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Bacterial β-glucan - Applicability for *in situ* enriched sourdoughs and molecular background of the biosynthesis and degradation

Julia Alexandra Bockwoldt

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I. List of abbreviations

approx.	Approximately
AZI09_xxxx	ORF number
bp	Base pairs
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CDM	Chemical defined medium
CFU	Colony forming unit
CPS	Capsular polysaccharide
dH_2O	demineralized H_2O
Da	Dalton
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleotide triphosphate
DY	Dough yield
EC	Enzyme commission (number)
ELISA	Enzyme-linked Immunosorbent Assay
EPS	Exopolysaccharide
g	Gram
GH	Glycosyl hydrolase
GRAS	Generally recognized as safe
GTF	Glycosyl transferase
h	Hours
HePS	Heteropolysaccharide
HoPS	Homopolysaccharide
HPLC	High-performance liquid chromatography
iBAQ	Intensity-based absolute quantification
kb	Kilo base pairs
kDa	Kilodalton
kg	Kilogram
L	Liter
LAB	Lactic acid bacteria
M	Molar
MALDI-TOF	Matrix assisted laser desorption ionization time of flight
MFS	Major facilitator superfamily
mg	Milligram
min	Minute
mL	Milliliter
mM	Millimolar
mMRS	Modified Man, Rogosa, and Sharpe medium

MP	Maltose phosphorylase
MS	Mass spectrometry
NCBI	National Center for Biotechnology Information
ng	Nanogram
nm	Nanometer
nt	Nucleotide
OD_x	Optical density measured at a wavelength of x nm
ORF	Open reading frame
*p*NP	para-nitrophenylphosphate
PCR	Polymerase chain reaction
rpm	Revolutions per minute
RT	Room temperature
s	Second
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
spp.	Subspecies
TMW	Technische Mikrobiologie Weihenstephan
UDP	Uridine diphosphate
UTP	Uridine triphosphate
V	Volt
v/v	Volume per volume
WT	Wild type
w/v	Weight per volume
w/w	Weight per weight
× g	Times gravity
µg	Microgram
µL	Microliter
µM	Micromole
µm	Micrometer

1 Introduction

1.1 Exopolysaccharides of lactic acid bacteria

The term lactic acid bacterium (LAB) conventionally includes i.a. the genera *Lactobacillus*, *Paralactobacillus*, *Pediococcus*. Zheng et al. (2020) recently reclassified the genus *Lactobacillus* into 25 genera including among others *Levilactobacillus*, *Lactiplantibacillus*, and *Furfurilactobacillus*. The *Lactobacillaceae* comprises Gram-positive, non-spore-forming, oxidative-negative, catalase-negative (except some pseudocatalase producing strains), fermentative and obligate saccharolytic cocci or bacilli, which produce lactic acid as the major end product of carbohydrate fermentation and further acetate, ethanol, carbon dioxide, formiat or succinate as by-products (Schleifer, 2009). The fermentation end products are the results of either the Embden-Meyerhof-Parnas (lactic acid as end-product) pathway of homofermentative LAB or the pentose phosphate or pentose phosphoketolase pathway of heterofermentative LAB (Patel et al., 2012).

In addition, some LAB and Bifidobacteria produce high molecular weight exopolysaccharides (EPS) which could be covalently or loosed attached to the cell surface as capsular polysaccharides (CPS) or released into the extracellular environment (Ryan et al., 2015, Ruas-Madiedo et al., 2002). EPS are in general classified into homopolysaccharides (HoPS) and heteropolysaccharides (HePS) depending on their monomeric composition (De Vuyst and Degeest, 1999, Monsan et al., 2001). Some LAB and Bifidobacteria can produce simultaneously HoPS and HePS or different HePS (Puertas et al., 2018, Llamas-Arriba et al., 2018, Salazar et al., 2012). The reasons for the formation of EPS and CPS were extensively discussed, which include protection against biotic and abiotic stress (e.g., temperature, pH, osmotic stress, or antimicrobial compounds), biofilm formation, cell-cell interaction, or to act as storage-compounds (Donot et al., 2012, Caggianiello et al., 2016, Ruas-Madiedo et al., 2008, Looijesteijn et al., 2000, Dertli et al., 2015, Russo et al., 2012, Salazar et al., 2016, Terrade et al., 2009).

1.1.1 Homopolysaccharides (HoPS)

HoPS are formed by glycosidic linkages of repeating units of the same monosaccharides, usually D-glucose, D-fructose, or D-galactose and the resulting polymers are glucans, fructans, or galactans, respectively (Monsan et al., 2001, Kavitake et al., 2016). The monomers are either α-linked or β-linked and mostly linearly arranged with possible ramifications. The molecular weight of HoPS is ≥ 10^7 Da (Abarquero et al., 2022). Glucans are further subdivided into β-D-glucans (Figure 1) represented by LAB isolated from alcoholic beverages and α-glucans which comprise varying glycosidic linkages named mutans (α-(1,3)), reuterans (α-(1,4)), dextrans (α-(1,6)), and alternans (altering α-(1,3) and α-(1,6) glycosidic linkages) formed by i.a. *Limosilactobacillus* (*Lim.*) *reuteri* ML1 (Kralj et al., 2004), *Lim. reuteri* VIP (Galle et al., 2012b), *Liquorilactobacillus* (*Liq.*) *hordei* spp. (Bechtner et al., 2020) and *Leuconostoc mesenteroides* NRRL B-1355 (López-Munguía et al., 1993), respectively. Fructans are also subdivided into levans with mainly β-(2,6)-linked fructofuranosyl residues produced by i.a. *Fructilactobacillus* (*Fr.*) *sanfranciscensis* (Rogalski et al., 2021) and inulin-type mainly β-(2,1)-linked produced by *Limosilactobacillus reuteri* LB 121 (Ozimek et al., 2006). An example of a galactan forming LAB is

Weissella confusa KR780676 which was described to produce an α-(1,6)-galactan (Kavitake et al., 2016). The biosynthesis of HoPS is commonly performed in the extracellular environment by glycoside hydrolases (GH) of sucrase-type enzymes, either fructansucrases or α-glucansucrases, both enzymes catalyze transglycosylation and lead to HoPS formation. This contrasts with the biosynthesis of β-D-glucan, which uses glycosyltransferases (GTF) as the key enzymes in polymerization, similar to the biosynthesis of HePS. A detailed presentation of the β-D-glucan pathway and further information is given in Chapter 1.2.

