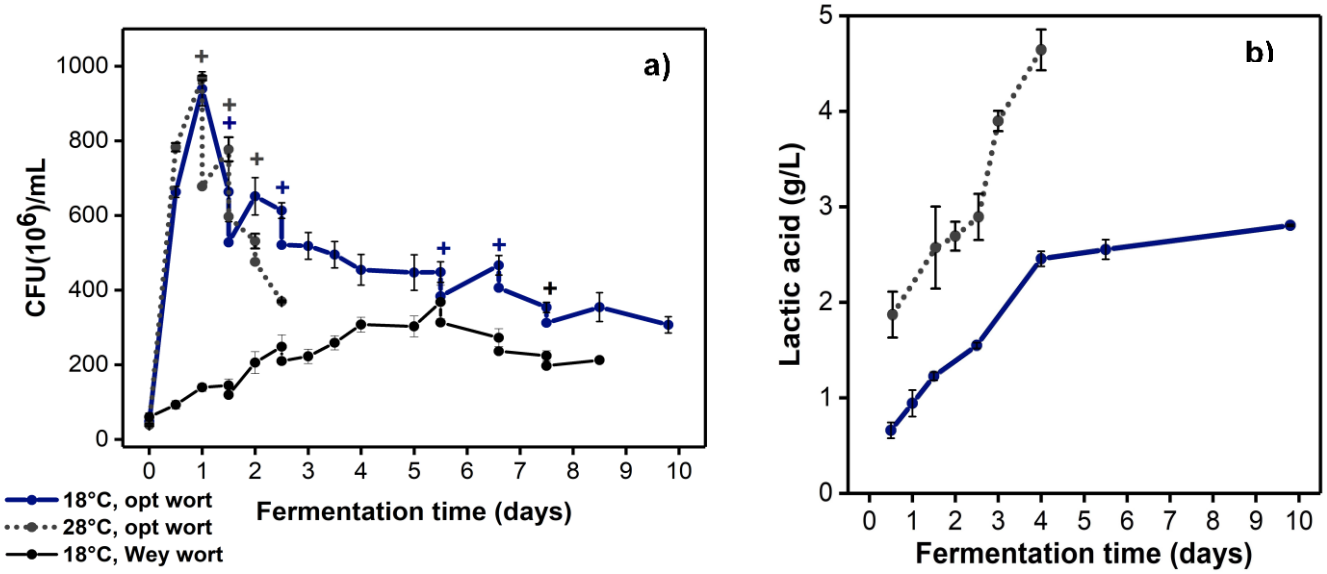

Figure 13. Growth behaviour of Lp.758 in optimized barley malt wort during combined fed-batch (at pH 3.5) and low temperature fermentation at 18°C as compared to 28°C and standard Weyermann malt wort (a) and consequent change in lactic acid content (b); opt wort: optimized wort; Wey wort: standard Weyermann malt wort; + time of wort feeding.

LAB viability extension obtained from these results is an important achievement to the set goal of this thesis when comparing with the drastic and rapid cell death observed earlier in

non-optimized fermentation. In sourdough fermentation, for instance, higher metabolites concentrations were produced upon the prolongation of fermentation duration (Ravyts and De Vuyst 2011).

