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Characterization of EPS-producing brewery-associated lactobacilli

Marion Elisabeth Fraunhofer

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Vorsitzender:	Prof. Dr. Horst-Christian Langowski
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Vorwort

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Abbreviations

%	percentage
°C	degree Celsius
× *g*	times gravity
A.	*Acetobacter*
aa	amino acid
AAB	acetic acid bacteria
ADP	adenosine diphosphate
AF4	asymmetric flow field flow fractionation
ATP	adenosine triphosphate
BADGE	BlAst Diagnostic Gene finder
BLAST	Basic Local Alignment Search Tool
bp	base pair
BRIG	BLAST Ring Image Generator
BSA	bovine serum albumin
BU	bitter unit
Cak	chloramphenicol
CDS	number of coding sequences
CFU	colony forming unit
chr	chromosome
comp	complete
COSY	analysis of correlated spectroscopy
cov	coverage
CPS	capsular polysaccharide
CO_2	carbon dioxide
D. melano.	*Drosophila melanogaster*
DGM	diagnostic marker gene

DNA	deoxyribonucleic acid
dH_2O	demineralized water
Da	Dalton
EPS	exopolysaccharide
FK	fructokinase
Fru	fructose
G.	*Gluconobacter*
Gal	galactose
GalA	α-galactosidase
GalE	UDP-glucose-4-epimerase
GalK	galactokinase
GalT	galactose-1-phosphate uridylyltransferase
Gal/Na^+ symporter	sodium solute symporter
GC-FID	gas chromatography-flame ionization detector
GC-MS	gas chromatography-mass spectrometry
Glc-6-P	glucose-6-phosphat
GlcU	glucose transporter
GK	Glucokinase
GPI	glucose-6-phosphat isomerase
gtf-2	glycosyltransferase-2 gene
gtf-2	glycosyltransferase-2 protein
h	Hour
H_2O_2	hydrogen peroxide
H_2SO_4	sulfuric acid
HAPEC	high performance anion exchange chromatography
HePS	heteropolysaccharide
HGAP 3	hierarchical genome-assembly process version 3

HoPS	homopolysaccharide
HPLC	High Performance Liquid Chromatography
HPMC	hydroxypropyl methylcellulose
HSQC	heteronuclear single quantum coherence
Illum	Illumina
kDa	kilodalton
kg	kilogram
L	liter
L.	*Lactobacillus*
Lc.	*Lactococcus*
LAB	lactic acid bacteria
LTA	lipoteichoic acid
M	molarity
MALDI-TOF MS	Matrix assisted laser desorption ionization-time of flight mass spectrometry
MALS	Multi-Angle Light Scattering
melB	melibiose carrier protein
MP	maltose phosphorylase
MDa	megadalton
MDR	multidrug transporter
MFS	major facilitator superfamily
Mg	magnesium
$MgCl_2$	magnesium chloride
MIC	minimal inhibitory concentration
min	minute
mL	milliliter
mm	millimeter
mmol	millimole

mMRS	modified de Man, Rogosa and Sharpe medium
Mn	manganese
Nis	nisin
NaCL	sodium chloride
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
nm	nanometer
NMR	nuclear magnetic resonance spectroscopy
no	number
O.	*Oenococcus*
OD	optical density
OG	orthologous group
P	permease
P.	*Pediococcus*
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGM	phosphoglucomutase
pl	plasmid
PMAA	partially methylated alditol acetate
pmf	proton motive force
RAST	rapid annotations using subsystems technology
Rep	plasmid replication protein
RI	refractive index
s	second
sp.	species
S.	*Streptococcus*
t	terminal

TCA	tricarboxylic acid
TFA	trifluoroacetic acid
TMW	Technische Mikrobiologie Weihenstephan
TOCSY	total correlated spectroscopy
UDP	uridine diphosphate
UGP2	UTP-glucose-1-phosphate uridylyltransferase
UK	UDP kinase
UTP	uridine triphosphate
V	volt
v/v	volume by volume
w/w	weight by weight
µg	microgram

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1. INTRODUCTION

1.1. The history and particularities of beer

With a history of approximately 5000 - 7000 years, beer represents one of the oldest beverages in the world. Its origin is suggested in Mesopotamia and Egypt, but it spread over all continents, resulting in one of the most famous beverages (Behre, 1999). Generally, beer is classified upon the yeast used for fermentation in top and bottom fermented beer, both including a variety of different beer types (Jelinek, 1946). In addition to the yeast and the particular fermentation conditions, the application of specific barleys and hops strongly influences the taste. Consequently, beer possesses a sustainable diversity and flavor spectrum.

Until the mid-age, beer was flavored additionally with certain ingredients like anise, caraway and vermouth. However, the additives also encompassed those with toxic and life-threatening properties such as nightshade and henbane. Consequently, Wilhelm IV released the German purity law in 1516, restricting the ingredients of beer to water, barley, and hop. Meanwhile, this law exists for more than 500 years, ensuring its traditional composition and safety.

The particularities of beer not only include its special aroma, but also its self-stabilizing properties. Due to its unique composition, beer possesses an intrinsic resistance against microbial spoilage as it contains ethanol, a low pH, a high content of carbon dioxide, a low oxygen amount, less nutrients and antimicrobial hop compounds (Suzuki, 2011). Each factor contributes to the stability of beer and reduces the viability of potential spoilage bacteria.

In this regard, ethanol, which results from the activity of the brewing yeast and ranges in most beer types from 3.5 to 5.0 % (v/v) (Vriesekoop, Krahl, Hucker, & Menz, 2012), inactivates bacteria by inhibiting cell membrane functions and rupturing the cell membrane (Ingram, 1990; Kalathenos & Russel, 2003).

The low pH (3.4 - 4.8) of beer, caused by the brewing process (Preedy, 2009), is responsible for the intrusion of small organic acids into the bacterial cell. Due to the high intracellular pH, these acids dissociate and induce an acidification of the cell. This not only destroys enzyme systems but also hampers the uptake of nutrients as the transmembrane pH gradient is dissipated, finally resulting in metabolic exhaustion (Suzuki, 2015). Beyond this direct action, the low pH boosts the antimicrobial properties of hop compounds. According to Simpson and Hammond (1991), a pH decrease of 0.2 increases the antibiotic hop action by up to 50 %.

The special gas atmosphere of beer further contributes to its microbiological stability. This atmosphere is characterized by a high carbon dioxide content, resulting from the fermentation and technological carbonization as well, and by a low oxygen content, referable to modern bottling techniques. Together, the low oxygen and high carbon dioxide values restrict the growth of aerobic bacteria. Carbon dioxide even acts in other manners as well, as it decreases the pH, affects the cell membrane, influences carboxylation and decarboxylation reactions, and directly inhibits bacterial growth (Vriesekoop et al., 2012).

In addition, the lack of nutrients caused by the metabolism of the brewing yeast further starves bacterial growth (Geissler, Behr, von Kamp, & Vogel, 2016; Suzuki, 2011).

The most important antimicrobial components of beer are hop compounds, namely α-acids (Vriesekoop et al., 2012). During the boiling of the wort, these acids isomerize to iso-α-acids, which not only contribute to the bitterness but to the stability of beer as well. Iso-α-acids act as ionophores, dissipating the transmembrane proton gradient (Simpson, 1993a, 1993b; Simpson & Fernandez, 1994). This abrogates the proton motive force and consequently inhibits nutrient uptake and essential enzyme reactions, resulting in cell death (Behr & Vogel, 2009; Sakamoto & Konings, 2003; Sakamoto, Margolles, van Veen, & Konings, 2001).

Finally, the increasing processing and hygienic standards, as well as the application of post-fermentative treatments like filtration, pasteurization, and cold storage further avoid the microbial contamination of beer and contribute to its purity and shelf life. Together, these intrinsic and extrinsic hurdles make beer to a stable beverage.

1.2. The spoilage of beer

1.2.1. **Beer-spoiling bacteria**

Although beer provides a variety of hurdles, certain bacteria developed strategies to life in this harmful environment. This includes gram-negative bacteria like *Pectinatus* and *Megsaphera* and gram-positive lactic acid bacteria (LAB). The latter represents the most relevant group of beer-spoiling bacteria, causing up to 90 % of the spoilage incidents (Back, 1994; Suzuki, 2011). 300 beer-spoiling LAB are described, including various strains and species of lactobacilli and pediococci. In detail, these are *L. brevis, L. linderi, L. (para)buchneri, L. rossiae, L. backii, L. (para)casei, L. acetotolerans, L. coryniformis, L. (para)collinoides, L. perolens* and *L. paucivorans* as well as *Pediococcus (P.) claussenii, P. damnosus* and *P. inopinatus* (Hutzler, Müller-Auffermann, Koob, Riedl, & Jacob, 2013).

1.2.2. **Mechanisms of beer spoilage by LAB**

The ability to counteract the detrimental effects of beer is a fundamental requirement for the survival in this ecological niche. Due to the intensive research in the last decade, the adaptive mechanisms behind beer/ hop tolerance of LAB are increasingly understood and are schematically illustrated in Figure 1.

One of the most important mechanism of resistance is associated with the cell envelope in order to increase the barrier function and in turn to decrease the intrusion of hops, ethanol and acids.

In this context, high molecular weight lipoteichoic acids (LTAs) are described to have barrier-increasing effects (Behr, Gänzle, & Vogel, 2006; Sakamoto & Konings, 2003; Yasui & Yoda, 1997). *L. brevis* strains exposed to hop bitter acids, were shown to increase the expression of LTAs accompanied by an improved barrier function (Behr et al., 2006). Those cell wall modifications are attributed to gene cluster like *horA* and *horC*, both well-known hop resistance clusters (Suzuki, Iijima, Sakamoto, Sami, & Yamashita, 2006).

Another possibility to increase the barrier function is the enhanced incorporation of saturated fatty acids such as C16:0 into the cell membrane, which reduce the membrane fluidity and protect the cell from acid and hop intrusion (Behr et al., 2006; Schurr, Hahne, Kuster, Behr, & Vogel, 2015).

The in this way improved barrier function reduces but cannot prevent the hop intrusion. Consequently, the cell has to find strategies compensating intracellular located hops.

One of the most intuitive ways to antagonize intruded hop bitter acids is to extrude them into the outer medium. The above-mentioned *horA* and *horC* cluster are described to participate in expelling hops out of the cell (Sakamoto et al., 2001; Suzuki, Sami, Kadokura, Nakajima, & Kitamoto, 2002). *HorA* encodes an ATP-dependent multidrug resistance transporter, *horC* a PMF-dependent one.

Another strategy to counteract intracellular hop is associated with cation homeostasis. The detrimental properties of hops strongly depend on the presence of cations, especially manganese (Geissler, Behr, Schmid, Zehe, & Vogel, 2017; Schurr, Behr, & Vogel, 2015). Binding this divalent cation conveys hops their full antimicrobial properties. Therefore, making manganese unavailable for hops represents an important mechanism of resistance. In this context, the afore-described LTAs are from importance again. They not only improve the

barrier function but also mediate cation homeostasis. Due to their polyanionic properties, LTAs are considered as reservoir for divalent cations, consequently reducing the content of available manganese and retaining it for essential cell functions (Behr et al., 2006; R. F. Vogel, Ehrmann, & Gänzle, 2002).

Another possibility of manganese homeostasis is its exchange with magnesium. According to Preissler (2011), hop stressed *L. brevis* TMW 1.313 increases the uptake of magnesium while releasing manganese. In contrast to manganese, magnesium strongly reduces the antimicrobial properties of hops (Behr & Vogel, 2009; Preissler, 2011; Simpson & Smith, 1992). A respective magnesium uptake system *CorA* (Kehres, Lawyer, & Maguire, 1998) is according to Geissler et al. (2017) present in many beer-spoiling LAB.

Although magnesium and manganese are exchangeable in many regards, some physiological functions strongly depend on manganese, as it is an essential trace element for LAB. To balance manganese homeostasis, beer-spoiling lactobacilli were found to encode *hitA*, a Mn^{2+}/H^{+} symporter and well-known hop resistance gene. *CorA* and *HitA* are often encoded on the same cluster, suggesting a concurrent regulation to ensure a balanced relationship of both cations (Geissler et al., 2017).

The hop- and pH-mediated intracellular acidification with the associated breakdown of the transmembrane gradient was found to be antagonized via an increased expression of ATPases that extrude protons out of the cell (Suzuki, 2015). In this context also other stress tolerance mechanisms, such as the ADI and the glutamate decarboxylase (GABA) system are upregulated (Behr et al., 2006; Schurr, Behr, & Vogel, 2013). Both pathways help to keep up the pH homeostasis by metabolizing protons. In addition to the consumption of protons, the ADI system also produces alkaline ammoniac and generates ATP, which can in turn be used to drive ATPases or other energy-requiring systems.

Finally, certain strains of beer-spoiling bacteria like *L. brevis* and *L. linderi* adapted to breweries were shown to reduce their surface area by shifting their morphology into smaller rods. Consequently, membrane-bound resistance mechanisms are suggested to be developed more efficiently (Asano et al., 2007).

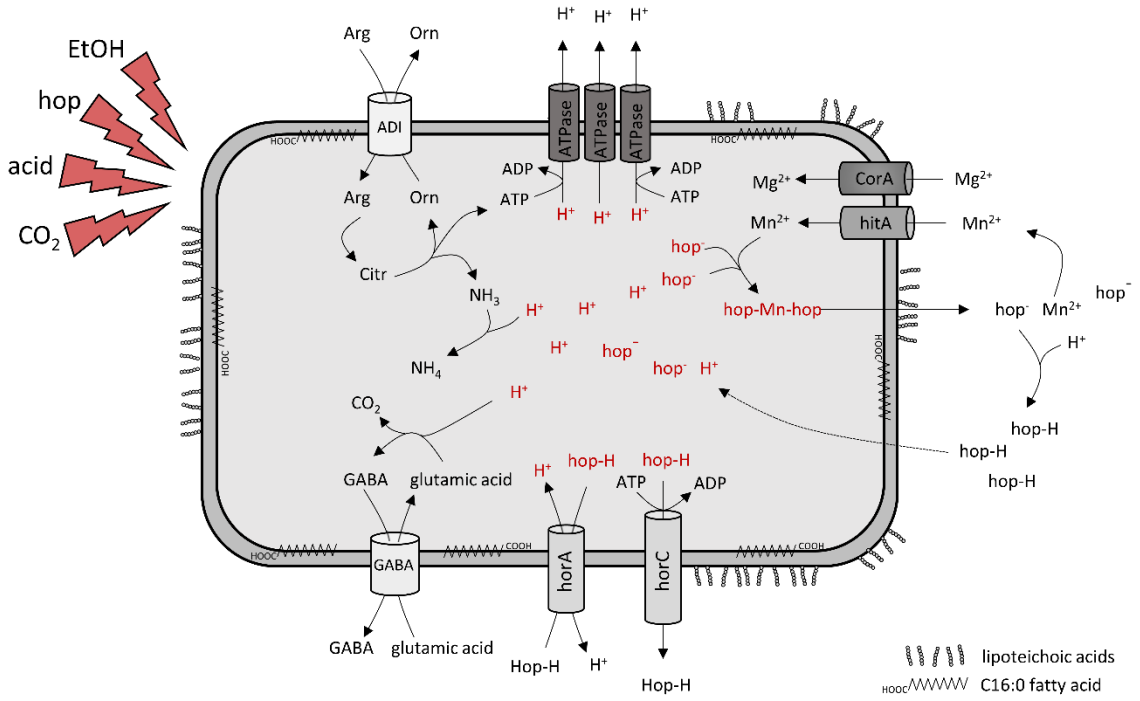

Figure 1 Overview of resistance mechanisms against beer hurdles in beer-spoiling LAB. The cell wall is modified with LTAs, the cell membrane with C16:0 fatty acids. Expression of *horA* and *horC* export intracellular hop compounds. *HitA* balances manganese homeostasis. Increased expression of proton-translocating ATPases antagonize intracellular acidification, supported by an upregulation of the ADI and GABA pathway. Arg: arginine, Orn: ornithine, Citr: citrulline, GABA: *gamma*-aminobutyric acid.

If LAB are able to modify their lifestyle in these respects, they can gain the ability to grow in and to spoil beer.

By performing continuous quality controls, breweries attempt to detect beer-spoiling bacteria as early as possible. Therefore, detection media, which enable the specific determination of relevant contaminants and PCR-based methods (mostly targeting hop resistance genes like *horA* or *horC*) are commonly used (Suzuki, Asano, Iijima, Kuriyama, & Kitagawa, 2008; Suzuki et al., 2006). Nonetheless, there are occasionally spoilage incidents.

Besides strains causing turbidity and off-flavor through growth and metabolite formation, respectively, beer-spoiling bacteria also comprise strains of lactobacilli, which produce slime that increases the viscosity of beer. These slimy, viscous properties are referred to the synthesis of exopolysaccharides (EPS).

1.3. Bacterial exopolysaccharides

In general, the synthesis of polysaccharides is from enormous importance for the bacterial cell. Upon their localization within the cell, polysaccharides are divided into three groups: cytosolic ones, which provide a carbon and energy source, those ones that build the cell wall like peptidoglycans, teichoic acids and lipopolysaccharides and finally polysaccharides exuded into the extracellular environment, called EPS (Donot, Fontana, Baccou, & Schorr-Galindo, 2012). EPS are high molecular weight polymers, which are produced by a variety of gram-positive and gram-negative bacteria (Sutherland). Due to the diverse nature of EPS-producing bacteria, the resulting polymers show an overwhelming diversity as well, affecting the size, structure, composition, linkage bonds and molecular weight. Generally, EPS are classified, in dependence of the monosaccharides involved, in homo- and heteropolysaccharides (HoPS, HePS), illustrated in Figure 2.

HoPS, like levan, curdlan, cellulose, dextran or mutan are characterized by containing just one type of sugar moiety, mostly glucose or fructose (Monsan et al., 2001).

HoPS are mainly synthesized extracellularly from energy-rich disaccharides such as sucrose, mediated by a single type of enzyme belonging to the glycosyl hydrolase family 68 or 70 (Torino, Font de Valdez, & Mozzi, 2015). These enzymes polymerize the HoPS out of respective disaccharides, which not only act as donor for the corresponding monosaccharide, but also for the energy required. After cleaving the energy-rich glycosidic bond, the energy released is used to transfer the sugar moiety to the growing end of the polymer. Accordingly, these enzymes possess a dual mode of action, combining a hydrolysis with a transferase reaction (Leemhuis, Pijning, Dobruchowska, Dijkstra, & Dijkhuizen, 2012; van Hijum, Kralj, Ozimek, Dijkhuizen, & van Geel-Schutten, 2006).

In LAB there is a second mechanism of HoPS synthesis described, namely for glucans, which bases on an intracellular glycosyltransferase-mediated synthesis. Although this mechanism is not yet fully understood, it is suggested to proceed via the polymerization of activated sugar nucleotides in form of UDP-glucose/ UDP-galactose (Karnezis, McIntosh, Wardak, Stanisich, & Stone, 2000; Torino et al., 2015; Werning et al., 2006). Consequently, this mechanism resembles more the HePS synthesis.

HePS are in contrast to HoPS structurally more complex, as they are composed of at least two different units, varying in their repeating unit from di- to heptasaccharides and possessing molecular weights of 10^4 to 10^6 Da (De Vuyst & Degeest, 1999). Thereby, not only

monosaccharides like e.g. glucose, galactose or rhamnose are involved, but also N-acetylated monosaccharides as well as organic and inorganic substituents (Mozzi et al., 2006).

Due to the complex structure, the synthesis is more complex as well, involving several enzymes (for the polymerization and secretion), mostly encoded in eps genes clusters, showing an operon-like structure (De Vuyst & Degeest, 1999; Jolly & Stingele, 2001). The HePS biosynthesis is characterized by an intracellular formation of repeating units, which are polymerized extracellular.

For the intracellular synthesis of a repeating unit, the respective monosaccharides are activated through the formation of sugar nucleotides like UDP-glucose or UDP-galactose and subsequently assembled at the membrane. Therefore, the sugar moieties are sequentially added to a growing repeating unit that is most probably anchored on a lipid carrier in the cell membrane. After completion of a respective unit, it is exported and polymerized extracellularly into the final HePS (De Vuyst & Degeest, 1999).

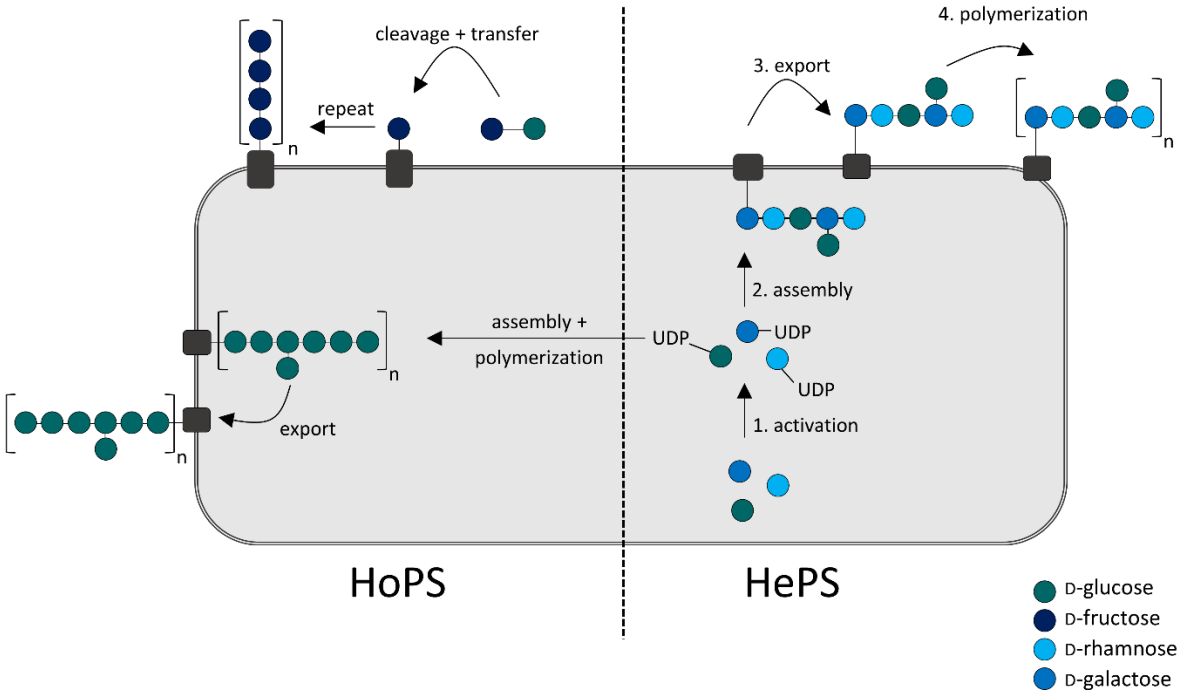

Figure 2 Schematic overview of the key steps of HoPS and HePS synthesis in LAB. HoPS synthesis is typically characterized by extracellular cleavage of energy-rich disaccharides, which are subsequently polymerized. Another mechanism of HoPS formation relies on the intracellular activation and polymerization of monosaccharides with a subsequent export. HePS synthesis also proceed via sugar nucleotides, which are polymerized extracellularly, though.

Both, HoPS and HePS can be secreted into the external environment or stay cell-associated as capsule (CPS).

Although the precise physiological function is still not fully understood in terms of different EPS types and producer strains, EPS usually confer the cell several benefits, supporting their resistance and survival. They are thought to protect bacteria against desiccation, phagocytosis, antibiotics, toxics and osmotic stress (Nwodo, Green, & Okoh, 2012). Moreover, EPS facilitate the adhesion to surfaces and play a crucial role in biofilms, which ensure the protected life of various other microorganisms (Lembre, Lorentz, & Di, 2012; Vu, Chen, Crawford, & Ivanova, 2009).

As EPS exhibit structure-giving properties, they are from great industrial interest. Bacterial polysaccharides like xanthan, gellan or curdlan are employed as additives in various food, pharmaceutical and cosmetic products to improve viscosity, texture and stability (Sutherland, 1990, 1998). Thereby, EPS are either isolated, purified and added, or appropriate bacteria are used as starter cultures, such as in dairy products, and produce the EPS *in situ* within the food matrix (Duboc & Mollet, 2001; Han et al., 2016).

Beside the rheological and stability improvements, EPS are claimed to have several health promoting properties as well (Caggianiello, Kleerebezem, & Spano, 2016).

As non-digestible food fraction, EPS act as prebiotic and exert beneficial effects on the human gastrointestinal tract (Salazar, Gueimonde, de Los Reyes-Gavilan, & Ruas-Madiedo, 2016). Moreover, EPS are suggested to possess antitumor (Demleitner, Kraus, & Franz, 1992; Oda, Hasegawa, Komatsu, Kambe, & Tsuchiya, 1983; Wang et al., 2014), antiulcer (Nagaoka, Hashimoto, Watanabe, Yokokura, & Mori, 1994) and immunomodulatory (Ciszek-Lenda, Nowak, Srottek, Gamian, & Marcinkiewicz, 2011; Kitazawa, Yamaguchi, Miura, Saito, & Itoh, 1993; Kulicke, Lettau, & Thielking, 1997) properties and are proposed to decrease blood cholesterol values (Nakajima, Suzuki, Kaizu, & Hirota, 1992; Tok & Aslim, 2010).

Due to this diverse and great potential, EPS and the producing bacteria received special attention and are under intensive research for various purposes.

However, not all industrial sectors benefit from EPS-producing bacteria. In the beverage industry, especially in breweries and wine manufactures, respective bacteria are feared contaminants, causing spoiled products with a slimy, viscous and thick texture.

1.4. EPS-producing beverage-spoiling LAB

1.4.1. **The slimy spoilage of wine**

150 years ago, Louis Pasteur firstly described the slimy spoilage of wine and referred this phenomenon to microbial origin (Pasteur, 1866). Meanwhile, the responsible bacteria are identified to belong to the species of *Oenococcus (O.)* (Dols-Lafargue et al., 2008; Ibarburu et al., 2007), *Lactobacillus* (Duenas-Chasco et al., 1998) and *Pediococcus* (Duenas-Chasco et al., 1997; Llauberes, Richard, Lonvaud, Dubourdieu, & Fournet, 1990).

Even if specific strains of these species are often beneficial and specifically used to refine the aroma and taste of wine via malolactic fermentation (Davis, Wibowo, Eschenbruch, Lee, & Fleet, 1985), their EPS-producing relatives are highly undesired.

The EPS produced by wine-spoiling LAB mostly possess a two-branched β-glucan structure, whose biosynthesis is mediated by a glycosyltransferase (Dimopoulou et al., 2014; Dols-Lafargue et al., 2008; Werning et al., 2006). This enzyme is supposed to polymerize activated monosaccharide precursors similar to the synthesis of HePS (Figure 2). In capsular form, the glucan was shown to increase the resistance of respective bacteria against the hurdles of wine like acid and ethanol (Dols-Lafargue et al., 2008).