The polymerization reaction of α-D-glucans and β-D-fructans are similar and starts with the cleavage of the glycosidic bond of sucrose. The cleavage energy is used for the next step when a glucose or a fructose unit is coupled to the growing polysaccharide chain. Although the polymerization processes are similar, the two enzyme groups are distinctly different. Fructansucrases are invertase-like enzymes (GH 68 family), found in various Gram-positive and Gram-negative bacteria. Glucansucrases are members of the GH 70 family and are predominantly represented by LAB (van Hijum et al., 2006, Drula et al., 2022).

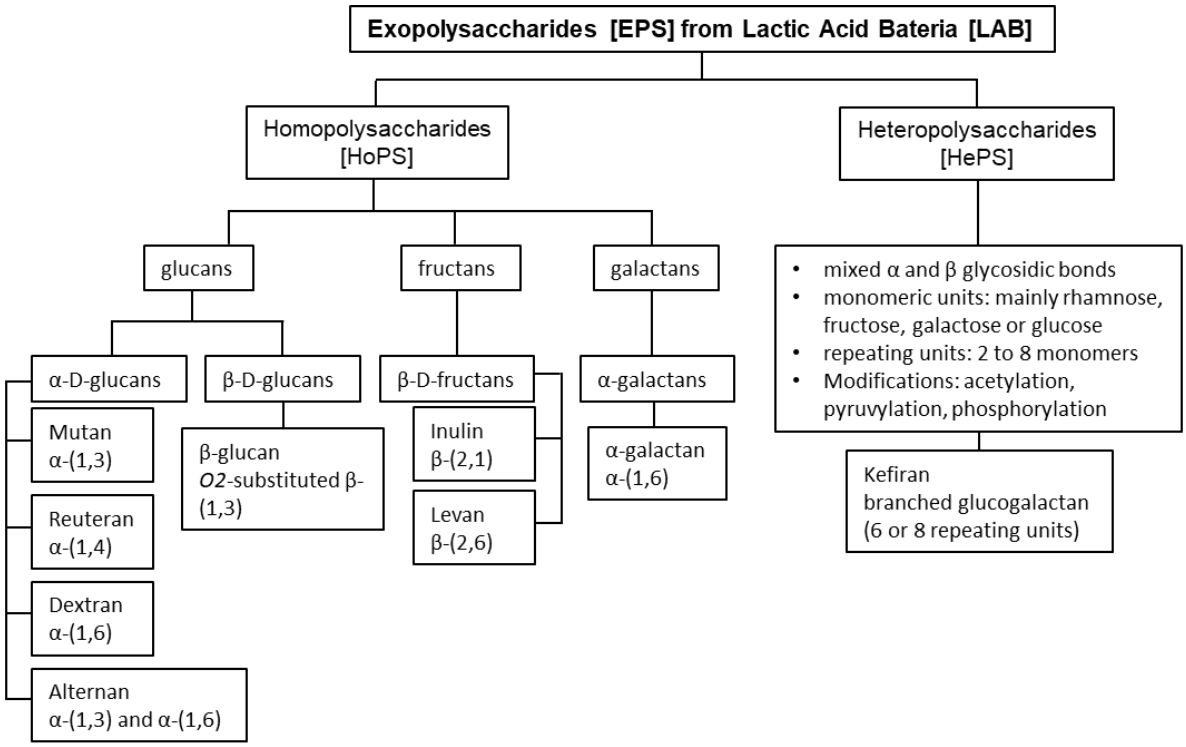

Figure 1. Classification of exopolysaccharides from lactic acid bacteria

The figure was adapted from Korcz and Varga (2021).

1.1.2 Heteropolysaccharides (HePS)

HePS, unlike HoPS, form a very diverse and complex polysaccharide group which makes further subdivisions difficult, except for kefiran (Figure 1) which is a glucogalactan (Moradi and Kalanpour, 2019). The monomeric composition of HePS includes D-glucose, D-galactose, D-fructose, and/or L-rhamnose, and even non-carbohydrate groups like phosphate, N-acetylglucosamine, or N-acetylgalactosamine. The diversity of HePS refers not only to their monomeric composition but also to

the versatile structures with repeating units varying from two to eight α-linked and/or β-linked monomers in a more branched manner than linearly arranged. LAB able to form HePS include among others *Streptococcus thermophilus* spp. (De Vuyst et al., 2003, Zhang et al., 2018) and *Lactobacillus kefiranofaciens* (Moradi et al., 2021).

Moreover, the biosynthesis of HePS is an energy-intensive multistep process that starts intracellularly and ends extracellularly. The pathway starts with the uptake of sugar, which is further processed until a sugar nucleotide precursor, e.g., glucose-1-phosphate, is obtained. In the next step, a priming glycosyltransferase (GTF) catalyzes the linking of a phosphorylated sugar to a carrier lipid which is attached to the membrane, and this is the first step of the polymerization process. Nucleotide-activated sugars are sequentially added by GTFs, catalyzing the glycosidic bonds of the repeating units. The repeating units are exported, probably with help of a flippase (Wzx) from the Wzy pathways, and the polymerization of the repeating units is performed extracellularly with the help of a polymerase (Wzy) (Notararigo et al., 2013, Islam and Lam, 2014, Whitfield et al., 2020, Zhou et al., 2019, van Hijum et al., 2006). Compared to HoPS, the HePS are smaller with molecular masses between 10^4 and 10^6 Da (Abarquero et al., 2022, Torino et al., 2015, De Vuyst and Degeest, 1999) and the yield of HePS (25-600 mg/L) is much lower compared to HoPS (1-10 g/L) due to their energy-consuming biosynthesis and the competitiveness with the peptidoglycan biosynthesis (Galle et al., 2011, Gobbetti and Gänzle, 2013, Badel et al., 2011, Salazar et al., 2016, Ruas-Madiedo et al., 2010).