Significant differences were observed in the aroma content between prolonged fermentation at 18°C and rapid fermentation at 28°C (Fig. 14). Esters content specifically was greater at 18°C fermentation. Furthermore, maturation of the resulting beverages was considered for aroma development. Under predefined forced-ageing conditions of 35°C for 31h, maturation revealed to significantly contribute to the aroma formation in LAFMB, regardless of the fermentation temperature. There, an increase in the key aroma content was observed (Fig. 14). Most importantly, there was a significant increase in the ester content of beverages upon force-ageing (Fig. 14b) whereby fermentation temperature reduction to 18°C significantly favored ester formation during maturation. Consequently, maturation delivered a fruity beverage to fulfil consumer's request for fruitiness in LAFCB. Furthermore, it was observed that changing a single ageing parameter significantly determined the flavor leading to beverages with a distinct and unique flavor. Further, the observed relevant contribution of LAB cells in the flavor development during forced ageing may derive from an uncontrolled liberation of enzymes from dead cells or the increased enzymatic reaction rate at high temperature. An earlier study demonstrated differences in the aroma composition of non-growing cells and those of living and growing cells of *Lc. lactis*, whereby non-growing cells degraded amino acids faster than the growing cells (van de Bunt *et al,.* 2014). In addition, it was suggested that the formation of aroma compounds during ageing was not stable but continuously and rapidly changing, which was reflected in the flavor. For these reasons, important research focus on the optimization of the ageing process of LAFMB will bring out very promising results. Then, this thesis proved for the first time that the maturation process is necessary for the flavor development in LAFCB.

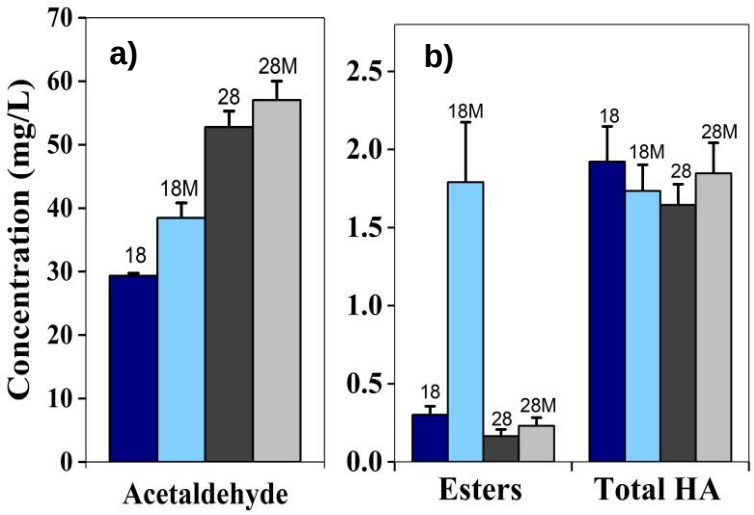

Figure 14. Increase in the acetaldehyde (a), total esters and higher alcohols (b) content upon maturation as compared to fresh beverages from 18°C and 28°C fermentation temperatures. 18M, beverage fermented at 18°C and force-aged; 28M, beverages fermented at 28°C and force-aged. Force-ageing was done at 35°C for 31h, esters are ethyl acetate and ethyl 2/3-methylbutanoate.

4. Conclusions

In LAFCB, the sensory aspect takes precedence over functional aspect. The problem outlined in this thesis is the poor flavor of LAFCB, which need to be improved. Therefore the main objective was to develop a process for flavor improvement of LAFCB focusing on existing processes brewing, fermentation, and maturation. This thesis has been based on six hypotheses, which structured and focussed on answering one research question on "How to improve and design the flavor of LAFCB by using existing processes and technologies?". The main findings led to the development of the process proposed in Fig. 15.

Specifically, the main findings are :

- The poor flavor of LAFMB is the main problem for consumer's acceptance and market development and remained understudied.
- The low concentration of key aroma compounds causes a poor flavor profile.
- The strain of *L. plantarum* Lp.758 was proposed as a starter culture for LAFMB production.
- Limitation in key amino acids, low buffering capacity and lactic acid accumulation cause poor fermentation performance of LAB in malt wort, which in turn are unfavorable to aroma formation.
- High-gravity fermentation proved to improve the fermentation performance through its high amino acid content and buffering capacity.
- Mashing process customization by protein rest time extension increased the amino acid content by 48.5%, and the buffering capacity.
- Combination of fed-batch and fermentation temperature reduction to 18°C significantly maintain LAB viability to up to ten days and significantly lowered lactic acid production.
- Maturation of the resulting beverages led to significant increase in esters and aroma compounds. Finally, maturation was the main flavor development phase in LAFMB.