In addition to the glucan, certain *O. oeni* strains were shown to synthesize another polysaccharide with HePS character (Ciezack et al., 2010). However, this EPS possesses soluble properties and does not contribute to the viscosity-increasing effects.

Compared to polysaccharides like dextran or cellulose produced by gram-negative acetic acid bacteria in concentrations up to 20 g/l (Sutherland), the glucan from slimy wine spoilers just accounts in small amounts around 200 mg/l (Llauberes et al., 1990; Velasco et al., 2006; Walling, Dols-Lafargue, & Lonvaud-Funel, 2005). Nonetheless, this content is sufficient to confer infested beverages a highly viscous texture.

1.4.2. **The slimy spoilage of beer**

The group of EPS-producing, beer-spoiling LAB comprises strains of *L. brevis, L. rossiae, L. (para)buchneri* and *P. claussenii* (Hutzler et al., 2013). Williamson (1959) described the phenotype of EPS-producing beer-spoiling bacteria as ropy on solid culture and viscosity increasing in liquid culture. However, in beer, this spoilage phenomenon moved out of the center of research and is still less explored.

Under consideration of the importance of a profound barrier function in beer-spoiling bacteria, EPS formation might reflect a mechanism of resistance. By enveloping the bacterial cell as protective layer, the antibiotic effectivity of beer ingredients could be weakened.

Unfortunately, the background and precise contribution of EPS formation in the context of breweries remain elusive so far, as the majority of EPS-forming beer-spoiling lactobacilli is not investigated.

2. MOTIVATION, HYPOTHESES AND APPROACHES

As one of the most popular alcoholic beverages, the global beer production makes up to approximately 2 billion hectoliter per year. However, spoiled batches occur occasionally. Although sustainable progress in microbial beer spoilage research have been made in the last decades, the viscous beer spoilage caused by EPS-producing lactobacilli is not yet included very well in this substantial knowledge. Since respective detection systems are missing as well, slimy spoiled beer is often just realized via consumers reject, associated with great image and economic losses of affected companies. Therefore, the understanding of the mechanisms involved in this spoilage phenomenon is essential in order to manage this problem. Under consideration of the following hypotheses, this study had the aim to gain more insights into the viscous spoilage of beer:

- Beer-spoiling bacterial species contain slime-forming strains.
- The slimy properties rely on EPS formation.
- EPS formation represents a mechanism of resistance against the antimicrobial hurdles of beer.
- Consequently, a capsular localization of EPS is assumed.
- The biosynthesis proceeds via certain key genes, which are exploitable as diagnostic marker genes.
- PCR primers can be derived, enabling the specific, fast and reliable detection of slimy beer-spoiling bacteria in breweries.
- The evolutionary development of (slimy) beer-spoiling bacteria occurred in another, natural ecological niche, which is much older than beer.

From these working hypotheses, the following approaches were derived:

- Lactobacilli from beer and from brewery surfaces should be screened on their ability to produce exopolysaccharides and their ability to spoil beer.
- The contribution of EPS formation on the bacterial cell should be determined by comparative physiological experiments.
- The localization, composition and structural properties of produced EPS should be identified via agglutination assay, HPLC-RI and NMR spectroscopy.
- Growth and slime formation should be studied under variation of the nutrient source to identify the requirements of EPS formation and to gain insights into the biosynthesis.

- The biosynthesis should be elucidated via whole genome sequencing and comparative genomics.
- PCR primers targeting specific key genes of EPS synthesis should be designed and proved for their specificity to detect slimy beer spoilers.
- The genomic particularities of beer-spoiling bacteria should be assessed to unravel the evolutionary development.

In this way, the here presented study intended to provide a comprehensive characterization of EPS-based, viscous beer spoilage.

3. MATERIAL AND METHODS

3.1. Bacterial strains, growth conditions and media

All media used for the cultivation of bacteria were autoclaved at 121 °C for 20 min to ensure sterility. In case of agar plates, 15 g/l agar (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) was added. Sugars were autoclaved separately from the medium to avoid Maillard reactions. Heat-sensitive ingredients were sterile-filtered (pore-size 0.2 µm, Sarstedt Ag & Co. KG, Nümbrecht, Germany) and supplemented after autoclaving.

3.1.1. ***Lactobacillus sp.***

83 strains of the species *L. brevis, L. rossiae, L. parabuchneri* and *L. plantarum* were (initially) used in this study. They were isolated from beer or brewery-associated surfaces and identified on species level using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Therefore, single colonies were smeared onto a stainless steel target (Bruker Daltonics, Bremen, Germany) and overlayed with 1 µl formic acid (70 %, Sigma-Aldrich GmbH, Darmstadt, Germany) and 1 µl α-cyano-4-hydroxy-cinnamic acid matric solution (Bruker Daltonics, Bremen, Germany). Mass spectra were generated with a Microflex LT MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser (λ = 337 nm) and operating in a linear positive ion detection mode under the control of Biotyper Automation Control 3.0 (Bruker Daltonics, Bremen, Germany) (Kern, Usbeck, Vogel, & Behr, 2013).

As working culture, lactobacilli were grown at 30 °C in mMRS (modified de Man, Rogosa and Sharpe) medium adjusted to pH 6.2. Growth medium composition (quantities per liter): 10 g peptone (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 5 g yeast extract (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 5 g meat extract (VWR GmbH, Darmstadt, Germany), 4 g K_2HPO_4 (Merck KGaA, Darmstadt, Germany), 2.6 g $KH_2PO_4 \cdot 3H_2O$ (Merck KGaA, Darmstadt, Germany), 3 g NH_4Cl (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 1 g Tween80 (Gerbu GmbH, Heidelberg, Germany), 0.5 g cysteine-HCl (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 0.2 g $MgSO_4 \cdot 7H_2O$ (Merck KGaA, Darmstadt, Germany), 0.038 g $MnSO_4 \cdot H_2O$ (Carl Roth GmbH & Co.KG, Karlsruhe, Germany), 5 g glucose (Merck KGaA, Darmstadt, Germany), 5 g fructose (Merck KGaA, Darmstadt, Germany) and 10 g maltose (Merck KGaA, Darmstadt, Germany). For experiments comparing the impact of low (4.3) and high (6.2) pH conditions, the preculture

was grown in mMRS with pH 5.25. For EPS isolation, a variant of the medium without yeast and meat extract, but supplemented with a vitamin mix (quantities per liter: 0.2 mg thiamin, 0.2 mg niacin, 0.2 mg folic acid, 0.2 mg pyridoxal, 0.2 mg pantothenic acid, 0.2 mg cobalamine; Sigma-Aldrich GmbH, Steinheim, Germany) was used. Modifications of the carbohydrate source are mentioned in the particular sections.

3.1.2. ***Lactococcus sp.***

Lactococcus lactis sups. *cremoris* NZ9000 TMW 2.772 was cultivated in GM17 medium ((M17 broth (BD Company, Heidelberg, Germany) supplemented with 1 % glucose; Merck KGaA, Darmstadt, Germany) at 30 °C.

Its genetically modified transformant *Lactococcus lactis* sups. *cremoris* NZ9000 TMW 2.2036, carrying a pNZ8048-gtf-2 plasmid, was standardly grown in $GM17_{Cak}$ medium (GM17 supplemented with 5 µg/ml chloramphenicol; Carl Roth GmbH & Co. KG, Karlsruhe, Germany), overnight at 30 °C, followed by subculturing 1 % (v/v) in fresh $GM17_{Cak}$. To induce heterologous gtf-2 expression on agar plates, a single colony was streaked onto $GM17_{Cak}$ plates supplemented with 5 ng/ml nisin ($GM17_{Cak\text{-}Nis}$; Sigma-Aldrich GmbH, Steinheim, Germany). In liquid culture, the target protein was induced by adding nisin (5 ng/ml) when the optical density at 590 nm (OD_{590}) reached 0.5. Cultures were maintained at 30 °C for 5 h before conducting experiments.

3.2. Dynamic of growth, slime and EPS formation

All experiments analyzing bacterial growth and the formation of slime/ EPS were performed in biological triplicates.

3.2.1. **Growth curves**

Growth curves were recorded in microtiter plates (Sarstedt Ag & Co. KG, Nümbrecht, Germany). Wells containing appropriate media were inoculated with a standard preculture to an OD_{590} of 0.1 and overlayed with paraffin oil (Sigma Aldrich GmbH, Steinheim, Germany). Growth curves were tracked using a plate reader (Spectrostar, BMG Labtech GmbH, Ortenberg, Germany) for five days at 30 °C and analyzed using MS Excel (Microsoft, Redmond, USA).

3.2.2. **Growth under stress conditions**

Growth challenge experiments were performed on solid and in liquid culture. The stress factors hydrogen peroxide, ethanol and acid were applied in different concentrations, given in Table 1. Heat-sensitive additives were supplemented, when agar/ medium temperature decreased to 40 °C. Both experimental setups were carried out with the same, standardly grown bacterial preculture which was set to an OD_{590} of 1. For stress tolerance assays based on solid medium, 10 µl cell suspension were dropped onto agar plates containing the respective stress factors. Visible growth was examined after incubating for three days at 30 °C. For stress tolerance assays based in liquid medium, 10 µl cell suspension were added to the wells of a microtiter plate (Sarstedt Ag & Co. KG, Nümbrecht, Germany) containing 290 µl of respective medium and were overlaid with paraffin oil (Sigma Aldrich GmbH, Steinheim, Germany). While incubating for five days at 30 °C, the OD_{590} was recorded regularly. Finally, minimal inhibitory concentration (MIC) values were determined which refer to the lowest concentration of a stress factor, preventing visible/ detectable growth of a bacterium.

Table 1 Stress factors and concentrations used for growth challenge experiments.

Stress factor	**Concentration**									
Ethanol [%]	0	2	4	6	8	10	12	14	16	18
H_2O_2 [mM]	0	0.5	1	1.5	2	2.5	3	3.5	4	4.5
pH	6.00	5.55	5.25	5.00	4.50	4.25	4.00	4.25	3.75	3.5

3.2.3. **Beer spoilage test**

The beer-spoiling potential was determined according to Suzuki, Iijima, Ozaki, and Yamashita (2005b) with slight modifications. 2 % of a standard preculture were inoculated in lager beer with pH 5.0 (adjusted with 6 M NaOH; Carl Roth GmbH & Co.KG, Karlsruhe, Germany) and incubated at 25 °C. After visible growth, the cells were transferred into 10 ml degassed test beer with a final concentration of $5x10^3$ cells/ml. As test beers, wheat, lager and pilsner beer with bitter units of 16, 21 and 30, respectively, were used, all produced from the same brewery. As control, the strong beer spoiler *L. brevis* TMW 1.313 and the non-beer spoiler *L. brevis* TMW 1.6 were included. While incubating at 25 °C, the test beers were checked regularly for macroscopic growth. After 60 days, the OD_{590} and the pH were measured. The experiment was conducted with biological and technical triplicates. Beer without inoculation served as control.

3.2.4. **Quantification of slime formation**

Slime formation was quantified using a flow cup (PCE Instruments, Meschede, Germany). The time a 50 ml bacterial culture needs to pass the cup was measured and the resulting flow time was used as estimation for the viscosity. Therefore, 50 ml cultures containing mMRS with different carbohydrates (concentration 20 g/l) at low (4.3) and high (6.2) pH were inoculated with 500 µl of a standard preculture and flow times were measured all 12 h over a period of five days. The flow time of medium without inoculation served as control.

3.2.5. **Determining fermentative pattern**

An API 50 CHL identification system (bioMérieux SA, Marcy-l'Etoile, France) was used to determine fermentable carbohydrates. The assay was performed in two versions: By using API medium, acid production was proven via color changes, by the use of mMRS medium growth was determined via an increased turbidity. According to manufacturer's instruction, an overnight culture was washed twice and resuspended in 10 ml API medium or mMRS medium, respectively. Each tube of the test strip was inoculated with 100 µl cell suspension and covered with a few drops of paraffin oil. The sugar fermentation pattern was assessed by colorimetric changes and turbidity after 24 h and 48 h at 30 °C.

3.2.6. **Biofilm formation assay**

The ability to build biofilms was investigated according to O'Toole (2011). Therefore, standard precultures were set to an OD_{590} of 1 and 100 µl transferred into the wells of a microtiter plate, covered and incubated for 12 h and 48 h at 30 °C. Afterwards, planktonic cells were removed by shaking the plate out and submerging into a water tray. The adherent cells were stained with 125 µl 0.1 % crystal violet (Merck KGaA, Darmstadt, Germany) for 10 min at room temperature. Subsequently, the plate was shaked out and washed two times as already described. The air-dried wells were covered with 300 µl acetic acid (30 % v/v) and incubated for 10 min at room temperature. Biofilm formation was quantified by determining the OD_{590}.

3.3. Characterization of exopolysaccharides

3.3.1. **Isolation of CPS and EPS**

To differentiate between cell-bound CPS and released EPS, two methods were used according to Tallon, Bressollier, and Urdaci (2003). Bacteria were cultured in dependence of

the carbohydrate source for 36 to 72 h at 30 °C in respective media. Subsequently, the cells were separated from the supernatant by centrifugation (12000 x *g*, 60 min). The EPS in the supernatant were precipitated with cold ethanol (2:1 v/v; CLN GmbH, Niederhummel, Germany) and kept at 4 °C overnight. After centrifugation, the resulting precipitate was dissolved in deionized water (dH_2O), dialyzed against dH_2O for three days and lyophilized. To isolate the cell-bound CPS, the cell pellet was washed twice with PBS buffer and dissolved in 1 M NaCl. To detach the polysaccharides from the cells, the pellet was sonicated (3 x 30 sec, power 90 %; HD-70/ Bandelin electronic, Berlin, Germany). Further processing of CPS was conducted as described for EPS.

3.3.2. **Monosaccharide composition of CPS and EPS**

The monosaccharide composition of EPS and CPS was determined with high performance liquid chromatography (HPLC). Freeze-dried EPS and CPS were dissolved in dH_2O to a concentration of 20 g/l and hydrolyzed with 15 % perchloric acid (70 %; Sigma-Aldrich GmbH, Schnelldorf, Germany) for 7 h at 100 °C. After centrifugation (12000 x *g*, 10 min) und filtration (0.20 µm syringe filters, Phenomenex, Torrance, USA), the supernatants were analyzed with a Rezec RPM column (Phenomenex, Aschaffenburg, Germany) coupled to a RI detector (Gykotek, Germany) according to the method of Brandt, Jakob, Behr, Geissler, and Vogel (2016). Sugar monomers were identified according to their retention time using external standard monosaccharides. The mobile phase was dH_2O with a flow rate of 0.6 ml/min.

3.3.3. **Structural characterization of CPS and EPS**

Glycosidic linkages of EPS and CPS were analyzed by methylation analysis as described previously (Wefers and Bunzel, 2015). Briefly, polysaccharides (3 mg) were dissolved in dimethyl sulfoxide (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and methylated by the addition of finely ground sodium hydroxide and methyl iodide (Sigma-Aldrich GmbH, Schnelldorf, Germany). The methylated polysaccharides were extracted into dichloromethane, dried, and subsequently hydrolyzed with 2 M TFA (Sigma-Aldrich GmbH, Schnelldorf, Germany) for 1.5 h at 121 °C. The solvent was evaporated, and partially methylated monosaccharides were reduced by the addition of sodium borodeuteride (Sigma-Aldrich GmbH, Schnelldorf, Germany). Following acetylation and extraction, the partially methylated alditol acetates (PMAAs) were analyzed by GC-MS and GC-FID (GC-2010 Plus, GCMS-QP2010; Shimadzu GmbH, Kyoto, Japan), both equipped with a DB-225 column

(30 m x 0.25 mm i.d., 0.25 µm; Agilent Technologies AG, Santa Clara, CA). Molar response factors according to Sweet et al. (1975) were used for semiquantitative estimation of the PMAA ratios.

Monosaccharide composition was determined after sulfuric acid hydrolysis (pretreatment in 12 M H_2SO_4 (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) for 2.5 h, dilution to 1.6 M H_2SO_4 and hydrolysis for 3 h at 100 °C) according to Saeman, Bubl, and Harris (1945) and methanolysis (16 h methanolysis with 1.25 M methanolic HCl at 80 °C, 1 h hydrolysis with 2 M TFA at 121 °C) according to Deruiter, Schols, Voragen, and Rombouts (1992). The hydrolysates were analyzed for their monosaccharide composition by high performance anion exchange chromatography as described previously (Wefers & Bunzel, 2015). To determine the absolute configuration, the exopolysaccharides were hydrolyzed with 2 M TFA for 30 min at 121 °C and their silylated (R)-2-octanol derivatives were analyzed by GC-MS as described previously (Wefers et al., 2014). For NMR spectroscopic characterization, samples (3 mg) were hydrogen-deuterium exchanged and subsequently dissolved in deuterium oxide (500 µL). NMR spectra were acquired at 298 K on a Bruker Ascend 500 MHz spectrometer equipped with a Prodigy cryoprobe (Bruker, Rheinstetten, Germany). Acetone was used as internal reference (^{1}H: 2.22 ppm, ^{13}C: 30.89 ppm) (Gottlieb, Kotlyar, & Nudelman, 1997).

3.3.4. **AF4-MALS-RI**

Asymmetric flow field flow fractionation coupled to multi-angle light scattering (AF4-MALS; Wyatt Technology, Dernbach, Germany) was used to separate EPS samples and to subsequently analyze their size distributions. Isolated EPS samples were dissolved in dH_2O by vigorous vortexing (0.1 mg/mL) and centrifuged at 14000 x *g* for 15 min to remove undissolved particles, which hindered accurate AF4 separations. For sample separations, 100 µL of the obtained supernatants were injected into the AF4-channel, respectively. The flow conditions and materials/equipment for separation of EPS samples were identical to those previously described by Ua-Arak, Jakob, and Vogel (2017). EPS separations were analyzed using ASTRA 6.1 Software (Wyatt Technology, Dernbach, Germany). Bovine serum albumin (BSA; Sigma-Aldrich GmbH, Steinheim, Germany) and levan produced by and isolated from *Gluconobacter albidus* TMW 2.1191 (Ua-Arak et al., 2017) were used as standards for molecular weight estimations.

3.3.5. **Immunological analysis**

Agglutination tests were performed with *Streptococcus (S.) pneumoniae* type 37-specific antiserum (Statens Serum Institut, Copenhagen, Denmark) as previously described by Walling, Gindreau, and Lonvaud-Funel (2005), with slight modifications. Briefly, single colonies were picked or overnight cultures were centrifuged (10000 x *g*, 30 min), followed by resuspending the cells in PBS buffer (Sigma-Aldrich GmbH, Schnelldorf, Germany). After blending the culture with antiserum or PBS (1:1 v/v) and incubating for 30 min at 4 °C, the mixture was analyzed microscopically at thousand-fold magnification (Axiostar Plus, Carl Zeiss AG, Oberkochen, Germany).

3.4. Molecular biological techniques

3.4.1. **DNA Isolation**

Total genomic DNA was isolated from LAB using the E.Z.N.A bacterial DNA Kit (Omega Bio-Tek Inc., Norcross, USA). To obtain high molecular weight DNA, the Genomic-tip 100/G kit (Qiagen, Hilden, Germany) was used according to the manufacture's instruction.

3.4.2. **PCR**

PCR amplification was performed with the *Taq* Core Kit 10 (MP Biomedicals, Santa Ana, USA). Primers were obtained from MWG Biotech AG (Ebersberg, Germany) and applied in an end concentration of 0.5 µM per reaction. All relevant primers are listed in Table 2. PCR amplification was performed with a reaction mixture of 1 µl DNA, 42.25 µl dH_2O, 0.25 µl of each primer, 0.25 µl Taq polymerase, 1 µl dNTPs and 5 µl 10x $MgCl_2$ buffer using a Mastercycler (Eppendorf AG, Hamburg, Germany). Respective PCR-programs are listed in Table 3 and Table 4.

Table 2 Oligonucleotide primers used in this study.

Primer	Sequence (5'→3')
Gtf-F	GAATCCGAACTAGCAATACTCGC
Gtf-R	ACTAGTGGAATGTGCAACAC TGG
NcoI-F	TATACCATGGTAAATGATAATGATTCAGAAC
XbaI-R	ATTATCTAGATTAATCATTCCAATCAACTGT

Restriction sites are underlined.

Table 3 PCR program used for *gtf-2* amplification.

Temperature [°C]	Duration [min]	Repeats
94	2	1
94	45	
60.6	60	32
72	54	
72	2	1

Table 4 PCR program used for *gtf-2* amplification with restriction sites for *Nco*I and *Xba*I

Temperature [°C]	Duration [min]	Repeats
94	2	1
94	20	
60	60	30
72	40	
72	10	1

3.4.3. Agarose gel electrophoresis

Nucleic acids and a 100-bp plus ladder (Thermo Scientific, Waltham, USA) were separated through gel electrophoresis in 1.3 % (w/v) agarose gels in 0.5 TAE buffer using an Electrophoresis Power Supply EPS 300 (Pharmacia Biotech, Uppsala, Sweden). Gels were stained with dimidium bromide (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), visualized with ultraviolet light (UVT-28 M transilluminator, Herolab, Wiesloch, Germany) and digitalized with a CCD camera (Intas Science Imaging Instruments GmbH, Göttingen, Germany).

3.4.4. Cloning and heterologous *gtf-2* expression

The *gtf-2* gene from *L. brevis* TMW 1.2112 was cloned into the expression vector pNZ8048 and transformed into *Lc. lactis* subs. *cremoris* TMW 2.772, according to King, Boes, and Kunji (2015). Figure 3 summarizes the experimental setup.

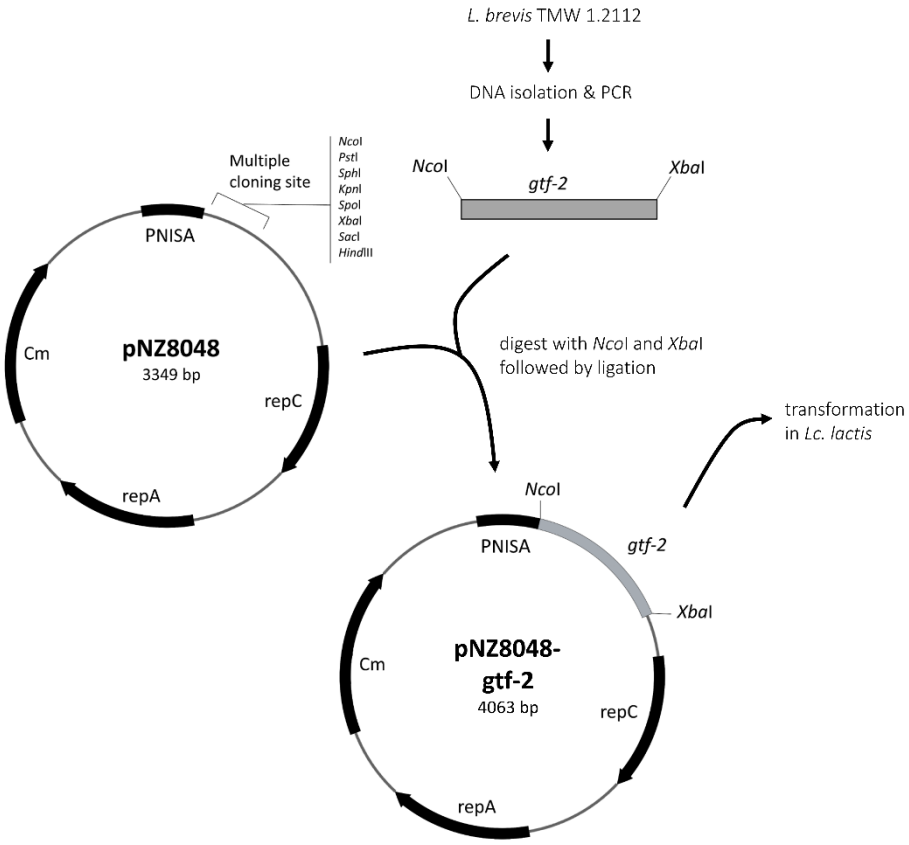

Figure 3 Schematic representation of pNZ8048-gtf-2 vector construction.

Briefly, total genomic DNA was isolated from *L. brevis* TMW 1.2112 using the E.Z.N.A bacterial DNA Kit (Omega Bio-Tek, Inc., Norcross, USA). Subsequently, the *gtf-2* gene was amplified with primers containing restriction sites for *Nco*I and *Xba*I (Table 2).

The expression vector pNZ8048 was isolated from an overnight culture of its carrying strain *Lc. lactis* TMW 2.774 using the plasmid preparation mini kit (Qiagen, Hilden, Germany). After digesting the *gtf-2* PCR product and the expression vector pNZ8048 with the respective restriction enzymes (*Nco*I and *Xba*I, Thermo Fischer Scientific Inc., Waltham, USA) (Table 5), ligation was performed overnight at 4 °C according to Table 6.

Table 5 Reaction mixture for restriction digest of the vector pNZ8048 and PCR-amplified *gtf-2* insert.

Reagent	**Volume [µl]**
CutSmart buffer	5
pNZ8048 plasmid / *gtf-2* PCR product	30
***Nco*I (10000 µ/ml)**	2
***Xba*I (10000 µ/ml)**	1
dH_2O	12

Table 6 Reaction mixture for ligation of the *gtf-2* insert into the pNZ8048 vector

Reagent	Volume [µl]
T4 DNA ligase buffer	2
pNZ8048 plasmid	50 ng (~ 1 µl)
Insert (*gtf-2* PCR product)	50 ng (~ 1 µl)
T4 DNA ligase	1
dH_2O	15

The resulting construct was transformed into *Lc. lactis* subs. *cremoris* TMW 2.771 cells via electroporation using a Gene Pulser Xcell^{TM} Electroporation System (Bio-Rad GmbH, München, Germany). Therefore, cells were grown overnight at 30 °C in M17 supplemented with 0.8 % glucose (Merck KGaA, Darmstadt, Germany) and 1.5 % glycine (Gerbu Biotechnik GmbH, Heidelberg, Germany), followed by inoculation to an OD_{590} of 0.1 in fresh media. After reaching an OD_{590} of 0.6, the cells were harvested, washed three times with and resuspended in washing buffer (dH_2O with 500 mM sucrose and 10 % glycerol). Electroporation was performed in cuvettes with 2 mm distance (Bio-Rad GmbH, München, Germany) with the following conditions: voltage: 2.5 kV, capacitance: 25 µF, low range resistance: 200 Ω, high range resistance: infinite. Immediately after pulsing, 1 ml recovery medium (GM17 with 500 mM sucrose, 20 mM magnesium chloride and 2 mM calcium chloride) was added and the cells allowed to recover for 3 h at 30 °C.