1.2 Beta-glucan

1.2.1 Lactic acid bacterial β-glucan – an extraordinary EPS

Beta-D-glucans are an exception among EPS; although the composition of solely D-glucose monomeric units places them in the HoPS group, their biosynthesis is similar to that of HePS. LAB known to produce β-glucans are i.a. *Levilactobacillus* (*L.*) *brevis* spp. (Fraunhofer et al., 2018a), *Pediococcus* (*P.*) *claussenii* TMW 2.340 (= DSM 14800^T, ATCC BAA-344^T) (Suzuki et al., 2020, Dobson et al., 2002), *Oenococcus* (*O.*) *oeni* spp. and *Pediococcus* (*P.*) *parvulus* IOEB 8801 (Dols-Lafargue et al., 2008). All these strains have something in common as they were isolated from alcoholic beverages such as beer, wine, or cider.

The β-glucans of LAB are mostly *O2*-substituted β-(1,3)-D-glucans (hereafter referred to as β-glucans) which means that D-glucose monomers are linked via 1,3-β-glycosidic bonds, and a glucose residue is branched via a 1,2-β-glycosidic bond on every other glucose monomer of the backbone (Llauberes et al., 1990, Fraunhofer et al., 2018a, Dueñas-Chasco et al., 1997, Dueñas-Chasco et al., 1998). The molecular masses of β-glucans from producing strains like *P. parvulus* spp. and *O. oeni* IOEB 0205, can be divided into two fractions: a low molecular mass fraction of 10^4 Da and a high molecular mass fraction with up to 10^7 Da (Werning et al., 2014, Ciezack et al., 2010, Dols-Lafargue et al., 2008, Garai-Ibabe et al., 2010a). As previously described for the biosynthesis of HePS, β-glucan is also synthesized from a nucleotide-activated sugar. Exemplary with maltose as the substrate: the sugar nucleotide precursor glucose-1-phosphate is phosphorylated by a maltose phosphorylase (MP) with simultaneous cleavage of maltose. This enzymatic reaction produces one glucose-1-phosphate molecule and one glucose

molecule, of which glucose-1-phosphate is activated by the transfer of a nucleoside diphosphate finally resulting UDP-glucose (Figure 2). The polymerization is performed by a transmembrane glycosyltransferase family 2 protein (Gtf-2) catalyzing the glycosidic bond between glucosyl residues and the growing chain while UDP is released (Werning et al., 2006, Fraunhofer et al., 2018a, Dols-Lafargue et al., 2008, Garai-Ibabe et al., 2010a).

The β-1,3 glucan synthase (EC 2.4.1.34) which is described as the key enzyme in β-(1,3)-D-glucan biosynthesis, belongs to the glycosyltransferase 2 family (Gtf-2) (Werning et al., 2006, Notararigo et al., 2014, Fraunhofer et al., 2018a, Walling et al., 2005b, Pittet et al., 2013). A study by Llamas-Arriba et al. (2018) demonstrated that particular gene sequences of β-1,3 glucan synthases are highly conserved, namely those from *Pediococcus* spp. i.a. *P. claussenii* TMW 2.340, *Oenococcus oeni* sp. and *Lactobacillus* spp. i.a. *L. brevis* TMW 1.2112. Moreover, the analyzed *gtf-2* genes were mainly plasmid-encoded except for *O. oeni* spp., as these strains exhibited chromosomal encoded *gtf-2*. The high plasmidome similarities of *L. brevis* spp. isolated from insects and *gtf-2* positive brewery isolates led to the assumption that insects could be involved in the spread of Gtf-2 in LAB (Fraunhofer et al., 2019).

Furthermore, LAB such as *L. brevis* spp., *P. parvulus* spp., *P. claussenii* sp., and *O. oeni* spp. are known to form β-glucan capsules. The encapsulation of the cells could provide an increase in protection against harsh environmental conditions like a low pH value, ethanol, hop bitter compounds, or polyphenols. All the strains were isolated from alcoholic beverages like beer, wine, or cider that exhibited a ropy or slimy character, which is a highly undesired feature for these products (Llamas-Arriba et al., 2018, Dols-Lafargue et al., 2008, Garai-Ibabe et al., 2010a, Fraunhofer et al., 2018a, Dobson et al., 2002, Lonvaud-Funel et al., 1993, Dueñas-Chasco et al., 1997, Dueñas-Chasco et al., 1998). However, the proteins involved in the formation of β-glucan capsules and thus the attachment to the cell surface are rather unknown. Still, the capsule formation of other EPS from LAB has been described as involving proteins of the Wzy pathways or potentially the LytR-Cps2A-Psr (LCP) protein family (D'Abrosca et al., 2018, Bitoun et al., 2012, Bitoun et al., 2013, Whitfield et al., 2020, Islam and Lam, 2014).

L. brevis spp. have been the most common beer spoilage organisms in Europe for years while *P. claussenii* spp. have a less important role (Suzuki et al., 2020). However, not all strains form EPS and thus spoil the beer. Even though these strains lack a polysaccharide capsule, they are still very well adapted against the harsh conditions like the presence of hop bitter compounds by plasmid encoded genes like *horA* (ATP-dependent multidrug transporter) and/or *horC* (proton motive force (PMF)-dependent multidrug transporter). These genes help to remove hop bitter compounds or high molecular weight lipoteichoic acids ratios improve the barrier function of the cell wall (Sakamoto and Konings, 2003, Suzuki, 2011). Fraunhofer (2018) described several LAB isolated from spoiled beer as forming capsular β-glucan, including *L. brevis* spp., *Furfurilactobacillus* (*F.*) *rossiae* spp., and *Lactiplantibacillus* (*Lp.*) *paraplantarum* sp.. Especially *L. brevis* TMW 1.2112 stood out, as this strain was able to increase the viscosity of the medium by a factor of 63. Moreover, a special growth characteristic was observed indicating a network-like cell structure due to the capsular β-glucan, as the cells diffused more over the entire liquid column than usual to accumulate on the bottom. This pattern disappeared after cells sedimented to a later stage in fermentation, assuming the utilization of β-glucan as carbohydrate source (Fraunhofer, 2018).