Thus, the results of this study show that the flavor of LAFCB can be improved and designed following the proposed process in Fig. 15. The process is simple and cost-efficient, where the development of a new concept of equipment is not necessary, the energy use is reduced through HGF and reduced temperature fermentation. Finally, this study enhances the market potential of LAFMB for brewing and beverage industries.

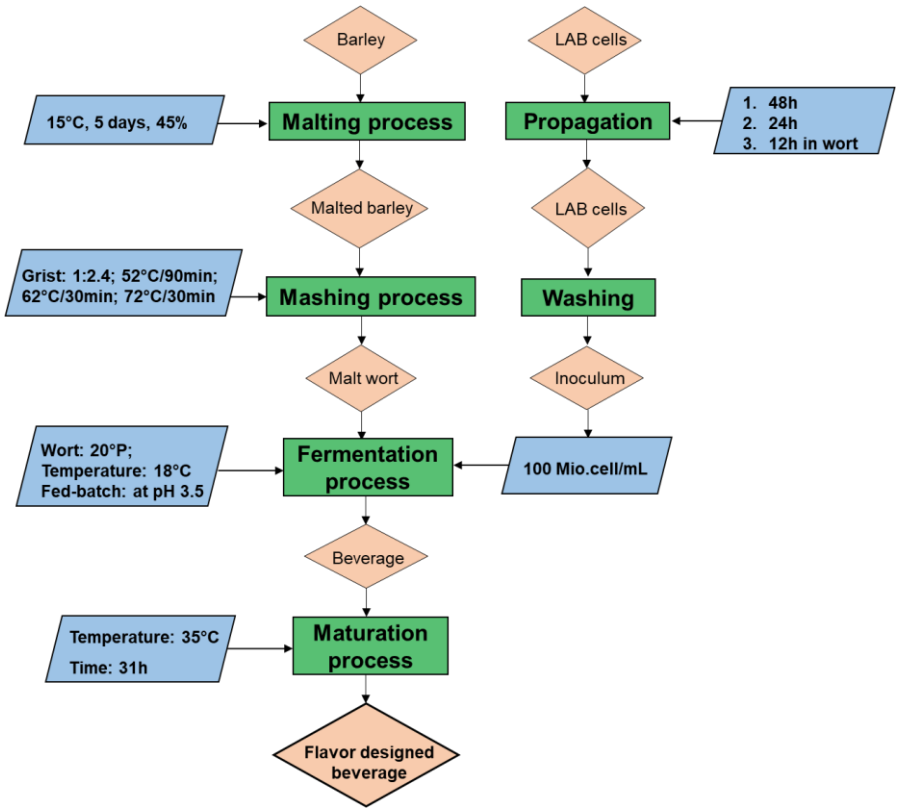

Figure 15. The developed and proposed process to produce LAFMB with improved flavor.

Implications of the study for practice and research

The main finding of this study responds to the study's research question and verifies the stated hypotheses, which helped to achieve the goal to develop a process for flavor improvement of LAFMB based on existing process and technologies. These findings have therefore far-reaching implications:

1. It was found that the low concentration of key aroma compounds is responsible for the poor flavor of LAFMB. That implies further research should focus on increasing the content of these compounds. Moreover, *L. plantarum* strain was proposed here as prominent for aroma formation. Besides its enzymatic complex package, it is known to resist the more to acidic stress as compared to other strains (Pieterse *et al,.* 2005), and is widely used for flavor development in cheese (Gobbetti *et al,.* 2015). Therefore, researchers and industrials should consider using *L. plantarum* strain to produce LAFCB.

2. Limitations that occur in LAF of cereal substrates to cause poor flavor profile were found to be amino acids exhaustion, the low buffering capacity of malt wort and lactic acid accumulation. The resulted impact was the significant and rapid cell death coupled with the degradation of metabolic activity. In the theory, researchers interested in LAFCB will find here an explanation of the short fermentation of LAB in their cereal-based substrates.