Cells harboring the plasmid were selectively cultivated on $GM17_{Cak}$ agar plates, followed by growth on $GM17_{Cak\text{-}Nis}$ plates to induce the expression of the target protein. Gtf-2-expressing clones were identified via their ropy phenotype and confirmed via colony-PCR and agarose gel electrophoresis. Therefore, a small amount of a single colony was added to a PCR reaction mixture, amplified according to Table 3 and separated through gel electrophoresis as described in chapter 3.4.3.

3.5. Genomics

3.5.1. Genome sequencing, assembly and annotation

Single-molecule real-time sequencing (PacBio RS II) was performed with high molecular weight DNA at GATC Biotech (Konstanz, Germany) (Eid et al., 2009). A library was prepared using one SMRT cell, followed by an assembly with the hierarchical genome-assembly process version 3 (HGAP 3) (Chin et al., 2013).

The genomes were completed by manual curation according to PacBio instructions (https://github.com/PacificBiosciences/Bioinformatics-Training/wiki/Finishing-Bacterial-Genomes) and annotated using the NCBI Prokaryotic Genome Annotation Pipeline and the Rapid Annotations using Subsystems Technology (RAST) (Angiuoli et al., 2008; Aziz et al., 2008; Overbeek et al., 2014).

3.5.2. **Acquisition of published genomes and procession**

Genomes were retrieved from NCBI GenBank as genbank files and downloaded from UNIX command line (wget) from ftp (Burks et al., 1985; Clark, Karsch-Mizrachi, Lipman, Ostell, & Sayers, 2016) before converting them into fasta files using genbank_to_fasta.py (Lee Bergstrand). To obtain consistent annotation, all genomes were additionally annotated with RAST (Aziz et al., 2008; Overbeek et al., 2014). Further, all NCBI proteins were analyzed with the tigr database using an in house analysis pipeline to provide further metadata and information about the annotation quality (Quackenbush, Liang, Holt, Pertea, & Upton, 2000).

3.5.3. **Plasmid prediction**

To predict the origin of contigs and scaffolds of draft genomes (assembly level contig or scaffold) to be chromosomal or plasmid-derived, a blast analysis was conducted against databases containing complete chromosomes and plasmids, respectively.

3.5.4. **Phylogenetics, phylogenomics and cluster analysis**

Phylogenetic and phylogenomic trees were built by performing sequence-based comparison of 16S rDNA, *recA*, *rpoB*, *gyrA*, fragmented all-against-all comparison, codon usage, amino acid usage and PanCore analysis. Therefore, DNA sequences of marker genes were aligned with Clustal W (Thompson, Higgins, & Gibson, 1994) and clustered using Treegraph2 (Stover & Muller, 2010). The reliability of inferred relations was tested with 1000 bootstrap replications (Retief, 2000).

Phylogenomic analysis of the chromosome and plasmidome was conducted via a fragmented alignment using Gegenees (Agren, Sundstrom, Hafstrom, & Segerman, 2012) applying the setting “fast” and a cutoff of 40 %. Data were exported as nexus files and trees built with TreeGraph2 (Stover & Muller, 2010). Chromosomes were analyzed additionally with mauve (A. C. Darling, Mau, Blattner, & Perna, 2004; A. E. Darling, Treangen, Messeguer, & Perna, 2007). Codon usage and amino acid usage were calculated with CMG Biotools, followed by heatmap construction and hierarchical clustering using R gplots package (Vesth, Lagesen,

Acar, & Ussery, 2013). PanCore-trees were also calculated with CMG biotools, applying standard settings. J species allowed the determination of the average nucleotide identity (Richter & Rossello-Mora, 2009). All chromosomal cluster studies included *L. koreensis* as outlier.

3.5.5. **Pan, core and accessory genomes**

To calculate pan, core and accessory genome on nucleotide level, the BlAst Diagnostic Gene finder (BADGE) was used (Behr, Geissler, Schmid, Zehe, & Vogel, 2016). By applying a 90/90 cutoff, genes were assigned to the same family, if 90 % of the alignment was identical and the length of the alignment was more than 90 % of the longest family member sequence. Prior analysis, all genomes were adjusted via an annotation equalizer, which is included into BADGE (Behr et al., 2016).

3.5.6. **Metabolic reconstruction and functional analysis**

Functional categorization was conducted via SEED subsystems (Overbeek et al., 2014). This method enables an assignment of predicted genes to three hierarchical levels – categories, subcategories and subsystems. However, it has to be mentioned that a given gene can be assigned to several subsystems. Function and annotation of genes of interest was studied in detail on protein level using conserved domain search (Marchler-Bauer et al., 2005; Marchler-Bauer et al., 2017; Marchler-Bauer & Bryant, 2004), NCBI BLASTp analysis (Altschul, Gish, Miller, Myers, & Lipman, 1990; Camacho et al., 2009), uniprot (C, Y, & P, 2013), string database (Snel, Lehmann, Bork, & Huynen, 2000; Szklarczyk et al., 2015) and KEGG mapper (H. Kanehisa & Fukunaga, 2014; M. Kanehisa & Goto, 2000; Okuda et al., 2008).

3.5.7. **Visualization of genome comparison**

Genomic analyses were visualized using the Anvio'o software (Eren et al., 2015) and BLAST Ring Image Generator (BRIG) (Alikhan, Petty, Ben Zakour, & Beatson, 2011).

4. RESULTS

4.1. Selection of strains

4.1.1. Identification of EPS-producing brewery-associated lactobacilli

66 strains isolated from beer and 17 strains from brewery surfaces were examined for their ability to produce EPS in liquid media. Therefore, macroscopic viscosity increases were taken into account as well as altered free falls from a pipette tip. This screening identified 25 EPS-producing strains from the species *L. brevis*, *L. rossiae* and *L. parabuchneri*, listed in Table 7.

Table 7 Characteristics of EPS-producing brewery-associated lactobacilli investigated in this study.

TMW no.	Species	Isolation source	Flow time [sec]	x fold viscosity increase*	Beer-spoiling potential
1.2144	*L. brevis*	wheat beer	22.7 ± 2.6	3.8	non
1.2145	*L. brevis*	strong beer	45.7 ± 0.5	7.6	strong
1.2107	*L. brevis*	wheat beer	27.7 ± 0.9	4.6	strong
1.2108	*L. brevis*	wheat beer	38.7 ± 1.7	6.4	strong
1.2147	*L. brevis*	wheat beer	44.0 ± 0.8	7.3	strong
1.2146	*L. brevis*	wheat beer	52.7 ± 0.5	8.8	strong
1.2148	*L. brevis*	wheat beer	31.0 ± 2.9	5.2	strong
1.2149	*L. brevis*	wheat beer	34.3 ± 1.7	5.7	strong
1.2150	*L. brevis*	wheat beer	49.7 ± 4.0	8.3	strong
1.2151	*L. brevis*	wheat beer	38.7 ± 2.6	6.4	strong
1.2111	*L. brevis*	wheat beer	48.7 ± 0.5	8.1	strong
1.2153	*L. brevis*	wheat beer	49.7 ± 3.7	8.3	strong
1.2154	*L. brevis*	lager beer	52.1 ± 0.6	8.7	strong
1.2110	*L. brevis*	wheat beer	58.0 ± 2.2	9.7	strong
1.2152	*L. rossiae*	wheat beer	13.7 ± 0.5	2.3	strong
1.2109	*L. brevis*	wheat beer	26.3 ± 1.2	4.4	strong
1.2112	*L. brevis*	wheat beer	120.3 ± 4.0	20.1	strong
1.240	*L. brevis*	beer	27.0 ± 1.4	4.5	strong
1.599	*L. brevis*	beer	42.7 ± 2.5	7.1	medium
1.1141	*L. parabuchneri*	beer	12.0 ± 0.0	2.0	medium
1.2155	*L. brevis*	beer	71.7 ± 2.4	11.9	strong
1.2156	*L. rossiae*	beer	8.0 ± 0.0	1.3	strong
1.2114	*L. brevis*	wheat beer	20.0 ± 0.8	3.3	strong

1.2115	*L. brevis*	wheat beer	15.7 ± 0.5	2.6	strong
1.2113	*L. brevis*	brewery surface	107.3 ± 2.4	17.9	weak

*Compared to the control (mMRS without inoculation)

4.1.2. **Quantification of viscosity-increasing properties**

The identified EPS-producing lactobacilli were characterized concerning their viscosity-increasing properties. For this purpose, the flow time of a bacterial culture through a flow cup was used as estimation for the viscosity. The maximal flow time of all strains within a cultivation period of five days is summarized in Table 7. Compared to the control (medium without inoculation), which caused a flow time of 6 sec, the bacterial strains caused 1 – 20 fold increases of viscosity. In this regard, *L. brevis* TMW 1.2112 represented the strongest slime producer with flow times of 120.3 ± 4.0 sec.

4.1.3. **Determination of beer-spoiling potential**

To prove the relevance of the identified EPS-producing lactobacilli for the spoilage of beer, their spoilage ability was determined in wheat, lager and pilsner beer. Consequently, a categorization into weak, medium and strong beer spoiler was possible, since the spoilage ability increases from wheat to lager to pilsner beer. The reliability of the assay was proven and confirmed by determining the growth of a well-known strong (*L. brevis* TMW 1.313) and a non-beer spoiling strain (*L. brevis* TMW 1.6), which showed, as excepted, growth in each or none of the test beers, respectively. 21 EPS-producing strains exhibited strong beer-spoiling abilities, while two strains possessed medium and one weak spoiling properties. Just one strain was not able to spoil any test beer (Table 7).

4.2. Physiological characterization of *L. brevis* TMW 1.2112

Since *L. brevis* TMW 1.2112 combined strong beer-spoiling abilities with strongest viscosity-increasing properties, this strain was selected for deeper characterization.

4.2.1. **The slimy phenotype of *L. brevis* TMW 1.2112**

The strain exhibited a slimy phenotype on solid and in liquid culture. Growth on agar plates resulted in ropy colonies forming long filaments when extending the colony surface with an inoculation loop, while growth in liquid cultures caused highly viscous liquids with macroscopically visible slime formation (Figure 4).

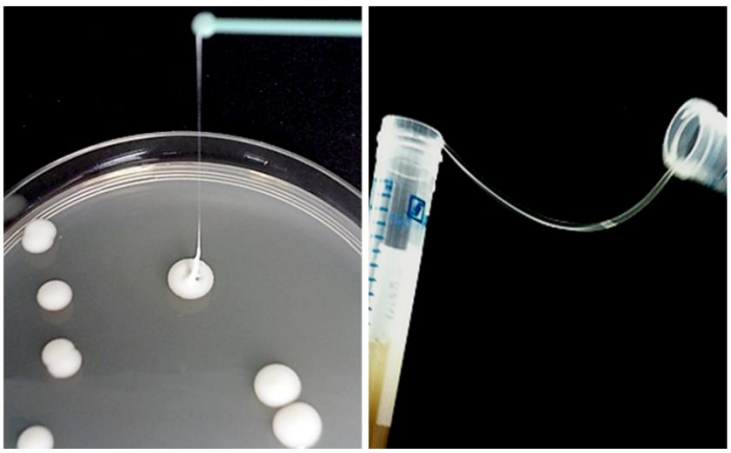

Figure 4 Ropy, viscous phenotype of *L. brevis* TMW 1.2112 on solid and in liquid culture, respectively.

The inoculation of cultivation tubes with *L. brevis* TMW 1.2112 caused diffuse growth and successive slime formation (Figure 5). At the growth maximum, bacterial cells as well as slime occurred all over the tube. Subsequently, the effect reversed, starting with the sedimentation of cells and the gradual disappearance of slime. The resulting cell pellet retained the slimy characteristics for several days. In contrast, the supernatant had lost all viscous properties. By shaking the cultivation tube, the cell pellet spiraled upwards like a thick mucoid string.

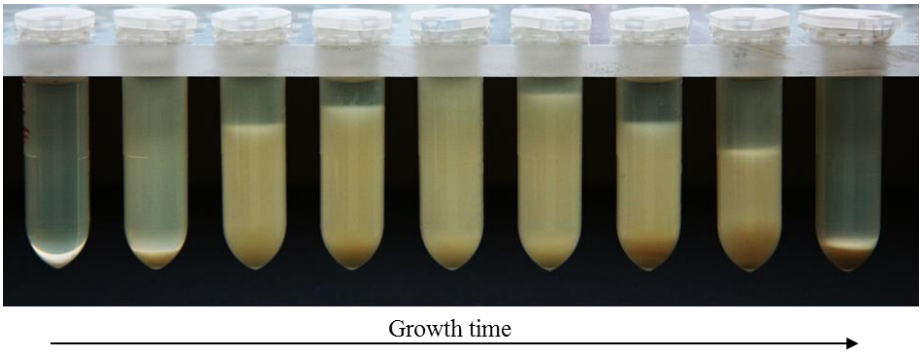

Figure 5 Growth and slime-forming dynamics of *L. brevis* TMW 1.2112 over a period of six days.

4.2.2. **Fermentation pattern**

The fermentation profile of *L. brevis* TMW 1.2112 was assessed using an API 50 CHL system. Out of 48 carbohydrates, 14 enabled bacterial growth (Table 8). This included the pentoses L-arabinose, D-ribose, D-xylose and methyl-βD-xylopyranoside as well as the hexose(-based) sugars D-glucose, D-maltose, D-melibiose, D-fructose, D-galactose and D-gentiobiose. Weak growth was also observed on the derivates of glucose methyl-αD-glucopyranoside, N-acetylglucosamine, potassium gluconate and potassium 5-ketogluconate.

Table 8 Fermentative pattern of *L. brevis* TMW 1.2112 evaluated with an API 50 CHL system.

Growth	No growth
L-arabinose, D-glucose, D-fructose, D-melibiose, gentiobiose, potassiumgluconate, methyl-αD-glucopyranoside, potassium 5-ketogluconate	glycerol, erythriol, D-arabinose, L-xylose, D-adonitol, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-αD-mannopyranoside, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-lactose, D-sucrose, D-trehalose, inulin, D-melezitose, D-raffinose, amidon, glycogen, xylitol, D-turanose, D-Xylose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium 2-ketogluconate

4.2.3. **Sugar- and pH-dependent growth**

Growth on API-positive carbohydrates, which can be present in beer, was investigated in more detail by recording OD-based growth curves in mMRS at low (4.3) and high (6.2) initial pH values. Three pentoses (D-xylose, L-arabinose, D-ribose), three hexoses (D-glucose, D- fructose, D-galactose) and two disaccharides (D-maltose, D-melibiose) were examined.

As expected, all conditions enabled growth, but with different efficiency (Figure 6). Both, the available sugar and the initial pH influenced the growth characteristics.

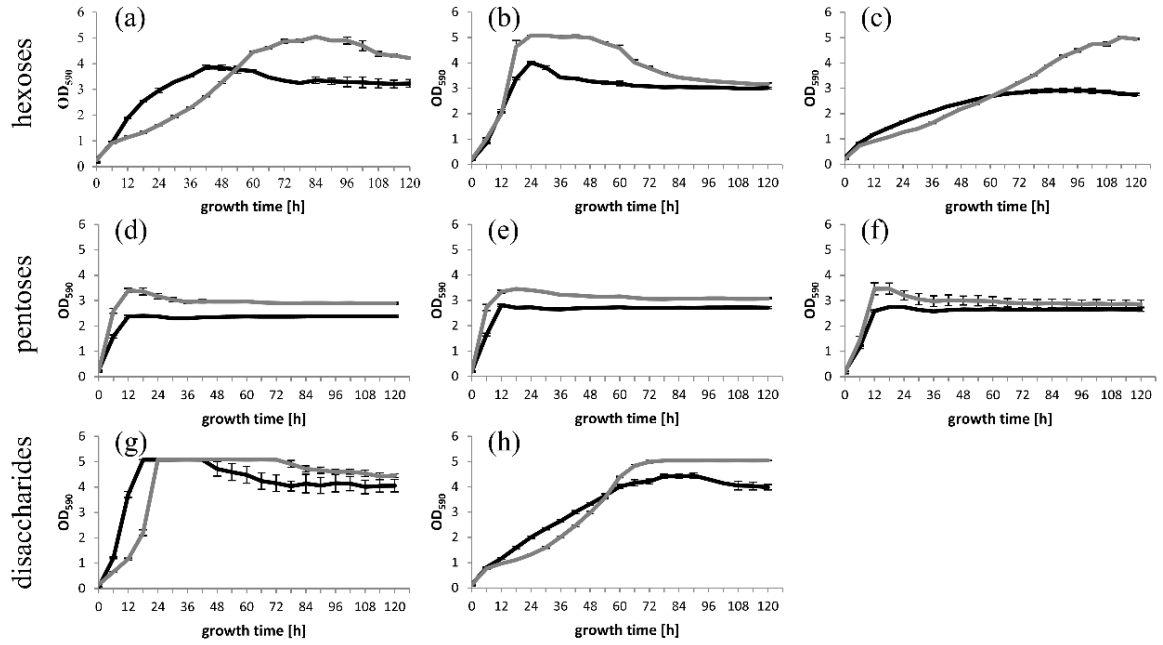

Figure 6 Sugar- and pH-dependent growth curves based on optical densities measured at a wavelength of 590 nm over five days. Black line: pH 4.3, grey line: pH 6.2. A: D-glucose, B: D-fructose, C: D-galactose D: D-ribose, E: L-arabinose, F: D-xylose, G: D-maltose, H: D-melibiose.

Regarding the impact of the different carbohydrate sources on the bacterial growth, D-maltose and D-fructose were the most favored sugars, enabling strongest and fastest growth (Figure 6(b)+(g)). D-melibiose and D-glucose caused a longer exponential growth phase, but led to comparatively high maximal optical densities (OD_{max}) as well. In contrast, D-galactose seemed to be a less favored sugar for growth (Figure 6(c)). In contrast to hexoses, all pentoses enabled a better growth at a higher initial pH of 6.2 (Figure 6(d-f)).

4.2.4. **Sugar- and pH-dependent slime formation**

In addition to the analysis of the influences of different initial pH and carbohydrate sources on cell growth, the impact of these factors on slime formation by *L. brevis* TMW 1.2112 was investigated. Therefore, the flow time of 50 ml cultures through a flow cup was measured and used as estimation for the viscosity of the cell-containing liquid. Flow times were determined all 12 h and maximum ones compared to each other (Figure 7).

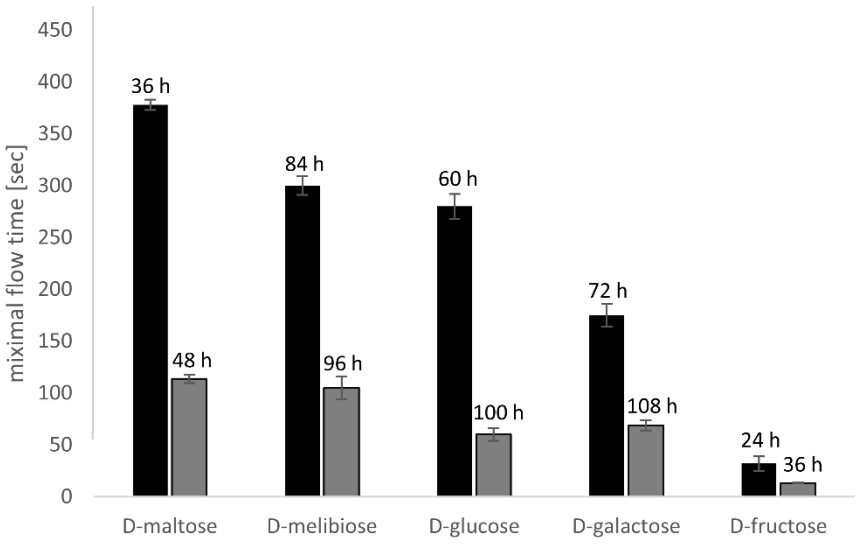

Figure 7 Sugar- and pH-dependent viscosities expressed as flow time. The time point of maximal viscosity is indicated. Black: pH 4.3, grey: pH 6.2.

The control (mMRS without inoculation) caused flow times of six seconds, independently of the pH of the medium (data not shown). The pentoses L-arabinose, D-ribose and D-xylose failed to induce slime formation and behaved similar to the control. In contrast, growth on all hexoses (D-glucose, D-fructose, D-galactose) and disaccharides (D-maltose, D-melibiose) caused slimy cultures.

Thereby slime formation strongly depended on both, the initial pH and the carbohydrate source.

Regarding the impact of the initial pH, low pH media resulted in all cases in stronger and faster slime formation than high initial pH medium. Low initial pH caused at least two fold higher viscosities, independently from the carbohydrate source.

D-maltose and D-fructose showed comparable growth characteristics (Figure 6), but they completely differed regarding their EPS-formation supportive properties. D-maltose represented the carbohydrate causing strongest slime formation (flow time 378 ± 5.23 sec), while fructose was the least supportive one (flow time 13 ± 0.47 sec). The residual sugars exhibited comparatively slight deviations with the tendency melibiose > glucose > galactose.

4.3. Characterization of EPS produced by *L. brevis* TMW 1.2112

4.3.1. Structural characterization of EPS and CPS

For the structural characterization of the exopolysaccharides formed by *L. brevis* TMW 1.2112, CPS from the cell pellet and EPS located in the supernatant were isolated and studied using multiple chromatographic and spectroscopic approaches. Methylation analysis was used to screen the glycosidic linkages of the polysaccharides (Table 9). CPS yielded only glucose-derived PMAAs, whereas the chromatograms of the EPS preparation additionally showed galactose- and mannose-derived PMAAs.

Table 9 Percentages of the partially methylated alditol acetates from the *L. brevis* TMW 1.2112 CPS and EPS preparation.

Glycosidic linkage	CPS	EPS
t-Glc*p*	50.7 %	54.6 %
1,3-Glc*p*	23.8 %	23.5 %
1,2,3-Glc*p*	25.5 %	21.9 %

t = terminal. Numbers indicate the substituted positions of a sugar unit.

Mannose and galactose were also detected by high performance anion exchange chromatography (HPAEC) after acid hydrolysis. Therefore, a control composed of precipitated growth medium was analyzed. Galactose and mannose as well as the detected galactose- and mannose-derived PMAAs were found in the control. In addition, both the control and the samples contained minor amounts of 1,4-substituted glucose, demonstrating that galactose, mannose, and (1→4)-linked glucose are medium-derived and most likely not exopolysaccharide constituents. Thus, it was possible to conclude that CPS and EPS contain

terminal-, 1,3-, and 1,2,3-substituted D-glucose units (the absolute configuration of glucose was determined by GC-MS after chiral derivatization). However, the high portions of terminal glucose residues suggest an underestimation of the 1,3- and/or the 1,2,3-substituted glucose units. Nevertheless, methylation analysis strongly suggests that both EPS and CPS of *L. brevis* TMW 1.2112 are are composed of (1→3)-linked glucans with branches at position *O*2. The ratios between the 1,3- and 1,2,3-substituted glucose units indicate a ramification at every second backbone unit.

To confirm these results and to assess the anomeric configuration of the monomeric units, one- and two-dimensional NMR spectroscopy was applied. Analysis of Correlated spectroscopy (COSY), Total correlated spectroscopy (TOCSY), and Heteronuclear single quantum coherence (HSQC) spectra of the polysaccharides allowed for the assignment of all 1H and 13C chemical shifts of the three structural units present (terminal, 1,3-, and 1,2,3-substituted glucose) (Table 14). The 1,3- and 1,2,3-substituted glucose units showed a characteristic downfield shift for their C3/H3 or, respectively, their C2/H2 and C3/H3 correlation peaks in the HSQC spectrum (Figure 8), indicating a substitution at this position. The ^{13}C chemical shifts of the unsubstituted ring protons/carbons showed chemical shifts comparable to the β-anomer of monomeric glucose, suggesting the presence of β-glucose residues.

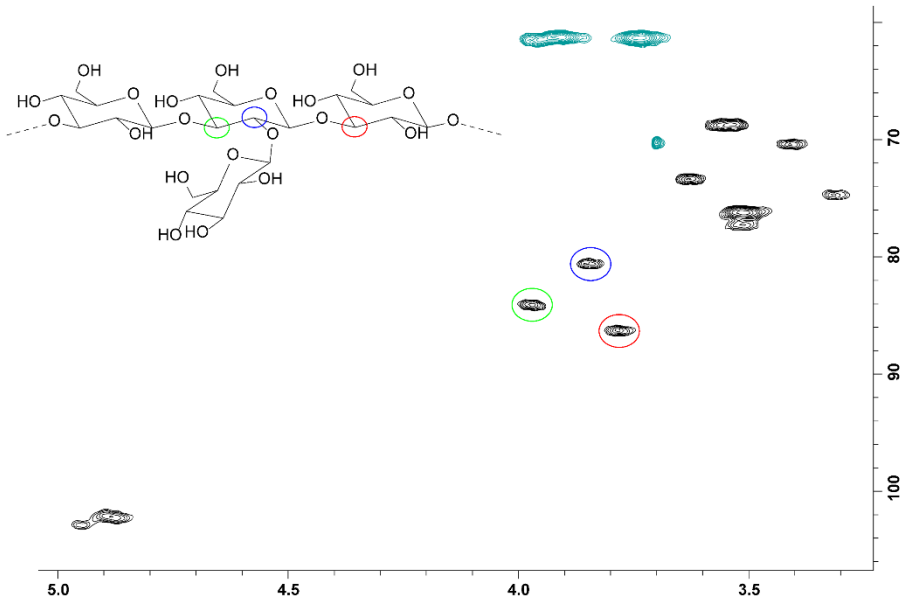

Figure 8 HSQC spectrum and proposed structure of the *L. brevis* TMW 1.2112 CPS preparation. The characteristic downfield shifts of the correlation peaks at the substituted positions are encircled.

In addition, all ^{13}C chemical shifts of the terminal glucose residues were in good agreement with the values reported for laminaribiose (β-(1→3)-linked glucobiose) (Roslund, Tahtinen,

Niemitz, & Sjoholm, 2008). On the other hand, slightly different chemical shifts were described for a β-(1→3)-linked glucan with branches at position *O*2 from *P. damnosus* (Duenas-Chasco et al., 1997). However, similar trends for the downfield/upfield shifts of the protons/carbons were observed but chemical shifts constantly differ by 0.12 ppm (^{1}H) or 1.5 ppm (^{13}C), respectively. Thus, the chemical shift discrepancies are likely due to varying solvents or different referencing. Taking all results into account, it can be concluded that the *L. brevis* TMW 1.2112 exopolysaccharides are composed of a backbone of β-(1→3)-linked glucose units, which are ramified with β-glucose residues at position *O*2.

As stated before, the 1,3- and 1,2,3-substituted glucose units were most likely underestimated by methylation analysis. Thus, volume integration was performed for the C2/H2 correlation peaks to get a semiquantitative estimate of the ratios between the structural elements. These signals should have roughly comparable $^{1}J_{CH}$ coupling constants and consequently an approximately comparable response. The ratio obtained for 1,3- and 1,2,3-substituted glucose units (1/0.8) indicated highly branched polysaccharides with ramifications at about every second backbone residue. In addition, terminal β-glucose residues were detected in amounts comparable to the branched backbone residues, suggesting that the terminal glucose units are mostly derived from ramifications.