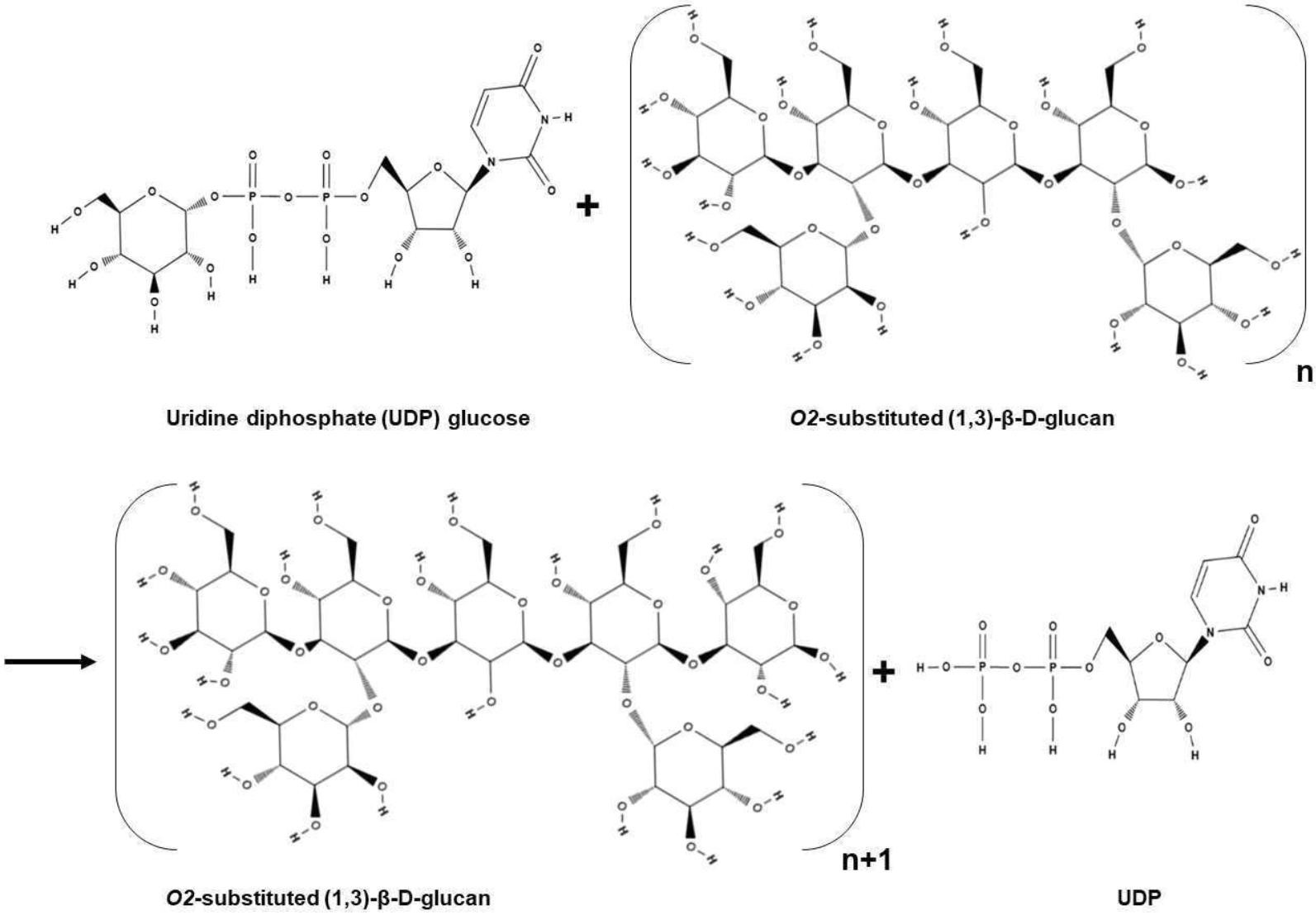

Figure 2: Polymerization of bacterial *O2*-substituted (1,3)-β-D-glucan from precursor UDP-glucose

1.2.2 Plant and fungal β-glucans

Beta-glucans from plants and fungi such as yeasts and molds have been studied for much longer in comparison to β-glucans from LAB. In plants, the polymer is to be found in the cell wall, and in cereal grains in the aleurone layer (Wood, 2007). Cereal β-D-glucans from oat and barley are described as linear polymers build of β-(1,3) and β-(1,4) linked glucose monomers (McCleary and Codd, 1991). The reported β-glucan contents of barley and oat are approx. 11.3% and 7.8%, respectively. In comparison wheat and rye contain less than 3% (Lazaridou and Biliaderis, 2007, Vinkx and Delcour, 1996, Gill et al., 2002, Rieder et al., 2012). Moreover, oat and barley-based products possess defined health claims by the US Food and Drug Administration (FDA) and European Food Safety Authority (EFSA) declaring that 3 g or more β-D-glucan from oats or barley per day support a normal blood cholesterol level and potentially reducing the risk of coronary heart diseases, and the EFSA further declared that at least 4 g per meal helps to reduce the rise in blood glucose levels after a meal (EFSA Panel on Dietetic Products and Allergies, 2011, EFSA Panel on Dietetic Products and Allergies, 2009, FDA, 2022).

Fungal β-glucans account up to 60% of the cell wall biomass and are formed mainly from β-(1,3) linked glucopyranosyl groups with β-(1,6) side chains (Kollár et al., 1997, Rop et al., 2009, Ene et al., 2015). However, the distribution and length of the side chains vary since yeasts have longer β-(1.6) side chains and mold having short β-(1.6) side chains. The β-(1,3) glucan makes up to 90% of the total cell wall β-glucan serving as basic framework for other cell wall components which are than covalently attached to the glucan (Bowman and Free, 2006, Grün et al., 2005, Bernard and Latgé, 2001). The molecular weight including chain lengths and degree of branching is considered to determine the biological activity of the β-glucan. *In vitro* and *in vivo* studies demonstrated that β-(1,3) and β-(1,6) glucans resulted in immunomodulating effects such as stimulation and inhibition of macrophage release or macrophage phagocytosis (Bohn and BeMiller, 1995, Gardiner, 2005, van Steenwijk et al., 2021, Chan et al., 2014). In contrast, cereal β-(1,3) and β-(1,4) glucans are considered as more resistant to absorption and digestion, resulting in lowering the serum cholesterol level or shaping the gut microbiome (Nakashima et al., 2018, Panahi et al., 2007, Hughes et al., 2008). Thus, it could be expected that the effects of *O2*-substituted β-(1,3)-D-glucans from LAB also have specific effects on human health which are described in more detail in Chapter 1.5.