This will serve them in decision-making to improve LAB fermentation performance in cereal-based substrates.

3. HGF increased malt wort amino acid content and buffering capacity that significantly improved LAB fermentation performance, the aroma yield and consumer's acceptance of LAFMB. Thus, HGF at 20 %w/w is recommended to produce LAFMB. Ultra-HGF may provide further amino acid and increased buffering capacity but, it should be taken with care because ultra-HGF at more than 20 %w/w has a slow-down effect on LAB growth rate instead.

4. To further optimize malt wort in amino acid content and buffering capacity, mashing process protein rest extension to 75 min was recommended. However, caution should be taken not to go beyond this time because reverse results would be obtained. Further, a high initial inoculum of 100 Mio. viable cells/mL was found to cope with the naturally occurring *Bacillus* spores that spoil wort. That implies the necessary step of wort boiling for sterilization should be undertaken but in addition to high inoculum fermentation. Temperature reduction and stepwise feeding of fermentation medium were recommended. Further, temperature reduction is cost-efficient for industries because of the reduction in energy use. Maturation of LAFMB was found to be the main step of flavor development in LAFCB, it does not require any specific equipment but should be customized.

The process proposed here is simple for application in any industry dealing with fermentation technology. That means the process not only improves the flavor of LAFMB but is also economical than the conventional fermentation process used so far in LAF of cereal-based substrates. The overall implication of this dissertation is that the improvement of the flavor of LAFCB will help industrials to develop LAFCB making it available on the market for consumers in need such as lactose intolerant and vegan people. That will ensure therefore a market success, industrial product portfolio enlargement, and energy saving.

Limitations of this study and recommendations for future research

Again, this dissertation attempts to improve the flavor of LAFMB but at the same time, it shows that further improvement is still necessary. First and foremost, this study focused on the sole use of barley, application to other cereals such as pseudo-cereals quinoa or amaranth that possess high nutritional values individually or in combination will probably continue this study. An additional limitation is that the maturation conditions of LAFMB proposed in this study did not consider large parameter ranges whereas optimum

conditions definition will bring a significant contribution to the flavor of LAFCB. Finally, the definition of a sensory evaluation schema for LAFMB will be helpful.

For LAFCB, consociation of high aminopeptidase and aminotransferase activity strains with functional strain for a multi-strain starter culture definition may help to bring together the flavor and functional asset in the resulting beverage. Studies on the techno-functionalities and toxicity of strain Lp.758 proposed as a starter culture in this study will complete its utilization in LAFCB. Finally, the mechanism of formation of agglomerate-like flocculation of LAB under acidic stress still to be understood.

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6. Appendix

a. Oral presentations

1. **Nsogning Dongmo, S**., Kollmannsberger, H., Becker, T.: Malzbasiertegetränke: High-Gravity-Fermentation zur Verbesserung des Aromaprofils. 49. Technologisches Seminar, Freising, Germany, 2016-02-18.
2. **Nsogning Dongmo, S**., Kollmannsberger, H., Sacher, B., Becker, T.: Impact of starter culture on the aroma profile and sensory attributes of barley malt beverages produced by lactic acid fermentation. FCT-Conference, San Francisco, USA, 2015-11-18.
3. Osen, R., **Nsogning Dongmo, S.,** Sacher, B., Procopio, S., Toelstede, S., Fritsch, C., Fischer, S.: Brauen mal anders-Entwicklung eines pflanzlichen Proteinerfrischungsgetranks für die vegane Ernährung. Jahrestagung Trend Vegan-Stand der Forschung, Freising, Deutschland, 2015-10-27.

b. Additional project contributions

1- Screening of Lactic acid bacteria to produce fruity aroma compounds and vitamins through extended exposition under acidic conditions – Carlsberg Project.

2- Wertsteigernde Nutzung von pflanzlichen Rohstoffen als proteinreiche, funktionelle Getränkegrundstoffe durch ein neuartiges kombiniertes Maische- und Fermentationsverfahren“ - AIF Project.