4.3.2. **Localization of EPS**

To confirm the possible capsular localization of the identified β-glucan, an agglutination test with *S. pneumoniae* 37-specific antiserum was carried out. The strain *L. brevis* TMW 1.2112 agglutinated in presence of the antiserum, thus demonstrating the presence of a β-glucan at the cell surface as CPS (Figure 9).

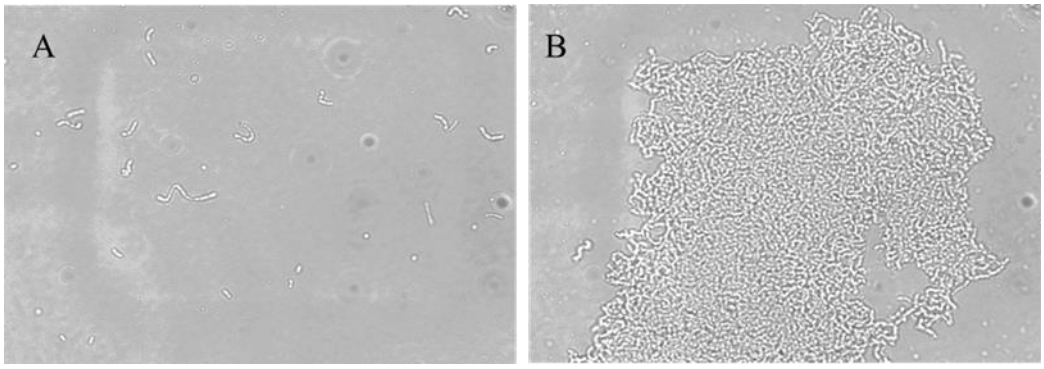

Figure 9 Agglutination induced by *S. pneumoniae* type 37-specific antiserum. *L. brevis* TMW 1.2112 without antiserum (A) and after antiserum addition (B).

4.3.3. **Influence of the carbohydrate source on EPS composition and size**

D-Glucose, D-fructose, D-maltose, D-galactose and D-melibiose represented those carbohydrates inducing slime formation of *L. brevis* TMW 1.2112 (Figure 7). The EPS produced by the strain growing onto these sugars at low pH conditions (pH 4.3) were extracted at time points of highest viscosities and analyzed with HPLC-RI and AF4-MALS to determine similarities and differences in its composition (and size).

Sugar monomer analysis revealed, that the carbohydrate source had no influence on the sugar composition of the resulting polysaccharide. The retention time of the detected monomer was in all cases consistent with the retention time of the standard D-glucose, showing the polysaccharide being a glucan (Figure 27), which is in agreement with the previous results.

Furthermore, all glucans produced from different carbon sources exhibited highly similar elution profiles during AF4-MALS separation/analysis (Figure 10).

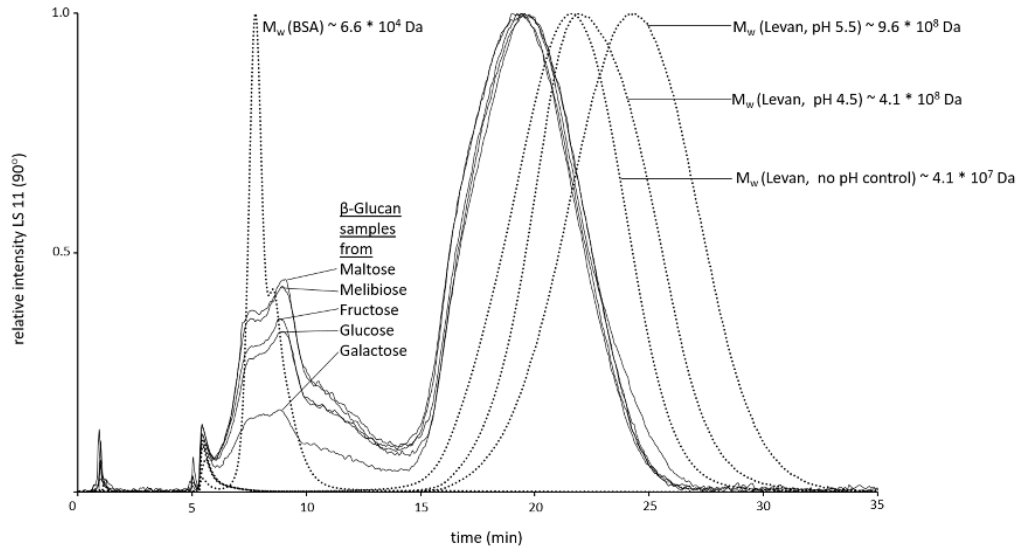

Figure 10 AF4-MALS elution profiles derived from light scattering (LS) detector 11 (90°). Bovine serum albumin (BSA) and levan (Ua-Arak et al., 2017) were used as standard compounds (dotted lines). Glucans produced from maltose, melibiose, fructose, glucose and galactose by *L. brevis* TMW 1.2112 are depicted in solid lines. Heavier molecules elute later during AF4 separation due to comparatively lower diffusion coefficients (Jakob et al., 2013; Nilsson, 2013).

While their molecular weights could not be determined due to non-usable concentration signals derived from refractive index and UV detections, all isolated glucans were composed of two main fractions ("low molecular weight" fraction of ~ 10^4-10^5 Da; "high molecular weight" fraction of ~ 106-107 Da), whose approximate molecular weight ranges were estimated from comparison to the elution profiles of the known standard polymers bovine

serum albumin (BSA) and levan produced by *Gluconobacter albidus* after 32 h with or without pH control (Ua-Arak et al., 2017) (Figure 10).

4.4. Genetic background of glucan synthesis

4.4.1. Genome sequencing and analysis

To gain more insights into the molecular background of β-glucan formation, genomic DNA was isolated from *L. brevis* TMW 1.2112 and sequenced via single-molecule real-time sequencing technology (PacBio RS II). General genome parameters and accession numbers are listed in Table 10.

Table 10 Strain characteristics, sequencing statistics, genome information, and accession numbers.

Strain	Source	BioSample no.[a]	Accession no.[b]	Cov.[f] (x)[c]	Size (Mb)	No. of contigs[d]	G+C content (%)	CDS[e]
L. brevis TMW 1.2108	beer	SAMN04517635	CP019734 - CP019742	148	2.92	9	45.29	2.582
L. brevis TMW 1.2111	beer	SAMN04517636	CP019743 - CP019749	341	2.88	7	45.32	2.440
L. brevis TMW 1.2112	beer	SMNO4517633	CP016797 - CP016802	396	2.67	6	45.72	2.537
L. brevis TMW 1.2113	brewery surface	SAMN04517634	CP019750 - CP019754	364	2.67	8	45.74	2.357

[a] All BioSamples are part of the BioProject PRJNA313253
[b] Accession numbers are listed for all contigs of each whole genome (as a range)
[c] Average coverage of HGAP assembly
[d] In chromosome plus plasmids and partial plasmids
[e] CDSs, number of coding sequences (total) based on NCBI PGAP
[f] Cov. = Coverage

On plasmid pl12112-4, a glycosyltransferase-2 (EC number 2.4.1.34, http://www.cazy.org/b8236.html) was identified (Figure 11), which was described as key enzyme for the synthesis of β-(1,3-1,2)-glucans (Karnezis et al., 2000) and belongs to the glycosyltransferase family 2 of carbohydrate active enzymes. The *gtf-2* gene contains 1704 nucleotides with a predicted protein product of 567 amino acids. Topological analysis indicates that the gene encodes a transmembrane protein with five helices (CBS prediction server; http://www.cbs.dtu.dk/services/TMHMM/). The other eight genes surrounding the *gtf-2* are not involved in sugar metabolism, but in the maintenance and transmission of the plasmid.

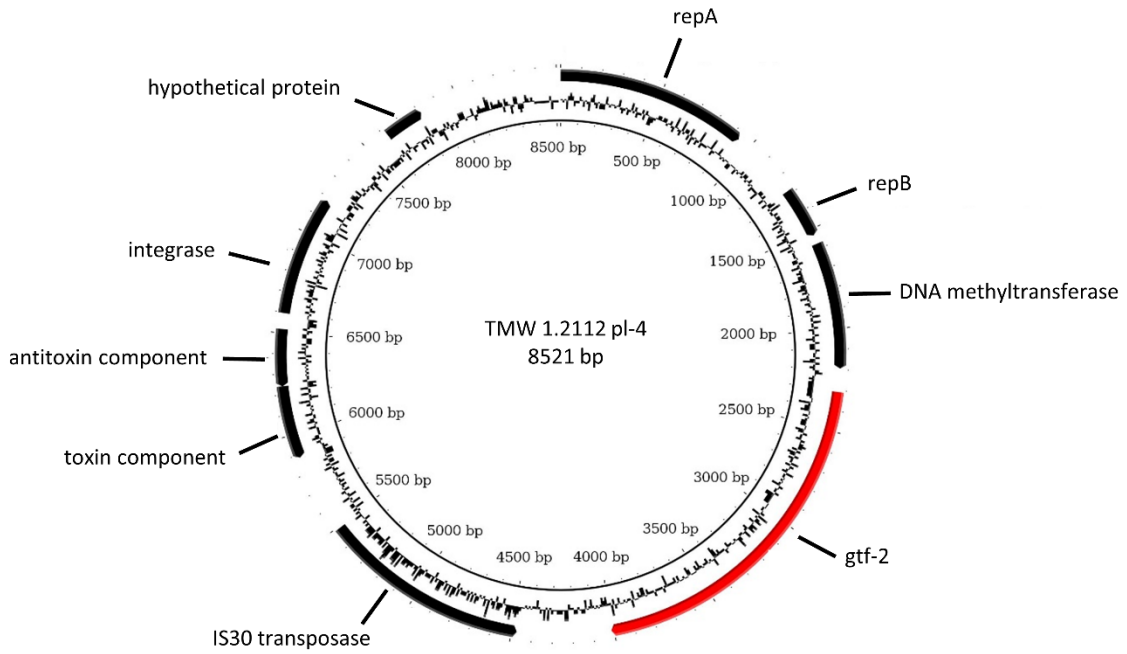

Figure 11 RAST-annotated plasmid pl12112-4 of *L. brevis* TMW 1.2112. The predicted ORFs are mapped on plasmid 4 using BRIG. Starting from inside: circle 1 shows the general position in kilobases; circle 2 depicts the G+C content; circle 3 presents genes encoded, in red the *gtf-2*. With exception of *gtf-2*, the genes are not related to EPS formation, only for plasmid conservation. repA/B: plasmid replication protein A/B.

4.4.2. *Gtf-2* comparison

The genomes of three further strains were sequenced additionally, namely from *L. brevis* TMW 1.2108 and *L. brevis* TMW 1.2111, both isolated from wheat beer, and *L. brevis* TMW 1.2113, which was derived from a brewery surface. The sequencing statics are given in Table 10. Comparing their genomes revealed the presence of a comparable *gtf-2*-carrying plasmid in all strains.

BLAST analysis identified further *gtf-2*-encoding LAB as well, all described to cause the slimy spoilage of beer, wine or cider. The public available plasmid or *gtf-2* sequences were mapped against the biggest *gtf-2*-carrying plasmid sequenced in this study, namely from *L. brevis* TMW 1.2108. Figure 12 illustrates the homologous regions of a certain strain to the reference plasmid. This shows the *gtf-2* gene to be highly conserved among all these strains, making it to a highly interesting diagnostic marker gene.

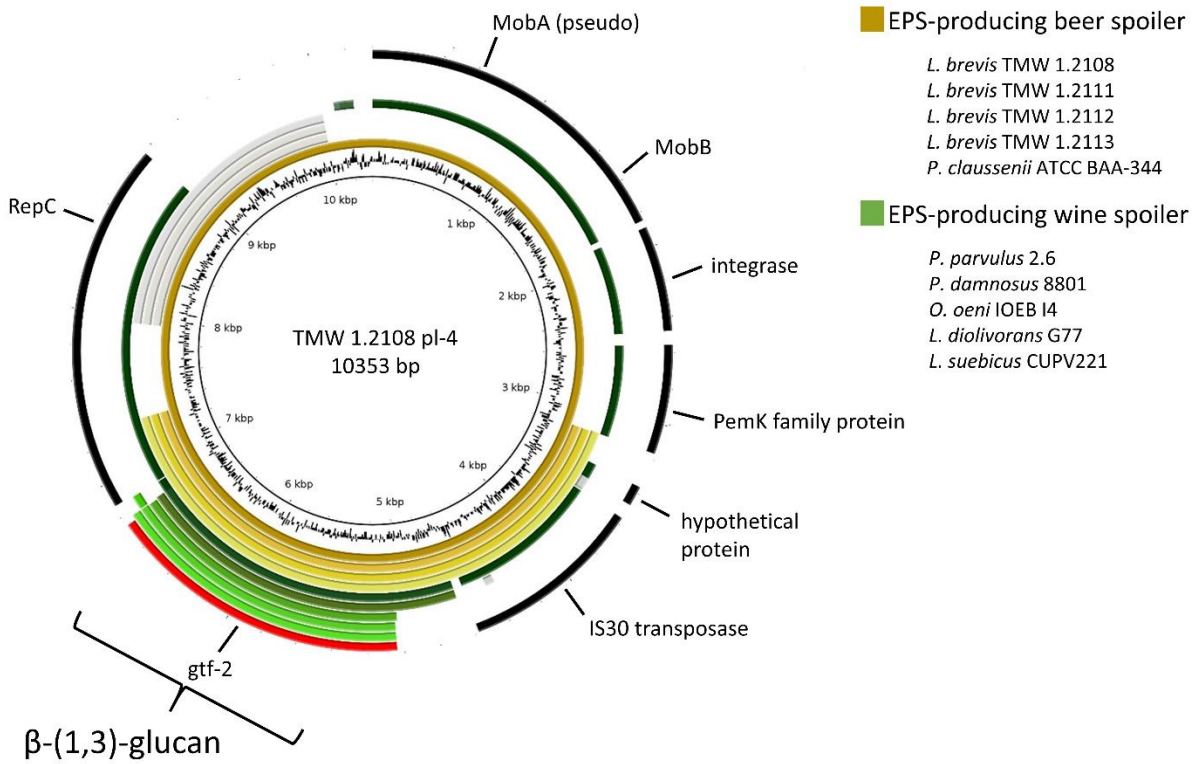

Figure 12 Similarities between plasmid pl12108-6 of *L. brevis* TMW 1.2108 and plasmids from other EPS-producing beverage spoilers. Starting from inside: circle 1 shows the general position in kilobases; circle 2 depicts the G+C content of *L. brevis* TMW 1.2108; circle 3 – circle 7 exhibit the corresponding genes of plasmids from beer-spoiling bacteria *L. brevis* TMW 1.2111, *L. brevis* 1.2112, *L. brevis* 1.2113, *P. claussenii* ATCC BAA-344; circle 8 – 12 exhibit the corresponding genes of plasmids from wine-spoiling bacteria *P. parvulus* 2.6, *P. damnosus* 8801, *O. oeni* IOEB I4, *L. diolivorans* G77, *L. suebicus* CUPV221; circle 13: presents genes encoded on pl12108-6 of *L. brevis* TMW 1.2108, in red the *gtf-2*.

Detailed information to the species and isolation source of these beverage-spoiling LAB as well as the gtf-2 particularities and locus tags are summarized in Table 11.

Table 11 Overview of EPS-producing beverage-spoiling lactic acid bacteria.

Strain	Origin	*Gtf* location	AA gtf	*Gtf* identity	Poly-saccharide	Locus tag*/ accession no.
L. brevis TMW 1.2112	beer	plasmid	567	-	β-(1→3)-glucan	AZI09_12770
L. brevis TMW 1.2113	beer	plasmid	567	99	β-(1→3)-glucan	AZI10_RS13125
L. brevis TMW 1.2111	beer	plasmid	567	98	-	AZI12_RS14330
L. brevis TMW 1.2108	beer	plasmid	567	98	-	AZI11_RS14540

P. claussenii ATCC BAA-344T[1,2]	beer	plasmid	567	99 %	β-(1→3)-glucan	PECL_RS09485
P. damnosus IOEB8801[3]	wine	plasmid	567	99 %	β-(1→3)-glucan	AF196967
P. parvulus 2.6[4]	cider	plasmid	567	99 %	β-(1→3)-glucan	AY999683
L. diolivorans G77[5]	cider	plasmid	567	99 %	β-(1→3)-glucan	AY999684
L. suebicus CUPV221[6]	cider	chr	567	99 %	β-(1→3)-glucan	GU174474
O. oeni IOEB0205[7]	wine	chr	567	97 %	β-(1→3)-glucan	EU556433
O. oeni I4[8]	cider	chr	567	98 %	β-(1→3)-glucan	AY999685

AA = amino acids; *Gtf* identity = sequence identity to *gtf-2* of *L. brevis* TMW 1.2112; chr = chromosome; * locus tag only used when available

[1] = (Pittet et al., 2012) [2] = Juvonen et al. (2015) [3] = Walling, Gindreau, et al. (2005), [4] = Werning et al. (2006), [5] = Werning et al. (2006), [6] = Garai-Ibabe et al. (2010), [7] = Dols-Lafargue et al. (2008), [8] = Werning et al. (2006)

4.4.3. *Gtf-2* screening

To verify the relevance of the *gtf-2* gene within the slimy spoilage of beer and to prove its suitability to serve as diagnostic marker gene, 50 lactobacilli, with or without beer-spoiling and with or without EPS-producing properties were screened for the presence of this gene (Table 7 and Table 11). Therefore, primers targeting a 900 bp region from the gene from *L. brevis* TMW 1.2112 were designed and used for PCR. All slime-forming strains generated the expected amplification signal (Figure 13), whereas amplification with DNA from non-slime forming strains remained negative.

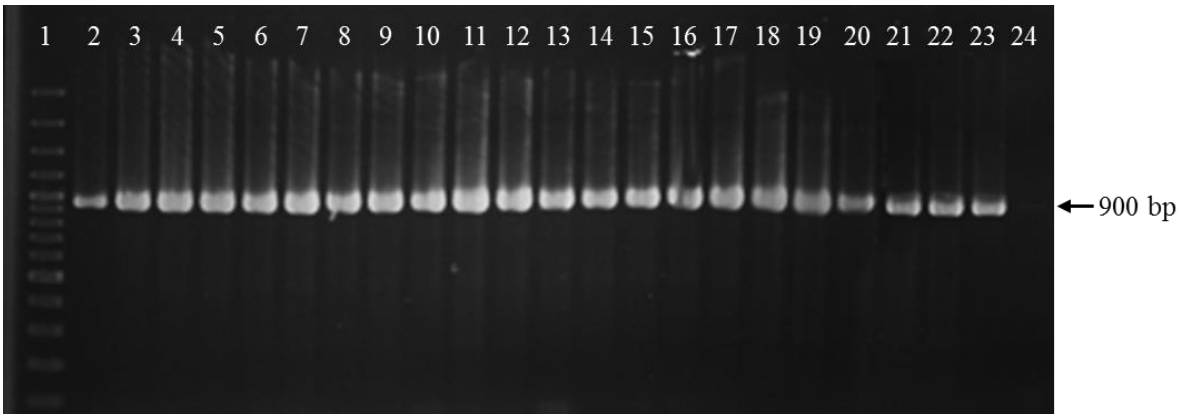

Figure 13 Gel electrophoresis of PCR amplicons generated with primers Gtf-F/Gtf-R on the following strains: *L. brevis* (line 2-20), *L. parabuchneri* (line 21), *L. rossiae* (line 22-23) – according to Table 7. Negative control (line 24), molecular mass standard (line 1). The length of the amplicon is indicated.

These results support the importance of the gtf-2 in the formation of glucans and suggest it as diagnostic marker gene for an early detection of EPS-forming beer-spoiling LAB.

4.4.4. Reconstruction of (sugar-dependent) glucan formation

To gain insights into the mechanism of glucan formation and into the metabolism of glucan-formation inducing carbohydrates (identified in chapter 4.2.4), the genomic data from *L. brevis* TMW 1.2112 were investigated regarding the putative metabolic steps from one specific carbohydrate to UDP-glucose, which is the substrate of gtf-2 for β-glucan biosynthesis (Llull, Garcia, & Lopez, 2001; Llull, Munoz, Lopez, & Garcia, 1999; McIntosh, Stone, & Stanisich, 2005; Walling, Gindreau, et al., 2005; Werning et al., 2006).

Glucose-1-phosphate (Glc-1-P) is suggested as key intermediate, as it represents the precursor of UDP-glucose. The glucose moiety from UDP-glucose is subsequently polymerized by the gtf-2 to glucan molecules and extruded. The presence of genes predicted to encode the enzymatic conversion of D-glucose, D-fructose, D-maltose, D-melibiose and D-galactose to UDP-glucose was checked within the genome of *L. brevis* TMW 1.2112. In this way, potential pathways leading to UDP-glucose were derived as displayed in Figure 14. The locus tags of the identified putative transporters and enzymes are described in Figure caption 14.

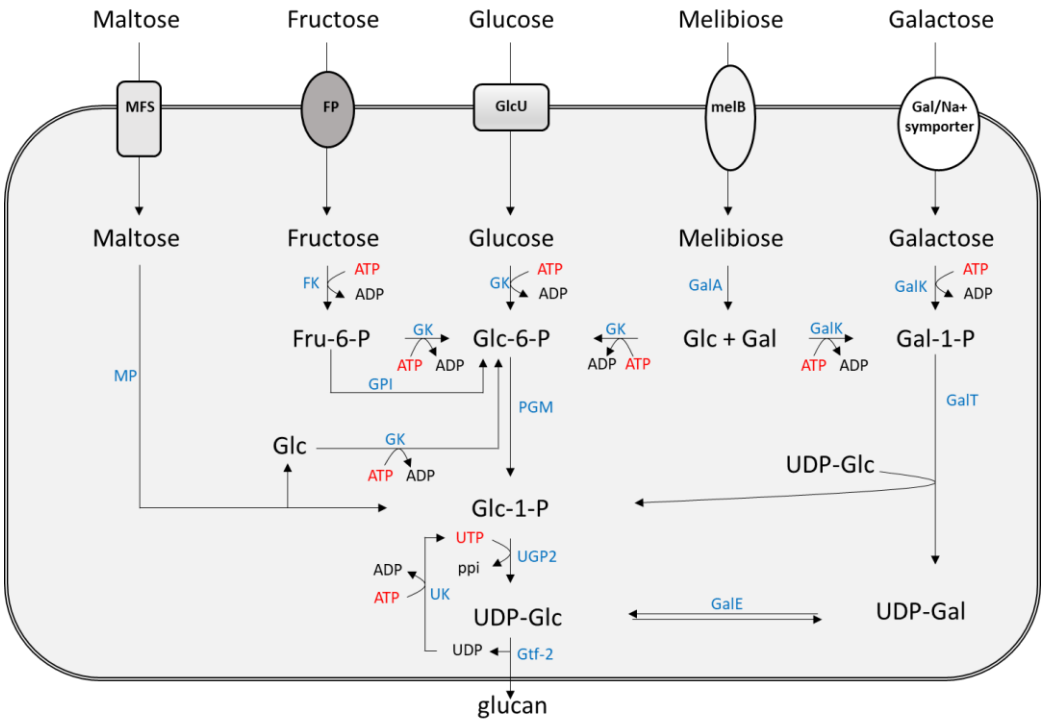

Figure 14 Proposed scheme for β-glucan biosynthesis of *L. brevis* TMW 1.2112 as derived from predictive genomic analysis depicting putative transporters for uptake and enzymes for intracellular conversion of different sugars. MFS: major facilitator superfamily transporter (AZI09_12515), GlcU: glucose transporter (AZI09_08395), P: fructose permease (AZI09_01055), melB: melibiose carrier protein (AZI09_11470), GK: glucokinase (AZI09_07205), FK: fructokinase (AZI09_01045), MP: maltose phosphorylase (AZI09_10320), PGM: phosphoglucomutase (AZI09_02415), GPI: glucose-6-phosphat isomerase (AZI09_08390), GalA: α-galactosidase (AZI09_08490), GalK: galactokinase (AZI09_02430), GalT: Galactose-1-phosphate uridylyltransferase (AZI09_02440), UGP2: UTP-glucose-1-phosphate uridylyltransferase (AZI09_08865), Gtf-2: glycosyltransferase-2 (AZI09_12770), GalE: UDP-glucose-4-epimerase (AZI09_02435) UK: UDP kinase (AZI09_05835), Gal/Na+ symporter: sodium solute symporter (AZI09_02425).

4.5. Role of gtf-2 in glucan formation

4.5.1. Heterologous gtf-2 expression

To investigate the assumed key position of the *gtf-2* gene in glucan synthesis, the respective gene was transformed heterologously from *L. brevis* TMW 1.2112 into *Lc. lactis* TMW 2.772. The genome of the resulting transformant strain *Lc. lactis* TMW 2.2036-Gtf^{+} consequently differed from its wild type just in the *gtf-2*-carrying plasmid.

Correct *gtf-2* incorporation and uptake was proven and confirmed via PCR and agarose-gel electrophoresis. After inducing gtf-2 expression by adding nisin, the strain showed a mucoid and ropy phenotype on agar plates, which formed long filaments when extending with a loop (Figure 15). This phenotype is identical to that one of *L. brevis* TMW 1.2112, while the wild type *Lc. lactis* TMW 2.772 did not show any conspicuous appearance.