1.3 Application of EPS in food

Most fermentation processes known today originate from spontaneous fermentations of raw materials from a time when microorganisms were not yet researched and their existence unknown. During that time, back-slopping, using small amounts of a ferment with desired features to inoculate the raw material, was performed to optimize and maintain food fermentation (Holzapfel, 2002). Today LAB are widely studied and known for their biotransformation of animal- and plant-based raw materials with a broad range of metabolites besides lactic acid, enhancing the development of flavors in fermented food and simultaneously reducing the risk of food spoilage. With the development of starter cultures, the industrial production of fermented foods and beverages with defined textures, tastes, and flavors was enabled. Starter cultures are preparations of microorganisms of at least one strain of bacteria, yeasts,

or molds, with high cell counts to be added to the raw material to ferment it producing fermented products with defined characteristics (Bintsis, 2018). In fact, many of the strains used from starter cultures also occur in spontaneous fermentations (Leroy and De Vuyst, 2004, Bintsis, 2018).

Next, to the organoleptic benefits, processes using functional starter cultures cause rapid acidification of raw materials, and the production of other metabolites like bacteriocins, enzymes, or EPS leads to improved shelf life, microbial safety, or improved texture of the products (Leroy and De Vuyst, 2004, Hati et al., 2013, Gerez et al., 2009). EPS producing LAB, except *Weissella* spp. or single strains, are generally recognized as safe (GRAS) and could be applied in food fermentations in the form of starter cultures (Galle and Arendt, 2014, Brandt et al., 2003, Korcz and Varga, 2021) and in the pharma industry (Saadat et al., 2019, Korakli and Vogel, 2006, Angelin and Kavitha, 2020) under *in vitro* or *in vivo* conditions (Fessard and Remize, 2017, Badel et al., 2011). Due to the consumers increasing demands for products without artificial additives, reduced sugar and/or fat content, and just more natural products in recent years, the *in-situ* product enrichment by EPS with an improvement of technological and sensory properties is an optimal solution. The benefits of LAB's EPS in industrial processes have already been extensively described and extend to different sectors (Table 1).

Table 1. Examples of EPS applications produced by LAB

Industry	Products	EPS	Benefit	References
Dairy	Yogurt, mozzarella, kefir	Inulins, kefiran, HePS	Water retention, bio-thickener, limit of syneresis, texture, fat reduction	(Duboc and Mollet, 2001, Moradi and Kalanpour, 2019, Badel et al., 2011, Zannini et al., 2016)
Cereal-based products	Sourdoughs: wheat, rye, gluten-free; cereal-mixtures: oat and rice	Dextran, reuteran, β-glucan, levan, HePS	Water-binding capacity, texture, delayed staling, taste, volume	(Brandt, 2007, Arendt et al., 2007, Korcz and Varga, 2021, Pérez-Ramos et al., 2017, Galle and Arendt, 2014)
Confectionary	Ice cream, chocolate mousse	Alternans, dextran, inulins	Thickener, gelling, sensory, stabilizer, sweetener	(Dertli et al., 2016, Cardarelli et al., 2008, Bounaix et al., 2009)
Meat	Raw fermented sausages, ham	Dextran, HePS	Texture, moisture, fat substitution	(Ryan et al., 2015, Loeffler et al., 2020)
Fermented beverages	Water kefir, tavern, tuba, aguamiel	Dextran	Kefir grains, increased viscosity, taste	(Pidoux, 1989, Bechtner et al., 2019, Cázares-Vásquez et al., 2021)
Therapeutics	Pre- and probiotics, drug carrier, anticoagulant	Alternan, dextran, β-glucan, levan, inulins, HePS	Improved LAB persistence, stimulating growth of host's colon microbiota, gut health, anti-inflammatory	(Salazar et al., 2016, Caggianiello et al., 2016, Notararigo et al., 2014, Pérez-Ramos et al., 2017, Varshosaz, 2012, Naessens et al., 2005, Angelin and Kavitha, 2020)

Among others, their advantages include acting as bio-thickening agents, their water-binding capacity, and substituting fat and sugar to a certain extent (Zannini et al., 2016, Loeffler et al., 2020, Korcz and Varga, 2021). Even though the EPS have no inherent taste, the texture changes of the products triggered by them can influence consumer taste experiences due to increased residence times in the mouth (Duboc and Mollet, 2001). In the dairy industry, starter cultures including EPS-producing strains have various functions in the production processes, e.g., as thickener and stabilizers, due to their water-binding properties the viscosity increase and the texture change. In addition, EPS could interact with micelles and proteins of the dairy product optimizing the stability of the casein network and moreover reducing syneresis (Daba et al., 2021, Hassan et al., 2004, Mende et al., 2016). In meat processing the application of EPS, especially dextran and HePS could help to reduce the fat content and still maintain the characteristic texture of the product (Loeffler et al., 2020). Further industrial sectors were described to benefit from EPS, e.g., fermented beverages contain dextrans to improve mouthfeel, taste, and the fermentation process itself (Cázares-Vásquez et al., 2021, Bechtner et al., 2019); in the processing of confectionary, i.a. inulins are used for textural improvement (Cardarelli et al., 2008); and the pharma industry uses various EPS for different applications i.a. as drug carrier matrices (Varshosaz, 2012).

1.4 Sourdough fermentation and the advantages of *in situ* EPS formation

Like all other food fermentations, sourdough fermentation is an ancient and traditional process. Sourdoughs are generated from a water-flour mixture colonized by LAB and yeasts. The fermentation of the mixtures enables the production of baked goods that not only have improved characteristics in taste, texture, and digestibility, but also in shelf life (Bartkiene et al., 2020). Wheat and rye flours are the most used flours in sourdough fermentation. In the case of rye flour, the fermentation is even necessary to enable its baking properties. Rye compared to wheat contains less gluten an important structural protein. The acidification of the rye flour-water mix increases the solubility of pentosans which enhances its water-binding capacity and, gas hydration and increases the bread volume (Arendt et al., 2007, Hammes and Gänzle, 1998, Damiani et al., 1996, Martínez and Devesa, 2000, Gobbetti et al., 2000). The metabolites of LAB contribute a large share to the positive effects by proteolysis regarding the formation of aroma, lactic acid, and acetic acid, with an optimal range between pH 3.5 and 4.3 (Esteve Collar et al., 1994, Arendt et al., 2007, Poutanen et al., 2009, Hammes and Gänzle, 1998). Both organic acids exhibit anti-microbial activity, however, at a specific concentration, acetic acid has better inhibition properties than lactic acid against yeasts, mold, and bacteria (Rocha and Malcata, 1999, Rocha and Malcata, 2016). Besides, a low pH during fermentation activates the proteolytic activity of the cereal enzymes which improves the nutritional value and bioavailability of the baked goods. Higher availability of minerals is achieved through the hydrolytic processes of phytate (Hammes and Gänzle, 1998, De Vuyst and Neysens, 2005, Cagno et al., 2002, Bartkiene et al., 2020). In summary, the advantages of sourdough fermentation for baked goods are broad and allow the development of versatile flavors, bread quality, and extended shelf lives.

The fermentation quotient (FQ) is described as an indicative value for bread flavor and quality and is calculated from the molar ratio of lactate and acetate. Various optimal FQ ranges were described in the

past, however, the FQ is more of a loose guideline as several factors affect the molar ratio, e.g., used flour type, microbiota, or used starter cultures (homo- and/or heterofermentative LAB), temperature, dough yield, external electron acceptors, pH, and fermentation time (Brandt et al., 2004, Gobbetti, 1998, Gerez et al., 2009, Barber et al., 1991, Spicher et al., 1986, Oura et al., 1982, Spicher and Stephan, 1987). In addition, the dough yield (DY) gives information about the hydration level of the sourdough (De Vuyst and Neysens, 2005) and is calculated by using the following equation:

$$\mathrm{DY} = \frac{\text{flour (g)} + \text{water (g)}}{\text{flour (g)}} \cdot 100$$

Furthermore, sourdoughs are divided into three different types according to fermentation temperature, DY, pH, microbiota, or later application field. Type I sourdoughs represent the traditional sourdoughs (pH 3.8-4.5 and fermentation temperature 20-30 °C) with a stable culture of bacteria and yeast. While type II liquid sourdoughs (pH ≤ 3.5 and fermentation temperature 30-45 °C) and type III dried sourdoughs are most likely industrial processed sourdoughs (Gobbetti, 1998, De Vuyst and Neysens, 2005, Brandt, 2019). Nowadays traditional bakeries often still use traditional sourdoughs for baking, but with the increasing demand for i.a. gluten-free products alongside the usual wheat and rye sourdough products, starter cultures are usually indispensable to meet these demands (Rühmkorf et al., 2012b, Arendt et al., 2007, Brandt, 2019, De Vuyst and Neysens, 2005).

The production of gluten-free baked goods is associated with losses in structure and texture due to the missing gluten or pentosan (Arendt et al., 2002). The gluten is involved in the viscoelastic properties, formation of the crumb, and gas-holding of the baked goods (Gallagher et al., 2004, Gallagher et al., 2003). The development of gluten-free baked goods is challenging as the improvement of the protein network and water-binding properties need to be addressed. Various approaches are already described for this purpose, such as the use of egg whites (Crockett et al., 2011), enzymes like transglutaminases (Renzetti, 2008), plant-based proteins from lupins, peas, soy, and legumes (Skendi et al., 2021), but also hydrocolloids from plants or microorganisms like xanthan produced by *Xanthomonas* sp. or gellan gum *Sphingomonas* sp. (Freitas et al., 2011, Gallagher et al., 2004, Schober et al., 2005). Compared to the yield of other EPS, the yield of xanthan ranges from 30 to 50 g/L and is much higher (Caggianiello et al., 2016). Enzymes and hydrocolloids were shown to be the key factors in the improvement of hydration and viscosity properties in gluten-free baked goods. However, the usage of such additives in manufacturing the doughs and finally baked goods usually increases the costs for the end consumer and does not meet consumers' demands for clean-label and natural products without any additives (Moroni et al., 2011). The increasing demand for clean-label products and the term 'clean-label' itself dates back to the 1980s when consumers increasingly avoided products with E-numbers because they were associated with negative health effects (Joppen, 2006). Until today, there is no established, objective definition of this term, yet it is associated to be free from artificial additives or ingredients and is considered more natural (Asioli et al., 2017).

The previously discussed EPS of LAB are already described as suitable hydrocolloids, improving technological properties of baked goods (Tieking and Gänzle, 2005). Moreover, by use of EPS-producers, the dough could be *in situ* enriched during fermentation, making the declaration as an additive redundant. The application of EPS in baked goods resulted in improved bread volumes as well

as water-binding capacities and further led to a delay in staling (Arendt et al., 2007, Brandt, 2007, Poutanen et al., 2009, Gänzle et al., 2007). The most frequently described EPS in sourdough fermentations belong to α-glucans, β-fructans, and HePS (Chen et al., 2016, Katina et al., 2009, Rühmkorf et al., 2012a, Tieking et al., 2005, Galle et al., 2012a). For example, *Fr. sanfranciscensis* sp. which is considered as the most predominant LAB in sourdough fermentations, produces HoPS (α-glucans and β-fructans) (Vogel et al., 2011, Gobbetti and Corsetti, 1997, Rogalski et al., 2021, Tieking et al., 2005, Zhang et al., 2019). However, sucrose must be added to initiate the formation of α-glucan and β-fructans by LAB which could lead to residual sweetness in the end product (Bounaix et al., 2009, Korakli et al., 2001). In addition, the sucrose addition can lead to increased acetate formation during sourdough fermentation (Kaditzky et al., 2007). Consequently, further LAB-derived EPS like HePS or β-glucans might be promising alternatives, also because of possible otherwise superior properties. Initial studies on the application of HePS in sourdough fermentation revealed that depending on the flour type the rheology could be influenced by *in situ* enrichment (Galle et al., 2011). Unfortunately, to date, there are only a few studies on the use of HePS in sourdough fermentation.