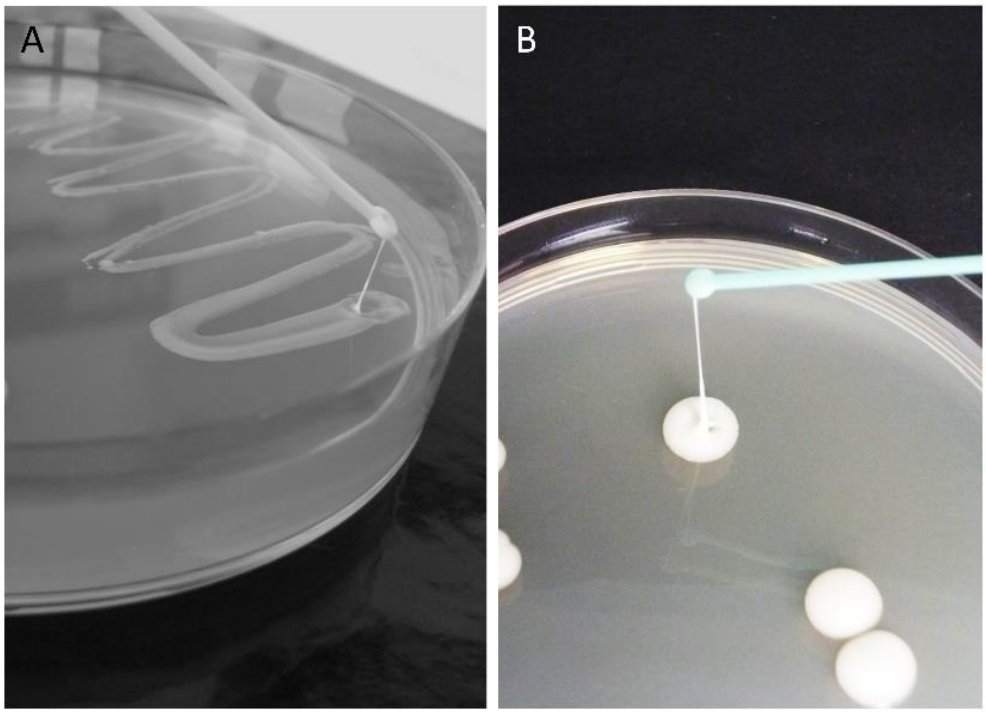

Figure 15 Ropy phenotype of the gtf-2 expressing *Lc. lactis* TMW 2.2306-Gtf^{+} (A) and the gtf-2 donor *L. brevis* TMW 1.2112 (B).

This confirmed that the *gtf-2* gene is sufficient to cause EPS synthesis and consequently to be the decisive gene for EPS synthesis in lactic acid bacteria. However, macroscopically observable EPS formation was restricted to solid culture, as liquid cultures did not show any viscosity increase or rheological alteration.

4.5.2. **Characterization of recombinant produced EPS**

To check the identity of the heterologously produced polysaccharide HPLC-RI, NMR spectroscopy and methylation analysis were used. This analysis revealed the presence of a β-(1,3-1,2)-glucan in the supernatant, which is identical to the polysaccharide produced by the *gtf-2*-host *L. brevis* TMW 1.2112.

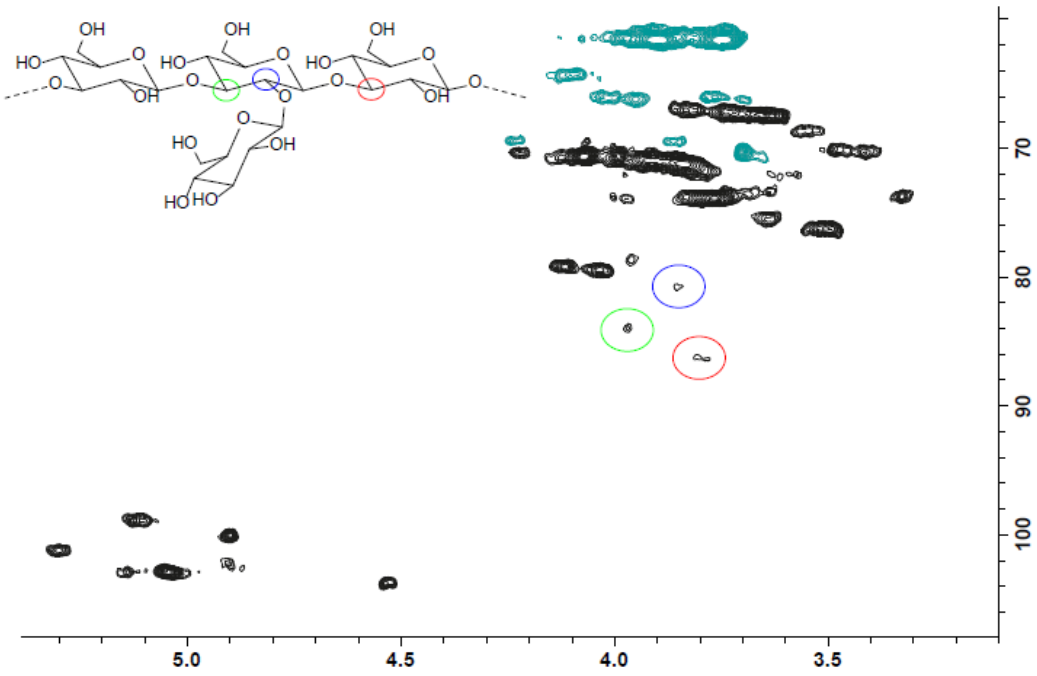

Figure 16: HSQC spectrum and proposed structure of the *Lc. lactis* TMW 2.2036-Gtf$^+$ EPS preparation. The characteristic downfield shifts of the correlation peaks at the substituted positions are encircled.

The exopolysaccharides produced in liquid and on solid medium were investigated in more detail concerning their localization by using an agglutination assay. CPS formation was found to be restricted to growth on agar plates. Performing the assay with liquid cultures did not cause any agglutination, demonstrating the EPS at least to be partially released into the surrounding medium (Figure 17).

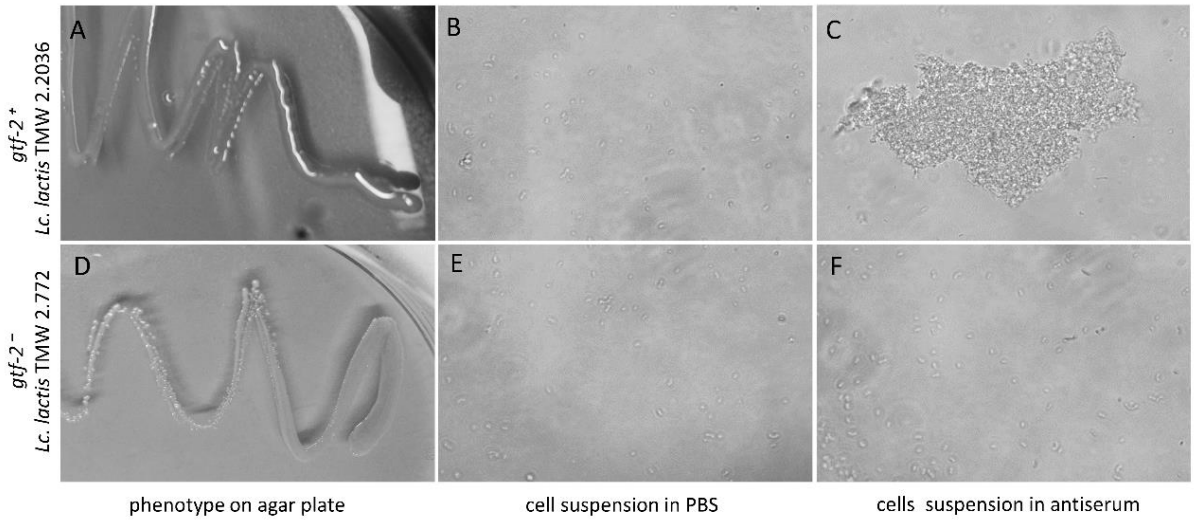

Figure 17 Phenotype of *Lc. lactis* TMW 2.2036-Gtf$^+$ (A) and *Lc. lactis* TMW 2.772 (D) on agar plate and in liquid culture (B, E). Addition of *S. pneumoniae* type 37-specific antiserum caused agglutination of *Lc. lactis* TMW 2.2036-Gtf$^+$ (C), but not of *Lc. lactis* TMW 2.772 (F).

4.6. Physiological contribution of glucan formation on the bacterial cell

4.6.1. Impact on stress tolerance

The impact of EPS formation on the growth in presence of stress factors like ethanol, acid and hydrogen peroxide was determined comparatively between *Lc. lactis* transformant TMW 2.2036-Gtf$^+$ and wild type TMW 2.772, on solid and in liquid media. In liquid culture, the gtf-2-expressing strain did not possess an increased tolerance against any hurdle, as all MIC values were identical between *Lc. lactis* TMW 2.2036-Gtf$^+$ and TMW 2.772. In contrast, growth on solid culture enabled the gtf-2-expressing transformant strain a higher resistance against all stress factors tested (Table 12). Comparing the MIC values of *Lc. lactis* wildtype versus (vs.) its CPS-forming transformant showed a repression by ethanol at 10 % vs. 12 %, by H_2O_2 at 1 vs. 1.5 mM and by pH at 4.00 vs 3.75.

Table 12 MIC values of *Lc. lactis* TMW 2.772 and 2.2036-Gtf$^+$ grown on solid culture.

Stress factor	*Lc. lactis* TMW 2.772 wildtype	*Lc. lactis* TMW 2.2036-Gtf$^+$ transformant
pH	4.0	3.75
H_2O_2 [mM]	1.0	1.5
EtOH [%]	10	12

4.6.2. Impact on biofilm formation

Investigating the ability to form biofilms in microtiter plates showed that both, the transformant and the wild type strain to be incapable to adhere to surfaces, as the washing steps following cultivation caused the removal of nearly all cell material. However, on solid culture, capsule formation of *Lc. lactis* TMW 2.2036-Gtf$^+$ caused an aggravated detachment of single colonies, showing at least increased adhesive properties.

4.7. The genomic diversity and niche-adaption of the *L. brevis*

The most potent glucan-producing *L. brevis* TMW 1.2112 is part of the most abundant beer spoiler species *L. brevis*, while it remains elusive how this species got into and adapted to this man-made environment, which is much too young for any directed evolution. The beer-spoiling abilities of *L. brevis* were postulated to generally reside in the plasmidome (Geissler et al., 2017)), as is also the case for most β-glucan producers. So this part (also) should provide a better insight into the pan-plasmidome of *L. brevis*. Therefore, all available *L. brevis* genomes were assessed under consideration of the isolation source.

4.7.1. General genomic features properties

26 genomes of *L. brevis* strains were retrieved from the NCBI public database to investigate the genomic diversity of this species. The strains originated from various sources of isolation and were roughly classified according to their isolation source in plant, diary, mammal, insect and brewery groups. Their genome size ranges from 2.3 to 2.9 Mb and is composed of a chromosome with or without an additional plasmidome. The strains possess a G+C content varying between 43.06 and 43.52 %, with the exception of *L. brevis* ATCC 27305 (40.20 %) and *L. brevis* BM-LB12908 (53.50 %). The G+C content of these two outliers strongly deviates compared to the remaining strains. Their membership to the *L. brevis* species was further disproven by BLAST analysis of conserved housekeeping genes (16s rDNA, *recA*, etc.). Consequently, the set was reduced to 24 strains for following investigations. General genomic parameters of all strains are summarized in Table 13.

Table 13 General features and genome statistics of *L. brevis* genomes.

ID	Group	Origin	Assembly	Sequ. tech.	Contigs		Length		GC [%]	
					Chr	Pla	Chr [Mbp]	Pla [Kbp]	Chr	Pla
TMW 1.465[1]	**brewery**	soft drink – brewery	scf	454	30	3	2.5	20.8	44.4	40.8
TMW 1.313[2]		beer	scf	454	1	10	2.6	166.1	44.0	36.8
TMW 1.2113[3]		brewery surface	comp	PacBio	1	4	2.5	130.5	45.9	41.9
TMW 1.2112[4]		beer	comp	PacBio	1	5	2.5	184.6	46.0	41.9
BSO 310[5]		brewery	scf	Illum	106	29	2.5	159.8	45.8	40.9
BSO 464[6]		brewery	comp	454	1	8	2.5	219.2	45.7	40.2
TMW 1.2108[7]		beer	comp	PacBio	1	8	2.6	351.9	45.8	41.4
TMW 1.2111[8]		beer	comp	PacBio	1	6	2.6	310.8	45.8	41.3
AG48[14]	**mammal**	sheep rumen	contig	Illum	4	5	2.4	227.1	46.1	41.4
DPC 6108[15]		feces (human)	contig	PacBio	3	8	2.7	234.5	45.8	39.0

47f[16]		feces (human)	contig	454	74	64	2.3	292.5	46.1	40.5
15f[17]		feces (human)	scf	454	75	22	2.4	77.1	46.2	39.9
Lb1595[18]		bovine teat (france)	contig	Illum	38	12	2.4	88.8	46.1	41.4
ATCC 14869[19]		GIT (human)	scf	Illum	86	0	2.5	-	45.9	-
DSM 20054[20]		feces	scf	Illum	11	0	2.5	-	45.9	-
TMW 1.6[21]		feces	scf	454	1	0	2.4	-	45.8	-
EF[22]	**insect**	*D. melano.*	contig	Illum	19	12	2.7	206.7	45.7	40.4
DmCS 003[23]		*D. melano.*	contig	Illum	48	46	2.6	301.6	45.8	41.4
EW[24]		*D. melano.*	contig	Illum	19	11	2.7	211.8	45.7	40.2
D6[13]	**dairy**	cheese	contig	454	63	60	2.4	243.3	46.1	40.9
TMW 1.1326[9]	**plant**	silage	scf	454	1	2	2.3	49.0	46.2	38.6
NPS-QW-145[10]		korean kimchi	comp	Illum	1	0	2.6	-	45.8	-
WK12[11]		Kimchi	contig	Illum	58	52	2.3	295.6	46.1	39.9
KB290[12]		fermented vegetable	comp	Sanger	1	9	2.4	192.7	46.1	38.9
BM-LB 13908[25]	-	beer	scf	Illum	417	0	2.8	-	53.5	-
ATCC 27305[26]	-	human	scf	454; Illum	178	0	3.2	-	40.2	-

Accession no: [1]JXUG01000004, [2]JXUF01000006,[3]CP019750, [4]CP016797, [5]LGIX01000001, [6]CP005977, [7]CP019734, [8]CP019743, [9]NC_008497, [10]NZ_CP015398, [11]NZ_BBOW01000110, [12]AP012167, [13]LQNG01000001, [14]NZ_JAGR01000001, [15]MDUA01000001, [16]LBHR01000001, [17]JXCD01000036, [18]LDEI01000001, [19]KI271195, [20]AZCP01000001, [21]JXUE01000020, [22]NZ_LPXV01000010, [23]JOKA01000074 , [24]NZ_AUTD01000001, [25]NZ_LTDY00000000, [26]NZ_ACGG00000000; Scf: scaffold; comp: complete; Chr: chromosome; Pla: plasmidome; Seq. tec.: sequencing technology; *D. melano.*: *Drosophila melanogaster*; Illum: Illumina.

1045 genes were found to be shared by all 24 strains and represent the core genome. 4444 genes were attributed to the accessory genome, resulting in a pan genome with 5489 genes. The respective Pancore analysis is illustrated by Figure 18.

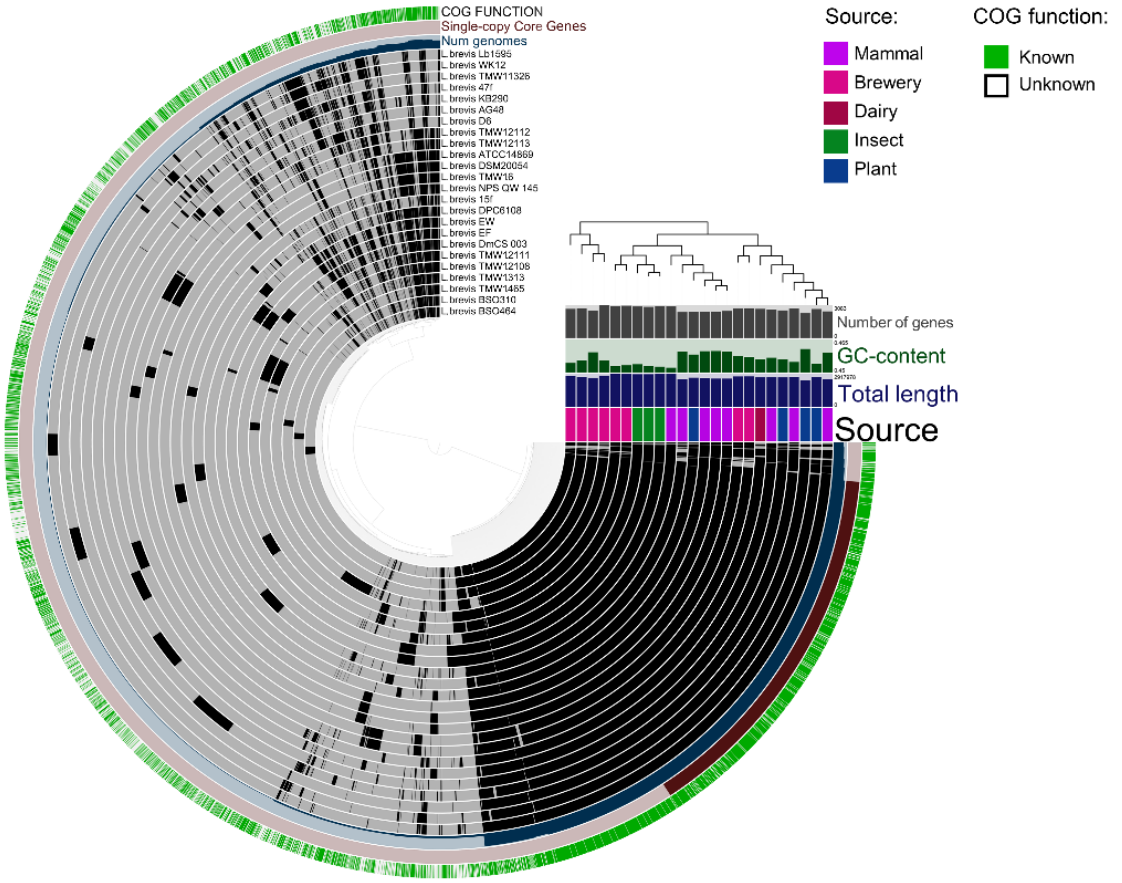

Figure 18 Genetic diversity of *L. brevis* displayed using Anvi'o software (Eren et al., 2015). The figure is showing the distribution of orthologous groups (OG) across this species. Each ring represents one strain, each layer shows the OG distribution (black: OG present, grey: OG absent). The top right panel provides further metadata about the strains. The clustering bases on grouping according to OG distribution. Each strain is coloured according to the origin of isolation.

The impact of this genetic diversity on metabolic traits was predicted by assigning pan, core and accessory genome in SEED categories (Figure 19) and conducting functional genome analysis.

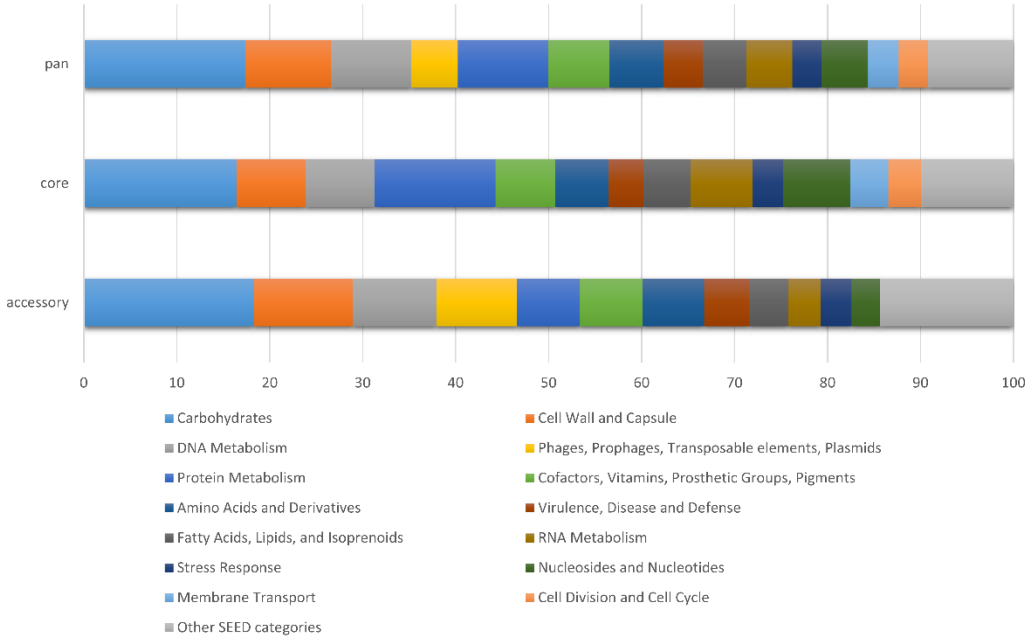

Figure 19 SEED category analysis of pan, core and accessory genome of all *L. brevis* genomes. All categories with a proportion less than 3 % were summarized as "Other SEED categories".

These investigations support the predicted metabolic diversity, which is reflected, among others, in the ability to metabolize citrate. According to their genetic constitution, only half of the strains are able to catabolize citrate and to use it as another carbon source for their growth and enhanced tolerance against acidic conditions (Laëtitia, Degraeve, & Yann, 2014). A further metabolic specification is displayed within three gastrointestinal-associated strains, which are characterized by the ability to utilize ethanolamine, an ability commonly associated with gut bacteria. This trait enables to use ethanolamine, a compound of cell membranes from mammalian and bacterial cells, as source of carbon and/or nitrogen (Garsin, 2010). The ability to reduce cytotoxic nitroaromatics while recycling pyridine nucleotides, mediated by a p-nitrobenzoate reductase, is restricted to 58 % of the investigated strains. Two strains possess an incomplete adenine deiminase pathway, possibly reducing their acid tolerance. Finally, three strains are lacking a pyrimidine reductase, playing a role in the biosynthesis of cofactors.

There are various other examples affecting all kinds of metabolic pathways. However, nearly all these metabolic capabilities are distributed within all strains regardless of their isolation source. There were no metabolic pathways, being unique for all strains of one source of isolation.

4.7.2. **Chromosomal comparison**

To investigate, if chromosomal similarity measures correlate to the isolation source and cause an environment-associated clustering, phylogenetic trees were constructed using different phylogenetic measures and marker genes.

Neither a clustering based on common housekeeping genes nor on 16s rDNA, codon usage and amino acid usage enabled a source-specific grouping. Further, the strains did not position together based on phylogenomic analysis of their chromosomes (Figure 20).

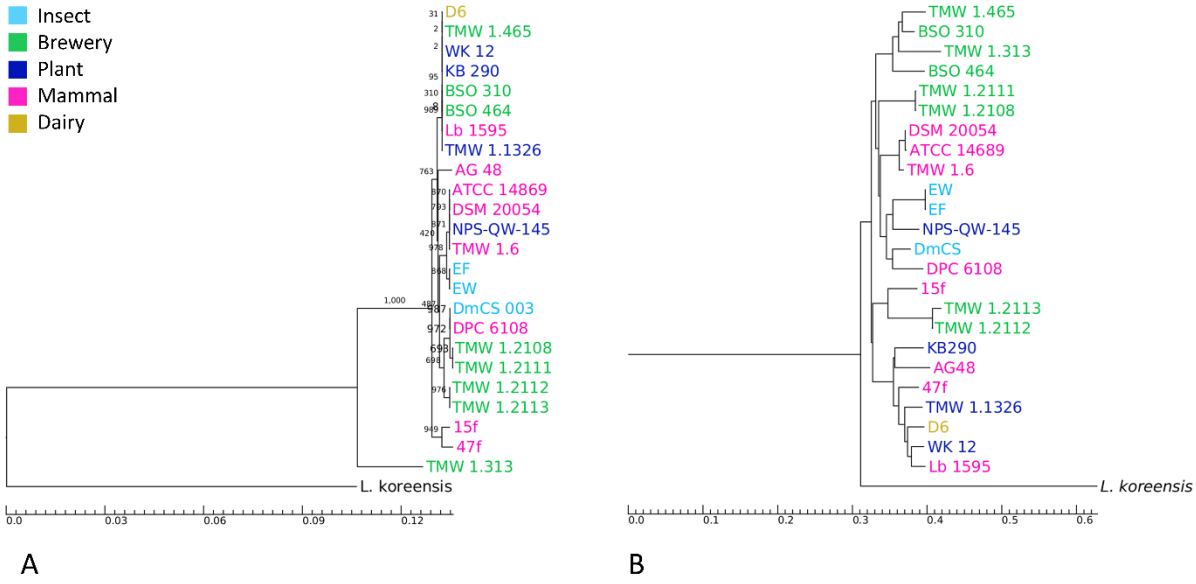

Figure 20 Similarity tree of *L. brevis* chromosomes based on the genetic marker *recA* (A) and a chromosomal alignment using mauve (A. C. Darling et al., 2004) (B). Each strain is color coded by origin of isolation. *L. koreensis* was included as outlier.

4.7.3. **Plasmidome comparison**

4.7.3.1. Phylogenomic comparison

While chromosomes constitute the more static part of the genome, plasmids are known as the mobile and dynamic part (Thomas & Summers, 2001). Therefore, phylogenomic analysis was performed on plasmidome level as well, using the all-against-all fragmentation algorithm of Gegenees.

This analysis enabled a grouping of brewery-derived and insect-derived strains in distinct clusters, both closely related (Figure 21). Strains belonging to the other environments still showed a mixed distribution.

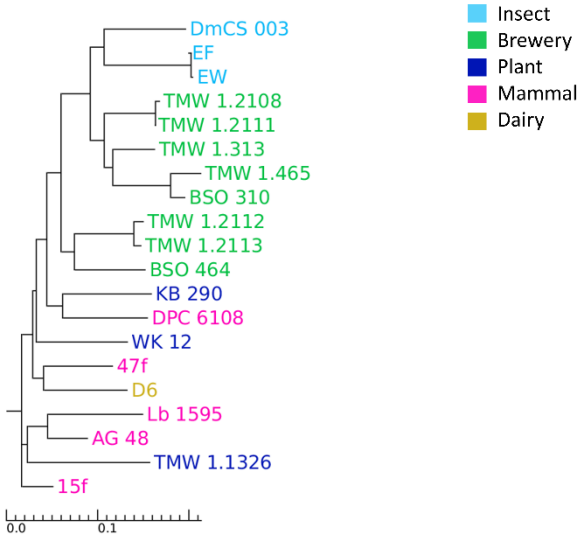

Figure 21 Phylogenomic tree of *L. brevis* plasmidomes based on a fragmented all-against-all comparison (Agren et al., 2012). Each strain is color coded by origin of isolation. *L. koreensis* was included as outlier.

To gain more insights into this clustering phenomenon, BADGE was applied to identify the plasmid-encoded, source-specific gene pool and to reveal genetic intersections to the other environments. This investigation showed the brewery-derived strains to be the most uniform ones. No other source shared as many source-specific genes as the brewery did (Figure 22), which of course could also be attributed to certain amount to the number of brewery genomes analyzed.