1.4.1 Application of β-glucan forming LAB in sourdoughs

The LAB strains able to produce β-glucans are in general isolated from alcoholic beverages (Fraunhofer, 2018, Dols-Lafargue et al., 2008, Lonvaud-Funel et al., 1993). Sourdough enrichment by bacterial β-glucan is similar to HePS less frequently described. Pérez-Ramos et al. (2017) analyzed the effects of the *in situ* β-glucan formation by *P. parvulus* 2.6 in cereal-based matrices and demonstrated that the rheological properties of oat mixtures were improved. In comparison Elizaquível et al. (2011) fermented yogurt, orange juice, and a mixture of orange juice and milk with the β-glucan producing stains, *P. parvulus* 2.6, *P. parvulus* CUPV22, and *Paucilactobacillus* (*Pa.*) *suebicus* CUPV221, which did not cause any changes in the food matrices. Further, the *in-situ* production of β-glucan in yogurt by the recombinant probiotic strain *Lacticaseibacillus paracasei* NFBC 338 [pNZ44-GTF^{+}] significantly improved texture properties (Kearney et al., 2011). Currently, the extent to which β-glucans from LAB influence dough matrices regarding structural and sensory properties, whether in wheat or rye sourdough breads, are missing.

Levilactobacillus, *Lactiplantibacillus*, *Furfurilactobacillus* and *Pediococcus* species are considered traditional components of the sourdough microbiota of wheat and rye fermentations (Bartkiene et al., 2020). Some strains of these genera have been identified to form β-glucans (Fraunhofer, 2018, Dobson et al., 2002, Llauberes et al., 1990). In contrast to other HoPS-producing strains, these β-glucan-forming strains use maltose as a substrate which is released during sourdough fermentation from starch by cereal amylases or exo-amylases activity of the sourdough microbiota (Fraunhofer, 2018, Arendt et al., 2007). The β-glucan yields were described to be low, ranging only between 18 and 243 mg/L. These low yields could be explained by the synthesis from nucleotide-activated sugars similar to the synthesis of HePS which reach similar yields. Alterations of sourdough fermentation parameters like fermentation temperature and time, flour type, carbon source availability, and cell counts might help to achieve higher EPS yields (Rühmkorf et al., 2012a, Garai-Ibabe et al., 2010a). But since initial studies showed that *in*

situ formed β-glucans in fact improved the rheological effect of cereal matrices, similar effects could be expected during sourdough fermentation (Pérez-Ramos et al., 2017). In summary, β-glucan-forming LAB could be used for sourdough fermentation to generate a clean label product with no residual sweetness, e.g., from sugar addition and excessive acetate formation, negatively affecting the quality of the baked goods, and thus with simultaneous enrichment of a potentially structure-improving EPS.

1.5 Health-promoting aspects of β-glucans

EPS formation by LAB in food fermentation offers technological as well as health beneficial benefits (Galle and Arendt, 2014, Chen et al., 2016, Poutanen et al., 2009, Russo et al., 2012, Pérez-Ramos et al., 2017). The described health beneficial effects include i.a. prebiotics properties acting as fermentable substrates for the intestinal microbiota, probiotics, immunoregulatory effects, the reduction of the serum cholesterol levels, and the lowering of the postprandial blood glucose level and insulin response (Salazar et al., 2016, Welman and Maddox, 2003, Liu et al., 2011, Laiño et al., 2016, Saadat et al., 2019). EPS utilized by LAB and Bifidobacteria as energy sources could be considered as prebiotics. Probiotics, on the other hand, are living microorganisms exhibiting health beneficial effects for the host when present in adequate amounts. In addition, probiotic microorganisms able to produce EPS could result in delayed passage of the gastrointestinal tract, attributed to the increased viscosity of the EPS-enriched food thus enabling temporary colonization of the host's intestine (Duboc and Mollet, 2001, Ayivi et al., 2020).

Especially the health-promoting effects of the LAB's *O2*-substituted β-(1,3)-D-glucans have already been extensively studied. Mårtensson et al. (2005) co-fermented oat-based products with *P. parvulus* 2.6 resulting in "*a significant reduction in total cholesterol by 6% […] in volunteers who had eaten the fermented, ropy, oat-based product compared with the control group*" and "*a significant increase in total bacterial count and Bifidobacterium ssp*." (p.429-430). Garai-Ibabe et al. (2010a) described the applicability of different β-glucan positive LAB as potential functional food producers with probiotic properties which could "*induce the production of inflammation-related cytokines by polarized macrophages*" (p. 9262). Russo et al. (2012) showed the prebiotic potential of *O2*-substituted β-(1,3)-D-glucans by feeding it to probiotic LAB and how it further "*increases the binding of the non-ropy Lactiplantibacillus plantarum WCFS1 strain to intestinal cells*" (p. 6036). The immunomodulatory properties of β-glucan from *P. parvulus* 2.6 were analyzed by Notararigo et al. (2014) concluding that this EPS "*activates macrophages and has an anti-inflammatory effect*." (p. 109). And as the last example Pérez-Ramos et al. (2017) suggested from the results of their study that β-glucan enriched cereal-based food "*could be used as vehicle matrices to increase the performances of probiotic bacteria.*" as it perhaps increases the tolerance of digestive tract stresses and acts as an energy source for probiotic strains (p.10-14).