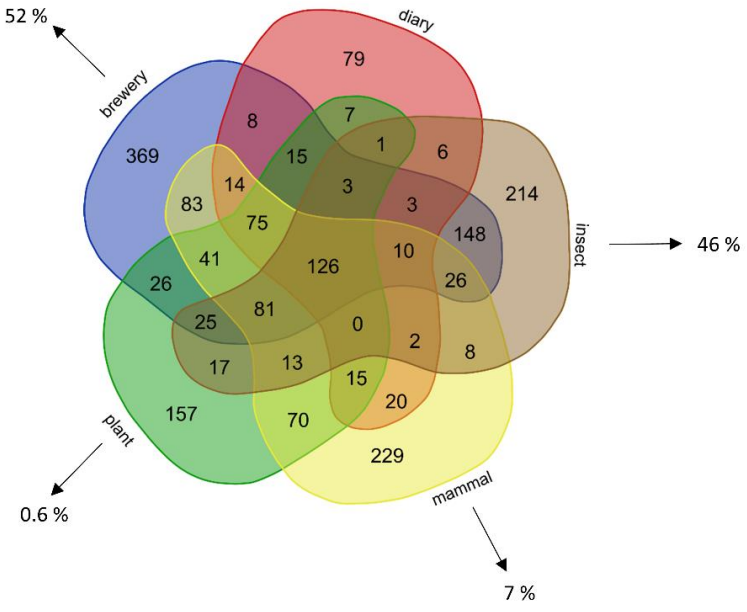

Figure 22 Venn diagram displaying the source-specific plasmidomes and respective intersections. The numbers denoted in plot indicate the number of genes, present in at least two strains of one habitat. (http://bioinformativs.psb.ugent.be/webtools/venn/).

Regarding source-specific genes being present in at least two strains of a respective environment, gave a value of 52 % for the brewery group. Within the insects, which also grouped together, this value reached 46 %. In contrast, genes being shared of minimum two mammal-derived strains just reached 7 %, within the plant group 0.6 %.

This remarkable gene sharing within the brewery- and the insect-associated strains explains their close neighboring within the phylogenomic cluster analysis.

4.7.3.2. Functional analysis

SEED categorization was conducted to get more insights into the possible functionality of the habitat-specific gene pools (Figure 23).

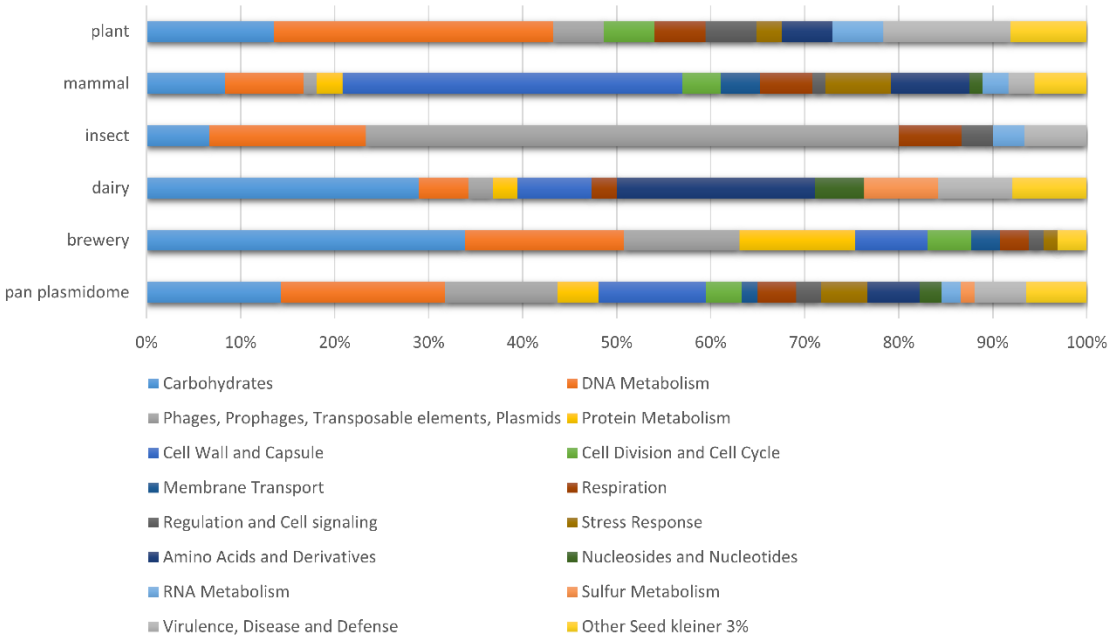

Figure 23 SEED category analysis of plasmid-encoded, habitat-specific genes. All categories with a proportion less than 3 % were summarized as "Other SEED categories".

Genes comprised by the *L. brevis* pan plasmidome are mainly predicted to be involved in carbohydrate and DNA metabolism. Regarding those categories making up the majority within the different groups, "DNA metabolism" showed to have highest prevalence within plants, "cell wall and capsule" within mammal and "phages, prophages, transposable elements, plasmids" within insects. Dairy- and brewery-associated strains shared "carbohydrates" as most abundant functional category. These abundances can at least partially be associated with adaptions to requirements of the respective ecological niche.

L. brevis D6 isolated from cheese is the only strain encoding a plasmid-located cluster involved in lactose and galactose metabolism. This cluster comprises, among others, gluco- and galactokinases and a 6-phospho-β-glucosidase (EC 3.2.1.86), which converts β-1,4-P-lactose to glucose and glucose-6-phosphate. This additional catabolic pathway might enable a more efficient metabolism of lactose, the predominant carbohydrate in dairy products. The abundance of genes involved in cell wall and capsule formation of mammal-derived strains might be referred to a lifestyle adaption as well. The gastrointestinal tract, which constitutes the precise isolation source of most mammal-derived strains, possesses a harsh environment. Acquisition of genes improving the cell wall barrier function could help the strains to deal with these conditions. Moreover, plasmid-located ethanolamine utilization proteins support the above-mentioned chromosomal encoded ethanolamine metabolism of three mammalian strains. As this pathway is common among gut bacteria and not found in other *L. brevis* strains, it can be considered as habitat adaption.

In brewery-associated strains, carbohydrates represented the most dominant SEED category. Beer is due to the yeast fermentation characterized by a low, but divers carbohydrate content, which differs from beer to beer and from brew to brew (Ferreira, 2009). This suggests the need for an efficient metabolism of each sugar and therefore the enrichment of carbohydrate-related genes.

4.7.4. **The brewery plasmidome**

The remarkable plasmidome-based shared gene pool makes the brewery-derived strains to the most consistent group and therefore to the most interesting one for further investigations. The respective strains have a plasmidome ranging in size from 20 – 350 kbp with a G+C content of 38.6 % to 41.9 %.

The brewery pan plasmidome comprises 2853 genes. Functional analysis did not exhibit brewery-associated benefits from non-brewery specific pan genes. Therefore, we focused on the brewery-specific ones, comprising 369 genes. Their distribution between the strains is given in Figure 24.

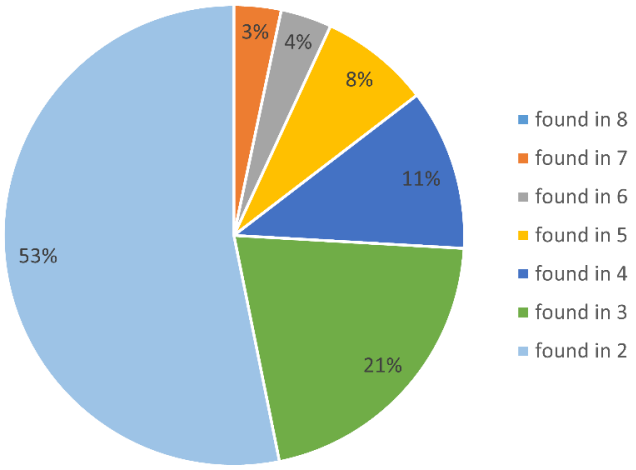

Figure 24 The shared gene pool of the brewery-specific plasmidome. All genes are considered, which were found to be encoded by at least two strains.

47 % of the genes were found to be present in only one strain, the rest was found in at least two different strains. 3 % (12 genes) were encoded by the plasmidomes of 7 from 8 brewery strains; however, no gene was present in all strains.

An assignment of all genes to functional categories allowed a view onto their possible metabolic contribution. But although the annotation and function was analyzed in detail on protein level using conserved domain search (Marchler-Bauer et al., 2005; Marchler-Bauer et al., 2017; Marchler-Bauer & Bryant, 2004), NCBI BLASTp analysis (Altschul et al., 1990; Camacho et al., 2009), uniprot (Consortium, 2017), string database (Snel et al., 2000; Szklarczyk et al., 2015) and KEGG mapper (M. Kanehisa & Goto, 2000; M. Kanehisa et al., 2014; Okuda et al., 2008), the large majority encoded unknown or hypothetical proteins. The functionality and abundance of the remaining genes is illustrated in Figure 25 as word cloud.

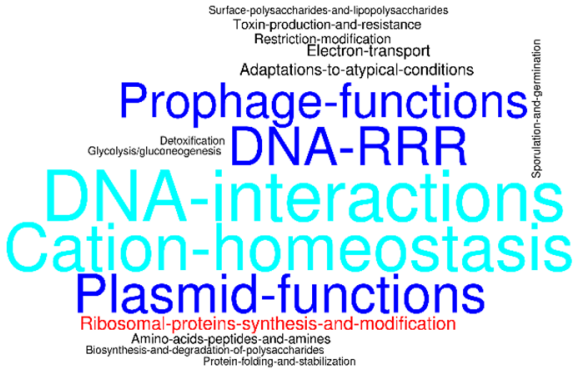

Figure 25 Wordcloud of brewery-specific, plasmid-encoded genes and functions. Font size correlates with gene abundance. Genes/ functions without informative biological function (e.g. hypothetical protein, mobile element protein) were excluded. DNA-RRR: DNA recombination, replication and repair.

Most of them were predicted to beinvolved in DNA metabolism and mobile element functions (plasmid and prophage functions), a common feature of plasmids in general. Traits resulting from the residual gene pool could be partially connected to an adaption to beer-typical hurdles.

This included the expression of like *horA* and *horC*, both well-known hop resistance cluster (Suzuki et al., 2006), encoded in 87 % and 62.5 % of the strains, respectively. The in this work well-studied *gtf-2* gene causing CPS formation was found in 50 % of the studied strains. 62.5 % of the strains encode *hitA*, a Mn^{2+}/H^{+} symporter and well-known hop resistance gene. The magnesium uptake system *CorA* (Kehres et al., 1998) was found in the majority of brewery-associated strains. *CorA* and *HitA* were mostly encoded on the same cluster.

Further, the brewery plasmidome exhibited an enrichment of ATPases, which are supposed to counteract acid stress and genes like the methionine sulfoxide reductase, which are predicted to be involved in the defense of oxidative stress, both well-known antimicrobial properties exerted by hop compounds (Behr & Vogel, 2010; Schurr, Hahne, et al., 2015). Moreover, a high abundance of genes predicted to be involved in carbohydrate metabolism were accumulated in brewery-associated strains (Figure 25). This was evidenced among other things by a gene cluster associated with maltose metabolism.

Unfortunately, there was a large gene pool remaining, whose contribution to the survival in beer stayed, due to missing or bad annotations, elusive.

4.7.5. **The shared gene pool of brewery- and insect-derived *L. brevis* strains**

L. brevis strains isolated from breweries and from insects constituted the only two groups, which built distinct clusters on plasmidome level (Figure 21). Both were closely related and shared the largest gene pool (Figure 22). 40 % of the brewery plasmidome were found in the insect plasmidome as well. In contrast, the plasmidomal overlap between brewery-mammal, brewery-diary and brewery-plant just accounted 22 %, 7 % and 2 %, respectively.

A closer view onto this shared gene pool revealed the presence of a truncated *horC* cluster in strains from both environments. The *horC* cluster, one of the major contributors to hop resistance can occur in two versions – a complete and a truncated one (Suzuki, Iijima, Ozaki, & Yamashita, 2005). While the complete one comprises seven genes, the truncated encodes just the MFS transporter *horC* and its putative regulator *horB*. Until now, both clusters were

considered as brewery-specific. To the best of our knowledge, this is the first time, a *horC* cluster was found in non-brewery associated strains as well.

5. DISCUSSION

Under consideration of the results of the current study, the initial hypotheses (see section 2) can be refined to the following theses:

- EPS-producing brewery-associated bacteria comprise strains of *L. brevis, L. rossiae* and *L. parabuchneri*.
- EPS formation of these bacteria results in viscous liquids.
- The increase in viscosity relies on a capsular β-(1,3)-glucan, which builds a complex network between the cells.
- This cell-bound glucan capsule increases the resistance against environmental stress and contributes to adhesive capabilities.
- Glucan formation is mediated by a plasmid-encoded, highly conserved glycosyltransferase-2, which polymerizes glycosyl residues from UDP-glucose.
- Consequently, glucan formation depends on the availability of hexose-based carbohydrates, which are convertible into UDP-glucose.
- The acquisition of the glycosyltransferase-2 gene is sufficient to create EPS-producing LAB.
- For brewery quality management, the glycosyltransferase-2 represents a suitable marker gene, which enables the specific detection of EPS-producing LAB via PCR.
- The species *L. brevis* possesses a sustainable genomic diversity, which allows the occupation of various ecological niches.
- The adaption to the brewery environment is a plasmid-encoded trait, causing a phylogenomical proximity of brewery-derived *L. brevis* strains on plasmidome level.
- Brewery- and insect-derived *L. brevis* strains show a significant plasmidomal overlap.
- The hop resistance gene *horC* is not brewery-specific as it was found in insect-derived strains as well.
- The evolutionary development of beer-spoiling bacteria occurred outside of beer and at least partially in insects.

These results are highlighted in more detail in the following sections.

5.1. Brewery-associated EPS-producing bacteria

A screening of beer- and brewery-derived lactobacilli (chapter 4.1.1) identified strains of *L. brevis*, *L. rossiae* and *L. parabuchneri* as EPS-producing beer-spoiling bacteria, whereby *L. brevis* constituted the most relevant species.

Under consideration of the strength of beer spoilage and viscosity increase, *L. brevis* TMW 1.2112 isolated from wheat beer was selected as representative strain for deeper characterization. This strain combined strongest beer-spoiling properties with strongest EPS formation (chapter 4.1). Nonetheless, most experiments were performed with other EPS-producing beer-spoiling lactobacilli as well to confirm the assumed transferability of the received results (chapter 9).

5.2. The slimy phenotype of *L. brevis* TMW 1.2112

The slime-forming ability of *L. brevis* TMW 1.2112 was assessed on solid and in liquid media, showing strong slime formation in both cases (Figure 4). Its phenotype is consistent with the characteristics of slime-forming beer-spoiling lactobacilli from Williamson (1959): growth on agar plates resulted in ropy colonies, forming long strings when picking with an inoculation loop followed by a jump onto the loop; growth in liquid culture caused an increased viscosity, whereby cells and EPS occurred all over the tube. Both, the appearance on agar and the behavior in liquid culture imply the formation of a CPS, connecting the cells in a sticky network.

5.3. Viscous spoilage of beer relies on capsular glucan formation

The assumed capsular localization of the polysaccharides produced by *L. brevis* TMW 1.2112 was proved and confirmed in this study via immunological analysis.

The structural characterization demonstrated that the capsule is composed of a β-(1→3)-glucan with ramifications at position *O*2. The PMAA ratios as well as the HSQC peak intensities suggested that about every second 1,3-linked backbone unit is ramified at position *O*2, which was also described for the β-glucans from *P. damnosus* (Duenas-Chasco et al., 1997), *Pediococcus sp*. (Llauberes et al., 1990), and *O. oeni* (Ibarburu et al., 2007). The polysaccharides isolated from the supernatant were identical to those isolated from the cell pellet. Thus, it can be concluded that the CPS is partially secreted into the surrounding medium.

The capsular localization of the polysaccharide was found to be essential for viscosity-increasing effects. A heterologously generated, glucan-producing *Lc. lactis* strain (which exhibited the same ropy phenotype as *L. brevis* TMW 1.2112 on solid culture) was not able to increase the viscosity, even if the presence of an identical β-(1,3-1,2)-glucan was proven. An agglutination test showed the glucan just on solid but not in liquid culture to be clearly cell-associated as capsular polysaccharide, suggesting the lactococcus strain to be unable to anchor the glucan tightly to its cell wall during planktonic growth, which caused its release. However, it has to be noted, that the isolatable EPS amounts were rather low in liquid media, why an inefficient β-glucan formation (in comparison to growth on agar plates) cannot be excluded.

Therefore, viscosity-increasing effects by β-glucan producers upon growth in liquids seem to be rather due to a tight capsular localization of β-glucan and a concomitant cell-network formation than to the produced and released polysaccharide itself.

5.4. Plasmid-endoced *gtf-2* gene mediates glucan formation

5.4.1. Genome sequencing and comparative genomics reveals *gtf-2* gene

To gain more insights into the molecular background of glucan biosynthesis, the whole genome of *L. brevis* TMW 1.2112 was sequenced and analyzed via comparative genomics. This investigation revealed a plasmid-located glycosyltransferase-2 as key gene for the formation of this glucan capsule. This type of glycosyltransferase was described to synthesize glucans by catalyzing the polymerization of glycosyl residues from UDP-glucose (Dols-Lafargue et al., 2008; Karnezis et al., 2000). Therefore, β-(1,3-1,2)-glucan formation by certain LAB resembles more the mechanisms of HePS biosynthesis than those of HoPS from energy-rich disaccharides such as sucrose (McIntosh et al., 2005; Torino et al., 2015).

Next to the *gtf-2* gene, the plasmid harbors eight other genes, all involved in the maintenance and transmission of the plasmid. The maintenance supposedly is ensured by a putative toxin-antitoxin system. This system is composed of two closely linked genes encoding a toxin and its cognate antitoxin, protecting the host against the toxic effect. Loss of the plasmid causes cell death since the unstable antitoxin is degraded earlier and the lasting toxin kills the cell (Yamaguchi, Park, & Inouye, 2011). Accordingly, EPS formation poses a stable phenotype of *L. brevis* TMW 1.2112.

Three other genomes of EPS-producing, beer-spoiling *L. brevis* strains (TMW 1.2108, TMW 1.2111 and TMW 1.2113) were sequenced as well and found to encode a similar *gtf-2*-carrying plasmid.

The *gtf-2* gene is also present on a plasmid of the slimy beer spoiler *P. claussenii* BAA-344 T. Moreover, all genes encoded on the plasmid of *L. brevis* TMW 1.2112 were found on the respective one of *P. claussenii* as well (Figure 26). Due to these similarities and the fact that all other genes encoded by these plasmids are not involved in sugar metabolism, we assume slimy beer spoilage to result mainly from the action of this plasmid-encoded glycosyltransferase-2.

Glucan-forming LAB not only affect beer, but also wine and cider. As in beer, viscous wine and cider are attributed to a gtf-mediated β-(1→3)-glucan formation (Dols-Lafargue et al., 2008; Duenas-Chasco et al., 1998; Ibarburu et al., 2007; Llauberes et al., 1990; Walling, Gindreau, et al., 2005; Werning et al., 2006). Moreover, the glycosyltransferase is species/genera-independently highly conserved among β-glucan-producing LAB.

Comparing the *gtf* gene between twelve slimy beverage-spoiling bacteria from the species *Pediococcus sp.*, *Oenococcus sp*. and *Lactobacillus sp*. showed sequence identities of at least 97 % (Table 11).

In *L. brevis* TMW 1.2108 and *P. parvulus* 2.6, responsible for viscous beer and wine spoilage, respectively, even the whole plasmid sequence is nearly identical.

These similarities in the genetic background and in the structure of the resulting polysaccharide imply a common origin and horizontal gene transfer to be responsible for the dispread of the *gtf* gene within beverage-spoiling lactobacilli and pediococci. This assumption is strengthened by the finding that the *gtf* gene is located on a mobilizable plasmid in *P. parvulus* IOEB8801 (Gindreau, Walling, & Lonvaud-Funel, 2001) and on a conjugative one in *P. parvulus* 2.6 (Werning et al., 2006). In addition, the respective gene of *P. parvulus* 2.6, *P. claussenii* ATCC BAA-344 T and all studied *L. brevis* strains is neighbored to a transposase (IS 30 family). This enzyme is able to cause a translocation of genes within the genome and an integration into the chromosome. This might explain the finding of chromosomal *gtfs* in *O. oeni* (IOEB0205 and I4) and *L. suebicus* CUPV221 which are in turn highly identical to the plasmid-located ones (Table 11). Indeed, the chromosomal *gtf* of *O. oeni* IOEB0205 is found in close proximity to a transposase.

5.4.2. **Heterologous expression confirms key role of gtf-2 in glucan formation**

To verify the assumed decisive role of the *gtf-2* gene in glucan formation, the gene was expressed from *L. brevis* TMW 1.2112 into *Lc. lactis*, strains commonly used for the manufacture of dairy products. The lactococcus strain was chosen as model host because of its amenability to genetic engineering. Another reason was the above-described inability of successful plasmid curing experiments, as the toxin-antitoxin system prevents the plasmid's loss in *L. brevis* TMW 1.2112.

In this way, the essential position of the *gtf-2* gene in glucan formation was confirmed, since this slight genetic alteration was sufficient to generate an EPS-producing strain (*Lc. lactis* TMW 2.2036-Gtf^{+}) with a ropy, mucoid phenotype on solid culture (Figure 15).

NMR spectroscopy of the heterologous produced EPS showed the structure to be identical to those from the *gtf-2*-donoring *L. brevis* strain, namely a β-(1,3-1,2)-glucan. Consequently, a highly specific mechanism of gtf-2-mediated glucose polymerization can be anticipated.

These results are in accordance to Llull et al. (1999) who showed a homologous glycosyltransferase termed "*tts"* to be the only gene required to drive β-(1,3-1,2)-glucan synthesis in *S. pneumonie*. In addition, Stack, Kearney, Stanton, Fitzgerald, and Ross (2010) showed the *gtf-2* gene from *P. parvulus* to be sufficient to induce glucan synthesis in *L. paracasei*.

Consequently, it can be assumed, that LAB acquiring/ getting access to this gene gain glucan-producing properties.

5.5. Mechanism and nutritional requirements of *gtf-2*-mediated glucan formation

To attain a deeper knowledge of the mechanism of glucan formation, the genomic data of *L. brevis* TMW 1.2112 were coupled to practical experiments studying the influence of the initial pH and carbohydrate source on growth, slime formation and EPS composition/ sizes.

Therefore, the fermentative properties of *L. brevis* TMW 1.2112 were studied and found to metabolize 14 different carbohydrates (Table 8). We focused on the growth performance and slime formation of pentoses (L-arabinose, D-ribose, D-xylose), hexoses (D-glucose, D-fructose, D-galactose) and hexose-based disaccharides (D-maltose, D-melibiose), which can be present in beer (Ferreira, 2009). Thereby a correlation between cell growth and slime

formation was found. In all cases, highest viscosities were determined when the stationary phase was reached. This suggests: the more cells, the more polysaccharide is produced.

5.5.1. **Influence of the initial pH on glucan formation**

Analyzing the influence of the initial pH on slime formation in more detail showed that low pH values caused increased slime formation, resulting in at least two fold higher degrees of viscosity than high pH conditions. Moreover, low pH enabled a faster slime formation. Both effects were independent from the carbohydrate source (Figure 7). This implies a comparatively higher activity of the responsible, outer membrane-anchored gtf-2 in an acidic environment, which is also typical for beer (pH 3.8-4.7) (Suzuki, 2011). As already mentioned, wine is affected from β-glucan producing lactobacilli as well formation (Dols-Lafargue et al., 2008; Duenas-Chasco et al., 1998; Duenas-Chasco et al., 1997; Llauberes et al., 1990; Walling, Gindreau, et al., 2005; Werning et al., 2006). As both beverages possess a low pH, the protection against acids is a fundamental requirement for the survival of microorganisms capable of growing in such acidic environments (Suzuki, 2015).

Since this study revealed β-glucan to be produced more efficiently at low pH values, it may be speculated, that harsh acidic environments therefore specifically induce slime formation to exert protective functions.

5.5.2. **Influence of the carbohydrate source on glucan formation**

In addition to the pH, the carbohydrate source influenced the extent of slime/β-glucan formation by *L. brevis* TMW 1.2112. EPS production was observed upon all studied hexose-based monosaccharides (D-glucose, D-fructose, D-galactose) and disaccharides (D-maltose, D-melibiose). D-maltose represented the most favorable carbohydrate for EPS synthesis, resulting in flow times of around six min for 50 ml cultures (63x higher than the control). On the contrary, the maximal flow time upon growth on D-fructose was solely 13 sec (2x higher than the control). Although D-maltose and D-fructose differed enormously in their EPS formation supportive properties, they showed comparable growth characteristics and represented the two carbohydrates, which enabled strongest and fastest growth. This discrepancy might be explained by the genetic constitution of *L. brevis* TMW 1.2112 discussed in the following paragraph.

5.5.3. **Reconstruction of gtf-2-mediated glucan formation**

Based on its genome sequence we proposed a metabolic scheme for the uptake and subsequent intracellular conversion of those sugars, from which β-glucan was produced by *L. brevis* TMW 1.2112 (Figure 14). Glucose-1-phosphate (Glc-1-P) is the predicted key intermediate for β-glucan synthesis. It represents the precursor of UDP-glucose, which is then polymerized to β-glucan chains (Llull et al., 2001; Llull et al., 1999; Walling, Gindreau, et al., 2005).

To obtain Glc-1-P all sugars have to be taken up from the environment via an active transport (Kim, Shoemaker, & Mills, 2009). Further processing as cleavage in case of disaccharides, phosphorylation and isomerization occurs intracellularly. The initial phosphorylation of D-glucose, D-fructose and D-galactose requires one molecule ATP, whereas D-maltose could be converted by a maltose phosphorylase, which enables the conversion of maltose to glucose and glucose-1-P without consumption of ATP (Ehrmann & Vogel, 1998). Moreover, it saves the isomerization from glc-6-P to Glc-1-P. Glc-1-P can then be directly used for EPS synthesis, and the remaining glucose can be phosphorylated to glc-6-P to enter catabolic processes to yield energy. Energy-conservation via maltose phosphorylase might explain the extent of cell growth and slime formation upon growth on D-maltose.

In contrast, D-fructose as carbohydrate source for *L. brevis* TMW 1.2112 resulted in the weakest slime formation. Nevertheless, the growth of the bacterial strain was strongly supported by this sugar. For the procession to Glc-1-P, fructose has to undergo two isomerizations after its phosphorylation. To avoid this cumbersome processing, the metabolization of fru-6-P via the central metabolism seems more favorable. Instead of producing large amounts of slime, the sugar is probably metabolized by glycolysis, which is completely encoded in the genome of *L. brevis* TMW 1.2112. Moreover, it can be speculated, that the bacterium needs fructose as electron acceptor which reliefs the cell from oxidative stress caused by hop-induced manganese losses (Preissler, 2011). Another hypothesis for the low glucan production upon growth on fructose is associated with cell wall biosynthesis. The starting point for N-acetyl-glucosamine, a major component of the cell wall is fructose-6-phosphate, and the enzymes involved are glutamine-fructose-6-phosphate aminotransferase, which catalyzes the formation of glucosamine-6-phosphate and UDP-N-acetylglucosamine diphosphorylase, which catalyzes the synthesis of UDP-N-acetylglucosamine.

Hence, any competition for a substrate for this glucan and cell wall biosynthesis would rather start from fructose than from maltose derived glucose or glucose-1-phosphate. This may explain lower amounts of glucan upon growth on fructose – because then glucan synthesis is in competition with growth.

D-melibiose was the second most supportive carbohydrate for EPS synthesis in *L. brevis* TMW 1.2112. The uptake and cleavage of this disaccharide provides two sugar molecules, glucose and galactose. This could explain its superiority over D-glucose and D-galactose as monosaccharide source. Moreover, the preference for this carbohydrate might be referred to an adaption to the environment "wheat beer", as the strain was isolated from this substrate. Yeasts used for wheat beer fermentation are not able to metabolize D-melibiose (Bokulich & Bamforth, 2013). Consequently, this sugar is available for potentially following spoiling bacteria. This theory is supported by the presence of a special transporter for this sugar.

In contrast to the hexose-based sugars, growth on different pentoses did not induce slime formation. Under consideration of the pentose catabolism, this fact seems obvious. The pentose-derived key intermediate xylulose-5-P is degraded to acetate and lactate, while in this pathway Glc-1-P does not represent an intermediate (Kandler, 1983). In contrast, Glc-1-P would have to be generated via the gluconeogenesis, which is a very energy consuming way. Moreover, gluconeogenesis starting from pyruvate is not possible, since the strain does not possess a pyruvate carboxylase. Therefore, pentoses just enabled cell growth without EPS formation.

Although the carbohydrate source had strong impact on the quantity of slime formation, it had no influence on the composition of the resulting polymer. Independently from the applied sugar, the polysaccharide always constituted a glucan (Figure 27). This shows a high specificity of the based mechanism.

Both, the pH and the carbohydrates promoting EPS formation correspond to the conditions found in beer (Ferreira, 2009). The kind and amount of the residual sugars such as maltose in beer are likely to have great impact on the susceptibility to become slimy after contamination with a β-glucan producer such as *L. brevis* TMW 1.2112. However, each sugar inducing slime formation enabled the production of capsular β-glucan to different extents. Even a "weak" capsular envelope causing no extreme ropiness of liquids could therefore be enough to exert protective functions against the harsh environment "beer".

To investigate this hypothesis and to determine the contribution of glucan formation onto the bacterial cell, *L. brevis* TMW 1.2112 and the heterologously generated, glucan-producing *Lc lactis* TMW 2.2036-Gtf^{+} were studied in this respect.

5.6. Physiological contribution of glucan formation on the bacterial cell

5.6.1. **Glucan formation as nutritional reserve**

The dynamics of slime formation of *L. brevis* TMW 1.2112 are characterized by a successively increase of viscosity, which however disappeared upon prolonged fermentation. Therefore, we suggest at least a partial degradation of the produced exopolysaccharide, which might serve as carbon source during starvation. Such an enzymatic EPS hydrolysis/degradation has been reported for several EPS-producing LAB strains (Cerning, Bouillanne, Landon, & Desmazeaud, 1992; Cerning, Desmazeaud, & Landon, 1988; Gancel & Novel, 1994; Pham, Dupont, Roy, Lapointe, & Cerning, 2000; Ricciardi et al., 2002). Unfortunately, a precise quantification of EPS was not possible, because the strong interaction between the cells and the capsule avoided a reliable quantitative isolation. Also the attempt to solve this network via the application of heat, dilution and detergents failed, underscoring the strong binding of CPS. Nonetheless, the genomic data of *L. brevis* TMW 1.2112 allow to hypothesize that the β-glucan could indeed serve as carbohydrate source under certain (possibly limited) growth conditions for *L. brevis* TMW 1.2112, as the strains encodes different (putative) β-glucan degrading glycosyl hydrolases, e.g. an endoglucanase (glycosyl hydrolase family 8; locus tag AZI09_02135) or a glycosyl hydrolase family 3 (locus tag AZI09_02175).

5.6.2. **Glucan formation as protective envelope**

Another reason for the encapsulation of *L. brevis* TMW 1.2112 could be an increased resistance against environmental stress factors. Beer and wine represent the most affected beverages in terms of slimy spoilage and both exhibit, due to the presence of antimicrobial hop compounds and sulfur dioxide, respectively, of a low pH and a quite high ethanol content, a harsh environment for bacteria.

Accordingly, forming capsular polysaccharides, which envelope the cell and act as an additional protective barrier might assist in bacterial tolerance.

To verify this assumption the gtf-2-expressing *Lc. lactis* TMW 2.2036-Gtf$^+$ was compared to its wild type concerning their resistance against ethanol, acid and hydrogen peroxide. These experiments revealed the gtf-2-expressing strain as more resistance to each growth challenge tested.

However, these results were restricted to growth on solid culture, where the EPS are cell-associated as capsule. As already mentioned, *Lc. lactis* TMW 2.2036-Gtf$^+$ is not able to keep the capsule in liquid culture, causing a release of the polysaccharides. Consequently, a protective function seems to rely on a capsular localization of the polysaccharides, which envelopes the bacterial cell.

In this manner, CPS formation might contribute to already described cell wall modification of beer-spoiling bacteria, aiming to reduce the intrusion of harmful hop compounds into the cell. Cell wall modifications are from critical importance for the survival of lactic acid bacteria in LAB beer and represent one of the most important resistance mechanism (Behr et al., 2006; Suzuki et al., 2006).

As *L. brevis* TMW 1.2112 keeps the polysaccharides cell-associated in every respect, the encapsulation could support the strain to handle harsh environments such as beer.

This assumption is in accordance to the results of Stack et al. (2010) and Coulon, Houles, Dimopoulou, Maupeu, and Dols-Lafargue (2012) who showed an increased resistance of CPS-forming LAB against different environmental stress like acid, heat and lysozyme. In contrast, Pittet, Morrow, and Ziola (2011) did not identify any differences in the ethanol tolerance of a *gtf-2*-deficient *P. claussenii* strain and its EPS-producing beer-spoiling paternal. Therefore, CPS formation by spoilage bacteria in sour and alcoholic environments can contribute to increased tolerances against acids and ethanol as also observed for acetic acid bacteria (Brandt, Born, Jakob, & Vogel, 2017), while some bacteria use additional strategies to overcome these harsh environmental conditions.

5.6.3. **Glucan formation as biofilm precursor**

Finally, capsules might facilitate the adherence to solid surfaces (de Palencia et al., 2009; Dols-Lafargue et al., 2008), suggesting an involvement of respective lactobacilli in (brewery-associated) biofilm formation.

However, *Lc. lactis* TMW 2.2036-Gtf$^+$ failed to build biofilms in liquid culture, presumably referable to the missing capsular localization of the glucan produced. Unfortunately, no

comparable assay relying on solid cultivation was available/ conductible. Nonetheless, the sticky phenotype on agar plates, which strongly aggravated to pick and detach single colonies from the plate, suggest at least increased adhesion properties. Dols-Lafargue et al. (2008) reported similar observations for a slimy, wine spoiling *P. parvulus*.

In consequence, such cells might resist cleaning flushing in breweries or other sectors more than non-CPS forming cells and could constitute a kind of biofilm precursors.

As biofilms in turn are hotspots for gene transfers from one species to another (Flemming & Wingender, 2010), the promotion of the development of gtf-positive lactobacilli or pediococci seems possible.

5.7. The *gtf-2* gene as diagnostic marker gene

The relevance of capsular glucan formation in in the survival of bacteria and the possible contribution to biofilm (precursor) formation clarifies the need and the importance for an early detection of such bacteria in breweries, in order to prevent spoiled beer and the associated economic losses of affected companies, as well as the dispread of this gene/ trait in the brewery environment. Due to its highly conserved nucleotide sequence, the *gtf-2* gene represents a promising diagnostic marker. By designing primers targeting the *gtf-2* gene, it was possible to detect slimy beer-spoiling LAB species-independently and to distinguish them from non-slimy ones. Thus, this gene represents an appropriate target for the reliable identification of these brewery contaminants with PCR.

5.8. The genomic diversity and niche adaption of *L. brevis*

As one of the most relevant beer-spoiling bacteria, *L. brevis* not only causes slimy beer spoilage but several other spoilage types as well. At the same time, *L. brevis* occupies a variety of other ecological niches and constitutes a beneficial strain with respect to medical, industrial and biotechnological purposes. This includes the use as probiotic to improve human body function (Annuk et al., 2003; Collins, Thornton, & O'Sullivan, 1998; Kishi, Kazuko, Matsubara, Okuda, & Kishida, 1996; M. G. O'Sullivan, Thornton, O'Sullivan, & Collins, 1992; Ramos, Thorsen, Schwan, & Jespersen, 2013; Takii, Nishimura, Yoshida-Yamamoto, Kobayashi, & Nagayoshi, 2013) or the utilization as starter culture in different dairy products (Gerez, Torino, Obregozo, & Font de Valdez, 2010; Jeyaram, Romi, Singh, Devi, & Devi, 2010; Mugula, Narvhus, & Sorhaug, 2003; Sesena, Sanchez, & Palop, 2005).

Therefore, the assignment of *L. brevis* as beneficial or harmful bacterium relies on its ability to occupy a respective habitat or upon contamination or deliberate application of cultures, respectively. The respective ecological niches differ considerably with respect to the kind and amount of nutrients as well as the specific stress factors and hurdles. While the gastrointestinal tract, beer and wine constitute a nutritionally variable environment with low pH and challenges like bile salts, hop compounds or sulfur components, respectively, ecological niches like dairy or sourdough are nutrient-rich and comply with comparatively "friendly" habitats.

The ability to deal with highly different terms and conditions implies an adaption and specialization enabling persistence and growth. When bacteria encounter new environments, they are forced to change and to adjust their physiological behavior in order to ensure growth and survival. For a transient persistence, adaptation may be sufficient via cellular regulatory networks. Long-term persistence and growth rather requires changes reflected in genome specializations (genomic plasticity) as functional units of heredity. Such specializations encompass the modification of existing genes via mutation (Feldgarden, Byrd, & Cohan, 2003; Giraud et al., 2001; Hottes et al., 2013; Sokurenko et al., 1998; Tenaillon, Taddei, Radmian, & Matic, 2001), the gain of beneficial genes (de Koning, Brinkman, Jones, & Keeling, 2000; Lawrence, 1999; McLysaght, Baldi, & Gaut, 2003; Ochman, Lawrence, & Groisman, 2000; Springael & Top, 2004) and the loss of useless, ancestral ones (Cole et al., 2001; Mirkin, Fenner, Galperin, & Koonin, 2003; Ogata et al., 2001). Those evolutionary processes promoting habitat adaptions are described for various lactobacilli (Cai, Thompson, Budinich, Broadbent, & Steele, 2009; Douillard et al., 2013; Kant, Blom, Palva, Siezen, & de Vos, 2011; Kant et al., 2014; O. O'Sullivan et al., 2009).

In this context, we used the genome sequences generated in this study as well as all other public available *L. brevis* genomes and performed (under consideration of the isolation source) comparative genomic investigations to unravel the ecologic versatility of this species and to identify niche adaptions, with focus on brewery-derived strains.

In this way, we found *L. brevis* possessing a broad genomic diversity displayed in its pan genome (Figure 18). The remarkable size of the pan genome is consistent with that one of other versatile and widely distributed species like *L. plantarum* (7107 pan genes) (Martino et al., 2016) , *L. casei* (5935 pan genes) (Broadbent et al., 2012) and *L. paracasei* (6000 pan genes) and *L. rhamnosus* (4893 pan genes) (Kant et al., 2014). In contrast, *L. sanfrancsicensis* or *L. lindneri*, which are restricted to sourdoughs (R. F. Vogel et al., 2011) or beer (Suzuki et

al., 2005), respectively, carry a much smaller pan genome as compared to ubiquititstic genera (Celano, Geissler, Minervini, Gobbetti, & Vogel, 2017; R.F. Vogel, Celano, Minervini, Gobbetti, & Geissler, 2017). This confirms a large pan genome to be distinctive for habitat generalists since the resulting genetic diversity enables the population of various habitats.

Analyzing the resulting metabolic traits in more detail failed to establish a correlation to a certain ecological niche, since all studied capabilities were distributed within all strains, regardless of their isolation source.

In contrast, for other lactobacilli precise genomic adaptions to specific niches are described. For example, the genomes of *L. paracasei* and *L. delbruckii* strains that settled in dairy products are characterized by a gene decay resulting in a high number of pseudogenes (Douglas & Klaenhammer, 2010; O. O'Sullivan et al., 2009). Consequently, the metabolism is simplified and traits being dispensable for this nutrient-rich environment are lost. Another example are gut-associated lactobacilli, which mostly possess broad metabolic capacities conferring them the ability to handle this dynamic and variable habitat (De Keersmaecker et al., 2006; Douillard et al., 2013; Kankainen et al., 2009; Koskenniemi et al., 2011).

We were not able to identify comparable genomic adaptions for the species of *L. brevis* and did not find metabolic capabilities being unique for all strains of one source of isolation. In this respect, it has to be emphasized, that the isolation source must not always correspond to the actual habitat of a bacterium, but can alternatively reflect a place of residence resulting from transient contamination of the respective ecological niche (Stefanovic, Fitzgerald, & McAuliffe, 2017). Nevertheless, Smokvina et al. (2013) described similar observations for *L. paracasei* and also Martino et al. (2016) failed to couple the genetic constitution of 54 *L. plantarum* strains to their respective habitat. The latter referred it to a nomadic lifestyle, in which the retaining of a universal set of genes enables a flexible life in many different environments.

As expected, also chromosomal similarity measures did not correlate to the isolation source and cause an environment-associated clustering, even when using different phylogenetic and phylogenomic investigations (Figure 20).

However, chromosomes are known as more static part, while plasmids are described as mobile and dynamic part. Plasmids are interchangeable between bacteria, often even between different species and genera, and mostly confer properties beneficial to their host, increasing its fitness and survival in a particular habitat (Rossi, Rizzotti, Felis, & Torriani, 2014). In

turn, mobile elements encoding useless functions are likely to be lost. This makes plasmids to a central engine of genetic diversification.

Indeed, phylogenomic analysis on plasmidome level enabled (at least) a grouping of brewery-derived and insect-derived strains in distinct clusters (Figure 21).

Deeper investigations showed the brewery-derived strains to be the most uniform ones. No other source shared as many source-specific genes as the brewery did (Figure 22), which of course could also be attributed to certain amount to the number of brewery genomes analyzed. Nevertheless, this remarkable gene sharing within the brewery- (and the insect-) associated strains explains their close neighboring within the phylogenomic cluster analysis.

5.9. The plasmidome of brewery-derived *L. brevis* strains

Analyzing the plasmidome of brewery-derived strains in more detail revealed several traits that could be partially connected to an adaption to beer-typical hurdles.

As the antimicrobial action of beer strongly relies on the presence of hop compounds, which cause cell death by acting as ionophore and dissipating the transmembrane proton gradient, the ability to counteract these detrimental properties is a fundamental requirement for beer-spoiling lactobacilli (Suzuki et al., 2006). In this regard, respective bacteria use two general strategies: (i) increasing the cell barrier function to reduce hop intrusion and (ii) counteracting intruded hop compounds.

An increased barrier function is mediated, among others, via the expression of LTAs, which is mediated by cluster like *horA* or *horC* (Suzuki et al., 2006). We found them in 87 % and 62.5 % of the strains, respectively. Another possibility to increase the barrier function is the synthesis of cell-wall bound CPS, coating the cell as protective envelope. The in this study well investigated *gtf-2* gene, which is responsible for capsular glucan formation, was present in 50 % of the studied strains.

Although hop intrusion is reduced by such mechanisms, it cannot be prevented entirely. Consequently, beer-spoiling LAB have to antagonize intracellular hops and the resulting detrimental effects.

The most intuitive way is the extrusion of intruded hops. In this respect, *horA* and *horC* are from importance again, as they encode an ATP-dependent multidrug resistance transporter and a PMF-dependent one, respectively. Both participate in expelling hops out of the cell (Sakamoto et al., 2001; Suzuki et al., 2002).

Another important trait to reduce the hazardous properties of hops is associated with cation homeostasis, which was in fact one of the most abundant functional categories within the brewery plasmidome (Figure 25). As the detrimental properties of hop compounds strongly rely on the presence and binding of manganese, a tight and efficient cation adjustment is critical.

From certain physiological points of view, manganese is exchangeable with magnesium, which in turn reduces the antimicrobial strength of hops (Behr & Vogel, 2009; Preissler, 2011; Simpson & Smith, 1992). The magnesium uptake system *CorA* (Kehres et al., 1998) and the manganese transporter *hitA* were identified in the majority of brewery-associated strains in close genomic proximity. This neighbored expression confirms the assumed concurrent regulation in order to ensure a balanced relationship of both cations (Geissler et al., 2017). As the afore-described LTAs located in the cell wall act as reservoir for divalent cations, they contribute to a reduced amount of available manganese and retaining it for essential cell functions (Behr et al., 2006; R. F. Vogel et al., 2002).

Moreover, hop compounds are known to cause an intracellular acidification, which has to be combated by the cell. This need is fulfilled in the here studied strains by an enrichment of ATPases, possibly counteracting the ionophoric effect of hops by expelling protons out of the cell and maintaining the transmembrane gradient.

Another particularity of the brewery plasmidome was the presence of genes like the methionine sulfoxide reductase, which are predicted to be involved in the defense of oxidative stress, another antimicrobial property exerted by hop compounds (Behr & Vogel, 2010; Schurr, Hahne, et al., 2015).

Finally, beer not only challenges the cell via specific antimicrobial actions, but also by providing just minor amounts of nutrients, especially carbohydrates. Moreover, the kind of carbohydrates differs from beer to beer and from brew to brew. Consequently, an efficient metabolism of various carbohydrates is from importance. As shown in Figure 25, genes predicted to be involved in carbohydrate metabolism are accumulated in brewery-associated strains. This is evidenced among other things by a gene cluster associated with maltose metabolism. Beer contains maltose as predominant carbon source (Moore & Rainbow, 1955; Wood & Rainbow, 1961), so this cluster might reflect an acclimatization to beer.

5.10. The shared gene pool of brewery- and insect-derived *L. brevis* strains

Beyond brewery- also insect-derived *L. brevis* strains constituted a close ecotype according to their plasmidome. Moreover, both plasmidomes are closely related as they share 40 % of the encoded genes.

A closer view onto this shared gene pool revealed the presence of a truncated *horC* cluster in strains form both environments, which was, so far, considered as brewery-specific. To the best of our knowledge, this is the first time a *horC* cluster was found in non-brewery associated strains as well. The activity of *horC* as MFS transporter not only confers resistance to hops, but also to multiple structurally unrelated drugs (Sakamoto et al., 2001; Suzuki et al., 2005), which could be of benefit for insect-derived lactobacilli as well.

For the overlapping plasmidomes in brewery and insect isolates, two explanations come into question. Theoretically, insects and breweries could present overlapping ecological niches characterized by similar environmental restrictions for bacteria selecting the respective strains independently. At first glance, these ecological niches do not appear as similar. However, some traits of the shared *L. brevis* brewery and insect plasmidome, e.g. those of *horC*-mediated multiple drug resistance, may also play an important role for their survival in insects feeding from many (plant) sources, which may contain toxic compounds. Another more convenient explanation suggests an exchange between both ecological niches. In this case, it needs to be considered whether both of these form habitats, or *L. brevis* were a contaminant only in beer or in insect. As insects constitute a natural, non-artificial habitat that was present long before brewing was developed, we suggest that *horC*, and possibly other shared "brewery" plasmidome functions, originally derive from insects, which contaminated beer with respective strains. This view is supported by the high improbability that insects fed on spoiled beer just before bacteria were isolated from them. In this context, the ancient beer brewing process has to be taken into account. This relied on open brewing kettles, which were readily accessible for insects bringing *L. brevis* as contaminant. This hypothesis is strengthened by the findings of Christiaens et al. (2014) who showed an ester production by yeasts to attract fruit flies, which in turn act as "yeast taxi" and promote their dispersal. In this process a simultaneous uptake and release of lactobacilli seems possible as well. Still, the maintenance of such functions require selective pressure and a respective role in insect as well as beer. While this is studied well in beer, it is widely unknown in insects. Furthermore,

functional units of the insect plasmidome could be transferred to different *L. brevis* strains to form brewery strains and vice versa by plasmid transfer. Therefore, no functional overlap could be found in the accessory chromosomes of strains from these habitats.

Due to this connection between insects and breweries, the above-discussed homologies (in the genetics of glucan formation) between slimy wine- and beer-spoiling lactobacilli also appear in a new glance. Insects have access to both, beer and wine kettles. Moreover, the sweet acid of wine strongly attracts fruits flies, making them to a well-known, undesired and annoying companion of open wine. Consequently, it can be speculated that insects are responsible for the *gtf-2* dispread among spoilage bacteria from both beverages. Maybe it is just coincidence, that no *gtf-2*-carrying insect-derived or *horC*-carrying wine-spoiling LAB have been genome sequenced so far.

6. SUMMARY

The unique composition of beer is not only responsible for its special taste but also for its stability. Beer is characterized by a low pH, the presence of ethanol, hop compounds and carbon dioxide and a depletion of nutrients and oxygen. In combination, these hurdles hinder most bacterial growth. Nonetheless, beer spoilage occurs occasionally. Due to the intensive research in the last decades, microbial beer spoilage is increasingly understood, with exception of EPS-producing lactobacilli causing beer with viscous, slimy properties.

83 beer- and brewery-derived lactobacilli were screened for their beer-spoiling and viscosity-increasing properties and identified 25 strains, which encompassed both characteristics. These belonged to the species of *L. brevis*, *L. rossiae* and *L. parabuchneri*, whereby *L. brevis* was the most relevant species.

Further investigations focused on the strongest slime-producer *L. brevis* TMW 1.2112, which was able to cause a 63-fold increase in viscosity. Thereby, the strain exhibited special growth and slime-forming dynamics, characterized by a successive viscosity increase, which enabled the cells to grow diffuse throughout the cultivation tube. After a maximum, the effect reversed, associated with sedimentation of the cells and a gradual disappearance of the viscous properties. This suggests a network between the cells and the polysaccharides. To gain more insights, respective polysaccharides were isolated from the cells (CPS) and from the supernatant (EPS) and analyzed via multiple chromatographic and spectroscopic approaches. Both were identical β-(1→3)-glucans, ramified with β-glucose residues at position *O*2. Therefore, we assume that this EPS is mainly produced as CPS and partially released into the surrounding medium. CPS formation was confirmed via an agglutination test.

By whole genome sequencing of four *L. brevis* strains and comparative genomics, a plasmid-encoded gylcosyltransferase-2 (*gtf-2*) gene was found as responsible for excess β-glucan formation. This gene possesses a species-independent highly conserved nucleotide sequence and is found in slimy wine-spoiling LAB as well. In all described cases, the resulting polymer constitutes the same β-glucan structure suggesting a highly conserved mechanism of polymerization. To gain more insights into the biosynthesis, the nutritional requirements of glucan formation were studied and coupled to the genomic data of *L. brevis* TMW 1.2112. This analysis revealed the need of hexose-based carbohydrates, which are convertible in UDP-glucose. This activated sugar nucleotide represents the key intermediate in bacterial

glucan formation as its glycosyl residue is directly polymerized by the gtf-2 action into glucan molecules.

The assumed key position of the gtf-2 in glucan formation was highlighted by expressing this gene heterologously in a *Lactococcus (Lc.) lactis* strain. The resulting transformant exhibited a ropy, mucoid phenotype on agar plates, while liquid cultures were not viscous as observed for the beer-spoiler *L. brevis* TMW 1.2112. Analysis of polysaccharides isolated from liquid cultures of recombinant *Lc. lactis* revealed the presence of a β-(1,3-1,2)-glucan, identical to that produced by *L. brevis* TMW 1.2112. An agglutination test showed the glucan just on solid but not in liquid culture to be clearly cell-associated, suggesting the lactococcus strain being unable to anchor the glucan properly to the cell surface during planktonic growth. Therefore, viscosity-increasing effects by β-glucan producers upon growth in liquids seem to be rather due to a tight capsular localization of β-glucan and a concomitant cell-network formation than to the produced and released polysaccharide itself.

The capsular localization of the glucan is not only necessary to cause viscosity increases, but also to contribute to the physiology of the cell. CPS formation conferred the here studied *Lc. lactis* strain two advantages – an increased stress tolerance and an increased adhesive capability. Although these benefits were due to the polysaccharide release in liquid culture restricted to growth on solid culture, the slimy beer spoiler *L. brevis* TMW 1.2112 might profit from both effects independently from solid or liquid environments, as the glucan is cell-associated in every respect. In the brewery environment both physiological contributions could promote the survival of the cell.

Another possible advantage brought by EPS formation is a reserve function during starvation periods. Prolonged fermentations caused the loss of the viscous properties and several glucancases were identified in the genomic data of *L. brevis* TMW 1.2112, which are proposed for glucan degradation. Therefore, we assume that the β-glucan could serve as carbohydrate source under certain (possibly limited) growth conditions for *L. brevis* TMW 1.2112.

Together, its decisive role within glucan formation and its conserved nucleotide sequence suggest the *gtf-2* gene as a promising diagnostic marker gene for an early detection of β-glucan-producing lactobacilli in breweries. In fact, PCR primers targeting this gene enabled the specific detection of respective bacteria independently from the precise species.

To conclude, this study provides a comprehensive characterization of the phenomenon of viscous beer spoilage.

Moreover, beer spoilage (caused by *L. brevis*) was confirmed to be generally a plasmid-encoded trait. Under consideration of the isolation source, all available *L. brevis* genomes were investigated via broad genomic comparisons. In this way, it was proven that the stringent ecological niche "brewery" demands an adaption which is reflected on plasmidome level. This plasmidome is phylogenomically closely related and the resulting traits can be connected to an adaption to beer-typical hurdles. Beyond a remarkably large gene sharing within this ecotype, brewery-derived strains shared 40 % of their plasmidome with insect-derived *L. brevis* strains. In this context, the brewery-specifity of the hop resistance gene *horC* was disproven, as it was present in strains from both sources. This leads to the final thesis that insects are responsible for the dispread of certain traits and possibly also for the *gtf-2* occurrence within the different habitats beer, wine and cider.

7. ZUSAMMENFASSUNG

Die besondere Zusammensetzung von Bier ist nicht nur für den speziellen Geschmack, sondern auch für die Stabilität verantwortlich. Bier zeichnet sich durch einen niedrigen pH-Wert, den Gehalt an Ethanol, Hopfen und Kohlensäure sowie einen Mangel an Nährstoffen und Sauerstoff aus. Obwohl diese Faktoren bakterielles Wachstum stark inhibieren, kommt es dennoch regelmäßig zu Verderbsfällen. Durch intensive Forschung in den letzten Jahrzehnten ist mikrobieller Bierverderb grundsätzlich immer besser verstanden, schließt allerdings nicht den schleimig-viskosen Verderb ein, der durch exopolysaccharid- (EPS-) bildende Laktobazillen verursacht wird.

Ein umfassendes Screening 83 brauereiassoziierter Laktobazillen hinsichtlich EPS-bildender und bierverderbender Eigenschaften identifizierte 25 Stämme die beide Charakteristika vereinten. Diese gehörten der Spezies *L. brevis*, *L. rossiae* und *L. parabuchneri* an, wobei *L. brevis* die relevanteste Spezies darstellte.

Die weiteren Untersuchungen fokussierten sich auf *L. brevis* TMW 1.2112, der mit einer 63-fachen Viskositätserhöhung den stärksten Schleimbildner darstellte. Dieser Stamm zeichnete sich durch eine besondere Wachstums- und Schleimbildungsdynamik aus, charakterisiert durch einen sukzessiven Viskositätsanstieg mit diffusem Zellwachstum. Nach einem Maximum reversierte sich der Effekt, wobei die Zellen sedimentierten und die viskosen Eigenschaften graduell abnahmen. Dies impliziert ein komplexes Netzwerk zwischen den Zellen und produzierten Zuckerpolymeren. Im Weiteren wurden Polysaccharide von den Zellen (CPS) und aus dem Überstand (EPS) isoliert und mit verschiedenen chromatographischen und spektroskopischen Methoden analysiert. Beide Isolate wiesen eine identische β-(1,3-1,2)-Glukanstruktur auf, wodurch anzunehmen ist, dass das EPS hauptsächlich in kapsulärer Form synthetisiert und partiell in das umgebende Milieu sekretiert wird. CPS-Bildung wurde mit einem Agglutinationstest bestätigt.

Durch die Sequenzierung des Genoms von vier *L. brevis* Stämmen und vergleichende Genomauswertung, wurde eine plasmidkodierte Glykosyltransferase als Schlüsselgen der Glukanbildung identifiziert. Dieses Gen ist durch eine speziesunabhängig hochkonservierte Nukelotidsequenz charakterisiert und ist auch in schleimigen Weinverderbern zu finden. In allen beschriebenen Fällen besitzt das resultierende Polymer dieselbe β-Glukanstruktur, wodurch ein hochspezifischer Mechanismus der Glukosepolymerisation anzunehmen ist.

Um tiefere Einblicke in die Biosynthese zu gewinnen, wurden die ernährungsassoziierten Anforderungen der Glukanbildung untersucht und mit den genomischen Daten von *L. brevis* TMW 1.2112 verknüpft. Diese Analyse zeigte eine Abhängigkeit von Hexosen, welche in UDP-Glukose metabolisierbar sind. Dieser aktivierte Zucker stellt das entscheidende Zwischenprodukt in bakterieller Glukansynthese dar, da seine Glykosylreste gtf-2-vermittelt polymerisiert werden.

Die Schlüsselrolle des *gtf-2* Gens in der Glukanbildung wurde durch die heterologe Expression des Gens in *Lactococcus* (*Lc). lactis* hervorgehoben. Der resultierende gtf-2-exprimierende Stamm zeigte einen schleimig-mukösen Phänotyp auf Agarplatten. Auch wenn in Flüssigkultur keine rheologischen Veränderungen festzustellen waren, konnte die Anwesenheit eines β-(1,3-1,2)-Glukans, welches identisch zu dem von *L. brevis* TMW 1.2112 ist nachgewiesen werden.

Mittels eines Agglutinationstestes wurde ermittelt, dass das Glukan nur auf festem, jedoch nicht in flüssigem Medium zellgebunden ist. Daher ist anzunehmen, dass *Lc. lactis* das Polysaccharid in planktonischem Wachstum nicht auf seiner Oberfläche verankern kann, und somit zu einer Freisetzung führt. Dies impliziert, dass die von β-Glukan-produzierenden Bakterien verursachte Viskosität auf einer Vernetzung der Zellen basiert und nicht auf ein sezerniertes Polysaccharid *per se* zurückzuführen ist.

Die kapsuläre Lokalisierung des Glukans ist nicht nur für viskositätssteigernde Effekte essentiell, sondern auch um die Physiologie der Zelle zu beeinflussen. CPS-Bildung ermöglichte dem untersuchten *Lc. lactis* Stamm zwei Vorteile: eine erhöhte Stresstoleranz und verbesserte adhäsive Eigenschaften. Auch wenn diese Effekte durch die Polysaccharid-freisetzung in flüssigen Umgebungen auf Agarplatten beschränkt waren, könnte der Bierverderber *L. brevis* TMW 1.2112 von beiden Effekten unabhängig von flüssigen oder festen Umgebungen profitieren, da das Glukan hier in jeder Hinsicht zellgebunden ist.

Zusätzlich könnte das Glukan einen weiteren Nutzen während Hungerphasen bringen. Da verlängerte Fermentation zum Verlust der viskosen Eigenschaften führte und im Genom zahlreiche Glukanasen identifiziert wurden die für einen Glukanabbau vorgeschlagen sind, könnte das Glukan unter bestimmten (möglicherweise begrenzten) Wachstumsbedingungen als Kohlenhydratquelle dienen.

Die Schlüsselrolle in der Glukansynthese und die konservierte Nukleotidsequenz machen das *gtf-2* Gen zu einem vielversprechenden diagnostischen Markergen für eine frühzeitige

Detektion von β-Glukan-produzierenden Laktobazillen in Brauereien. Die Entwicklung geeigneter PCR-Primer ermöglichten die spezifische und speziesunabhängige Detektion entsprechender Bakterien.

Zusammenfassend liefert diese Studie eine umfassende Charakterisierung des viskosen Bierverderbs. Darüber hinaus wurde (durch *L. brevis*-vermittelter) Bierverderb im Allgemeinen als plasmidkodierte Fähigkeit bestätigt. Unter Berücksichtigung der Isolationsquelle wurden vielseitige Vergleiche aller verfügbaren *L. brevis* Genome angestellt und zeigten, dass die stringente ökologische Nische „Brauerei“ eine Adaption fordert und diese auf Plasmidomlevel wiederzufinden ist. Dieses Plasmidom ist phylogenomisch nah verwandt und die resultierenden Fähigkeiten konnten einer Anpassung an biertypische Hürden zugeordnet werden. Neben einem bemerkenswert großen gemeinsamen Genpool innerhalb dieses Ökotyps, teilten brauerei-assoziierte *L. brevis* Stämme 40 % ihres Plasmidoms mit aus Insekten stammenden *L. brevis* Isolaten. In diesem Kontext wurde die Brauereispezifität des Hopfenresistenzgens *horC* widerlegt, da es in Stämmen beider Habitate gefunden wurde.

Dies führt zur abschließenden These, dass Insekten für die Verbreitung bestimmter Fähigkeiten verantwortlich sind und möglicherweise auch das *gtf-2* Vorkommen in den Habitaten Bier, Wein und Cider verursachten.

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9. APPENDIX

9.1. Additional figures and tables

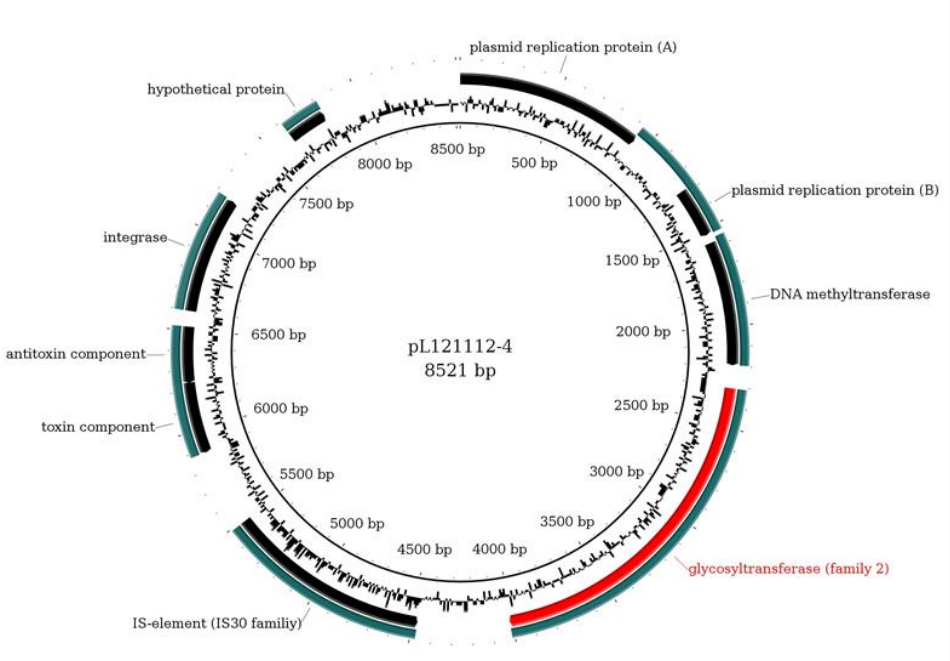

Figure 26 Similarities between plasmid pl12112-4 of *L. brevis* TMW 1.2112 and plasmid pPECL7 of *P. claussenii* ATCC BAA-344 T. Starting from inside: circle 1 shows the general position in kilobases; circle 2 depicts the G+C content of *L. brevis* TMW 1.2112; circle 3 presents genes encoded on pl12112-4 of *L. brevis* TMW 1.2112, in red the gtf-2. Circle 4 exhibits the corresponding genes of plasmid pPECL7 of *P. claussenii* ATCC BAA-344 T.

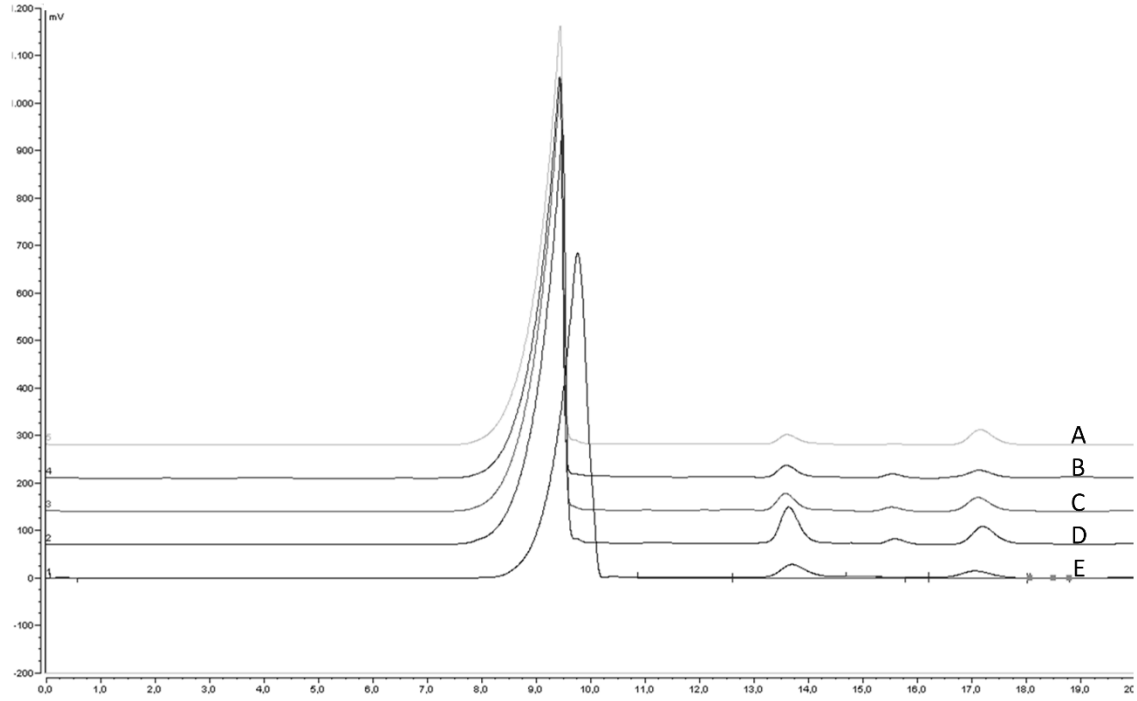

Figure 27 HPLC-RI chromatogram of EPS produced by *L. brevis* TMW 1.2112 in mMRS media with different carbohydrate sources (melibiose (A), fructose (B), glucose (C), maltose (D), galactose (E)). Retention time of detected peaks consistent with external standard glucose (13.5 sec). Peak at retention time 17.5 sec represents medium trace.

Table 14 ^{1}H and ^{13}C chemical shifts of the three structural elements present in the CPS and EPS of *L. brevis* TMW 1.2113. Chemical shifts are given in ppm.

Structural element		**1**	**2**	**3**	**4**	**5**	**6**
1,3-Glc*p*	^{1}H	4.90	3.63	3.79	3.55	3.51	3.74/3.91
	^{13}C	102.24	73.35	86.30	68.71	76.26	61.30
1,2,3-Glc*p*	^{1}H	4.88	3.84	3.97	3.55	3.51	3.74/3.91
	^{13}C	102.26	80.60	84.11	68.71	76.26	61.30
t-Glc*p*	^{1}H	4.95	3.31	3.51	3.40	3.51	3.74/3.95
	^{13}C	102.95	74.77	77.24	70.42	76.26	61.52

1,3-Glc*p* = 1,3-substituted β-glucopyranose, 1,2,3-Glc*p* = 1,2,3-substituted β-glucopyranose, t-Glc*p* = terminal β-glucopyranose bound to position *O*2 of a 1,3-substituted β-glucopyranose.

9.2. Additional strains used in this study

Non-EPS-producing lactobacilli used for *gtf-2* primer evaluation are listed in Table 15.

Table 15 Lactbobacilli used for *gtf-2* primer evaluation.

Species	TMW no.	Source of isolation
L. brevis	1.100	Sourdough
L. brevis	1.1205	Sourdough
L. brevis	1.230	Beer
L. brevis	1.302	Brewery
L. brevis	1.313	Brewery
L. brevis	1.315	Brewery
L. brevis	1.317	Brewery
L. brevis	1.1282	Brewery
L. brevis	1.465	Brewery
L. brevis	1.473	Brewery
L. brevis	1.485	Brewery
L. brevis	1.507	Brewery
L. brevis	1.474	Brewery
L. brevis	1.507	Brewery
L. brevis	1.1369	Honey fermentation
L. brevis	1.1370	Honey fermentation
L. brevis	1.6	Faeces
L. brevis	1.1326	Silage
L. brevis	1.328	Brewery
L. brevis	1.336	Brewery
L. brevis	1.318	Brewery
L. brevis	1.308	Brewery
L. brevis	1.228	Brewery
L. rossiae	1.164	Beer
L. backii	1.2073	Brewery
L. plantarum	1.1308	Brewery
L. linderni	1.2006	Brewery

9.3. Verification of capsular β-(1,3-1,2)-glucan formation

All experiments performed with *L. brevis* TMW 1.2112 were conducted with other strains, which were identified in the initial screening. These investigations confirmed the assumed transferability of the results to other EPS-producing, brewery-associated lactobacilli.

9.3.1. **EPS localization - Agglutination assay**

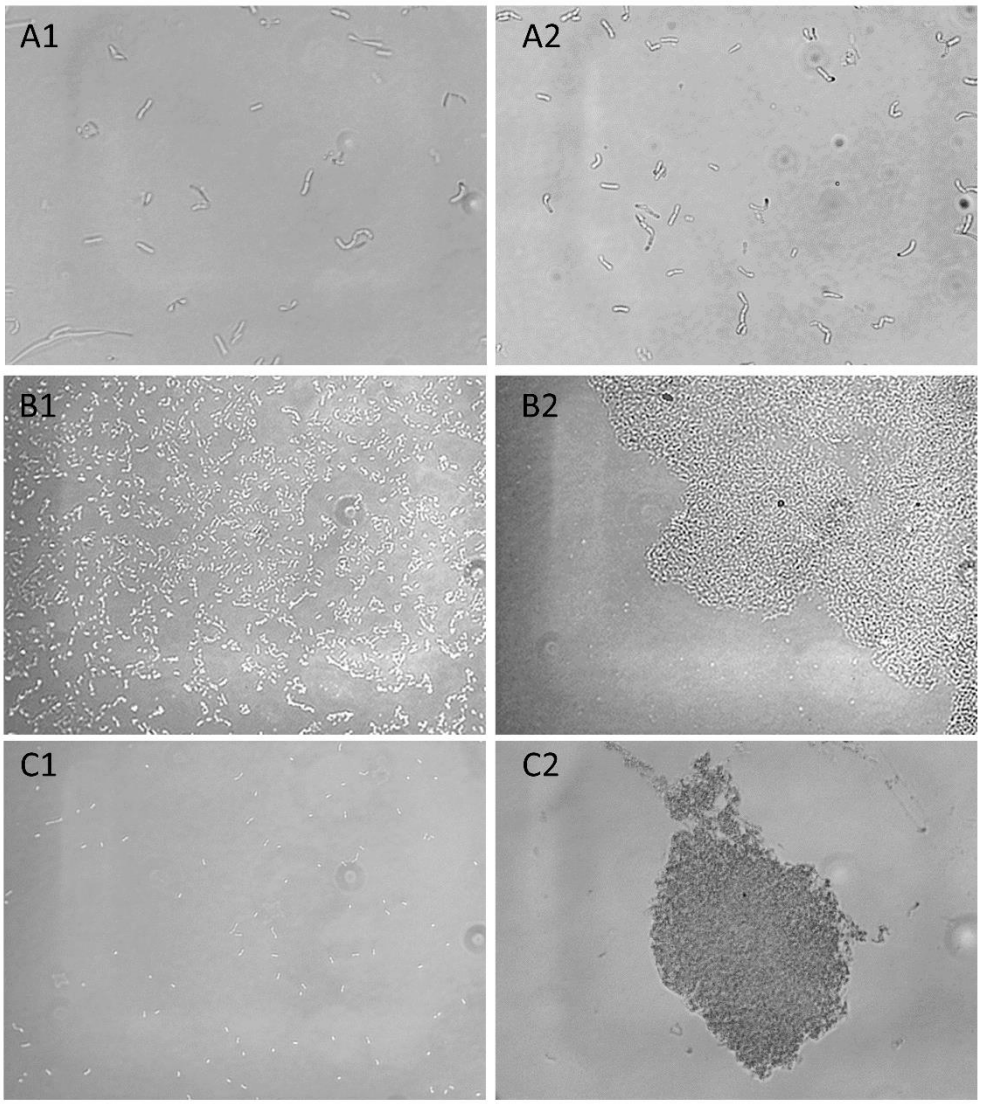

Figure 28 Agglutination test performed with *L. brevis* TMW 1.313-Gtf$^-$ (A), *L. brevis* TMW 1.2111-Gtf$^+$ (B) and *L. parabuchneri* TMW 1.1141-Gtf$^+$ (C). 1: cell suspension in PBS buffer, 2: cell suspension in *S. pneumonia* type 37-specific antiserum.

9.3.2. **EPS composition - HPLC-RI**

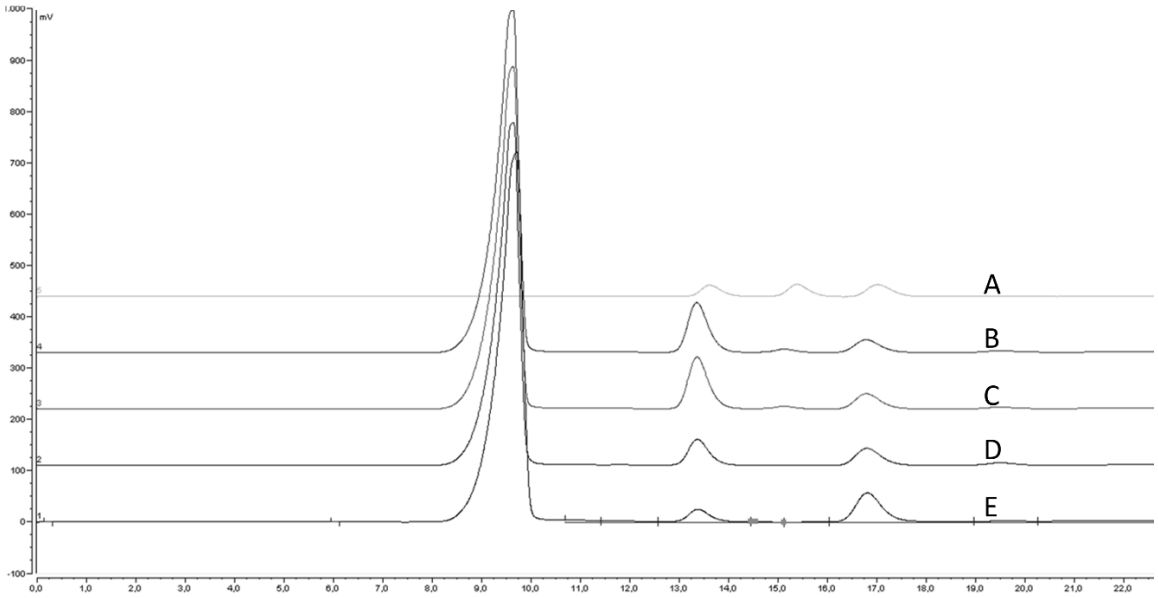

Figure 29 HPLC-RI chromatogram of lyophilized EPS from different lactobacilli. A: external standard composed of D-glucose (13.5 sec), D-galactose (15.2 sec) and D-mannose (17.2 sec). B: *L. brevis* TMW 1.2108, C: *L. rossiae* TMW 1.2155; D: *L. parabuchneri* TMW1.1141; E: control (precipitated and dialyzed growth medium).

9.3.3. **EPS structure - NMR spectroscopy**

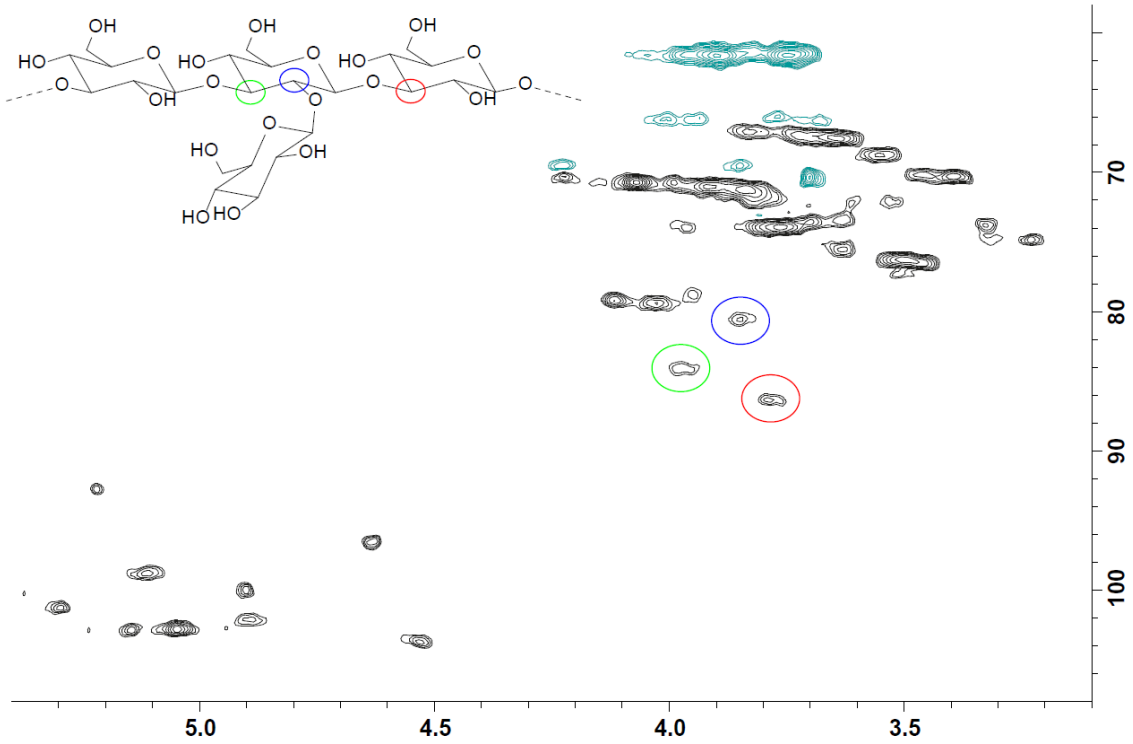

Figure 30 HSQC spectrum and proposed structure of the *L. brevis* TMW 1.2113 EPS preparation. The characteristic downfield shifts of the correlation peaks at the substituted positions are encircled.

10. LIST OF PUBLICATIONS DERIVED FROM THIS WORK

Peer-reviewed journals

- Fraunhofer, M.E., Geissler, A.J., Jakob, F., Vogel, R.F. **Multiple genome sequences of exopolysaccharide-producing, brewery-associated *Lactobacillus brevis* strains**. Genome Announcments 2017, 5 (26) e00585-17. doi: 10.1128/genomeA.00585-17.
- Fraunhofer, M.E., Geissler, A.J., Wefers, D., Bunzel, M., Jakob, F., Vogel, R.F. **Characterization of beta-glucan formation by *Lactobacillus brevis* TMW 1.2112 isolated from slimy spoiled beer.** Int J Biol Macromol. 2017. pii: S0141-8130(17)32535-7. doi: 10.1016/j.ijbiomac.2017.09.063.
- Fraunhofer, M.E., Jakob, F., Vogel, R.F. **Influence of different sugars and initial pH on exopolysaccharide formation of beer-spoiling *Lactobacillus brevis* TMW 1.2112.** Curr Microbiol. 2018. doi: 10.1007/s00284-018-1450-z.
- Fraunhofer, M.E., Jakob, F., Wefers, D., Bunzel, M, Vogel, R.F. **Characterization of heterologous β-glucan formation by *Lactococcus lactis* NZ9000.** Submitted for publication.
- Fraunhofer M.E., Geissler, A.J., Behr, J., Vogel, R.F. **Comparative genomics of *Lactobacillus brevis* reveals a significant plasmidome overlap of brewery and insect isolates.** Submitted for publication.

Oral presentation

Fraunhofer, M.E., Geissler, A.J., Wefers, D., Bunzel, M., Jakob, F., Vogel, R.F. (2016) **Characterization of beta-glucan formation by *Lactobacillus brevis* TMW1.2112 isolated from slimy spoiled beer.** World brewing Congress, Denver, Colorado, U.S.A.

11. CURRICULUM VITAE

Personal data

Marion Elisabeth Fraunhofer

Born on 24.06.1987 in Bad Aibling, Germany

Education

05/2014 – 11/2017 Doctoral thesis at the Chair of Technical Microbiology, Technical University of Munich, Germany

03/2011 – 10/2013 Master Study Biological Chemistry at the University of Vienna, Austria

10/2007 – 11/2010 Bachelor Study Nutrition Science at the University of Vienna, Austria

09/1998 – 06/2007 Gymnasium Bad Aibling, Germany