The health beneficial effects of cereal-based β-D-glucans in the human diet have already aroused attention a long time ago as they can have positive effects such as reducing serum cholesterol levels, reducing the risk of cardiovascular diseases, lowering postprandial blood glucose, and insulin response (Wood, 2007, Panahi et al., 2007, Smith et al., 2008, Bell et al., 1999, Behall et al., 2006), and being preventive to colon cancer and anti-inflammatory activities (Zhu et al., 2016, Zhu et al., 2015). Especially

oat and barley-based products carry health claims approved by the US FDA and EFSA declaring that those products can support a normal blood cholesterol level and potentially reducing the risk of coronary heart diseases, and help to reduce the rise in blood glucose levels after a meal (EFSA Panel on Dietetic Products and Allergies, 2011, EFSA Panel on Dietetic Products and Allergies, 2009, FDA, 2022). Although the plant-based β-D-glucan concentration of barley (2.5-11.3%) and oat (2.2- 7.8%) is higher compared to wheat (0.4-1.4%) and rye (1-3%), the application or substitution of β-D-glucan richer cereals in doughs usually affects the volume, texture, and firmness of baked goods (Lazaridou and Biliaderis, 2007, Vinkx and Delcour, 1996, Gill et al., 2002, Rieder et al., 2012). These findings further substantiate a possible health benefit of consuming baked goods enriched with β-glucan.

1.6 EPS degradation by LAB

Considering industrial applications low EPS yields or even the degradation of *in situ* synthesized polymers are very undesirable (De Vuyst et al., 2001). In general, LAB do not use their synthesized EPS as carbohydrate storages for later energy production and rather as a kind of protective shield against abiotic or biotic stress. However, a decrease in the EPS concentrations in LAB cultures was observed with progressive fermentation times, attributed either to enzymatic activity or physical effects. It is mostly still unclear why the LAB degrade the EPS, although they do not use it for energy production (Cerning et al., 1992, Vuyst and de Ven, 1998, Dierksen et al., 1995, Zannini et al., 2016, Degeest et al., 2002). Regarding the degradation of β-glucans by the producing strains themselves, so far this was only observed by Fraunhofer et al. (2018a), with a reduction of slimy and viscous properties. Further, it was assumed that the EPS could be used as a carbon source during starvation (Fraunhofer et al., 2018a), and as already described before, EPS could be used from Bifidobacteria or LAB-including probiotics as energy sources and thus act as prebiotics (Mårtensson et al., 2005, Russo et al., 2012).

Enzymes that are able to degrade EPS are members of the glycoside hydrolases (GH) family, also called glycohydrolases, that comprise among others α-D-glucosidases, β-D-glucosidases, α-D-galactosidases, β-D-galactosidases, or exo- and endo-glucanases (Drula et al., 2022). Some of these enzymes were previously described to be involved in EPS degradation which was observed as a reduction of the culture broth viscosity (Degeest et al., 2002, Pham et al., 2000). Till now, 170 GH families that are further associated with the numerical classification EC 3.2.1.- are described. The GH families in general possess hydrolytic activity leaning towards glycosidic bonds including carbohydrate compounds and compounds with non-carbohydrate fractions (Henrissat and Bairoch, 1993, Henrissat, 1991, Lombard et al., 2014). In summary, the hydrolytic activities of β-glucan-producing LAB needs to be analyzed in more detail to uncover the exact background to this process and to better understand the intrinsic value of this EPS. By acquiring this knowledge, the possible applications in sourdough fermentations can be better explored and, if necessary, adapted.

2 Motivation and Hypotheses

The increasing demand for the consideration of clean labels avoiding excessive additives, health-related or even health-promoting aspects in food processing, runs through all branches of industry, including baked goods. However, the use of cereals with known health-promoting effects, like oats or barley, in sourdough fermentation and bakery products is usually limited due to technological and sensory drawbacks (Lazaridou and Biliaderis, 2007, Rieder et al., 2012). Therefore, wheat and rye are the most frequent choices for sourdough fermentation, despite their content of health-promoting β-glucan being much lower. The main representatives in the sourdough microbiota are LAB and yeasts. Some LAB representatives produce EPS which can introduce structural, sensory, and even health benefits to fermented products (Bartkiene et al., 2020, De Vuyst and Neysens, 2005, Galle and Arendt, 2014, Gobbetti et al., 2008). The β-glucan, as a representative of LAB's homopolysaccharides (HoPS), is of particular interest here because, unlike other HoPS (α-glucans and β-fructans), it does not rely on the addition of sucrose. The maltose or glucose from starch hydrolysis could be used as a substrate for β-glucan formation (Fraunhofer, 2018, Arendt et al., 2007, Bounaix et al., 2009). The application and benefits of β-glucan in cereal-based foods have only been described superficially. In contrast, health-promoting benefits of bacterial β-glucans have already been extensively described (Pérez-Ramos et al., 2017, Mårtensson et al., 2005, Notararigo et al., 2014, Russo et al., 2012).
This study investigates, the applicability and persistence of β-glucan-producing LAB, e.g., *Levilactobacillus brevis* and *Pediococcus claussenii* isolated from spoiled beer, in wheat and rye sourdough fermentation. The circumstances that influence the formation and possible degradation and therefore the yield of the polymer, as well as the intrinsic factors acting on the dynamics, are identified for process optimizations. Moreover, the rheological and sensorial effects of the β-glucan and to some extent the physiological effects are analyzed.

The present work discusses the following working hypotheses:

- Beta-glucan producing LAB are competitive in the fermentation of β-glucan-deficient cereals like rye and wheat and enable β-glucan enrichment of the sourdoughs by simultaneous renunciation of sucrose addition.

- Exogenous factors affect the β-glucan formation by LAB and alterations of the fermentation conditions facilitate higher yields in sourdough fermentation.

- Sourdoughs enriched with bacterial β-glucan exhibit improved structural and sensorially characteristics and will contribute to health benefits in humans.

- The formation and degradation of bacterial β-glucan are regulated processes and are associated with expression of enzymes in distinct phases of microbial growth.

Based on the working hypotheses the experimental approaches of this study were derived:

- Pilot scale fermentation of flour-water mixtures are inoculated by β-glucan positive LAB to determine their ability to persist against the endogenous microbiota during sourdough fermentation and to enrich the dough by *in situ* β-glucan formation.

- By altering fermentation parameters (temperature, time, etc.), the formation of the polymer can be influenced and increased.

- Performing rheological analyses and sensorial analyses to support the identification of features attributed to β-glucan in sourdough and sourdough bread.

- Potential health-associated effects are analyzed from isolated and processed sourdough and bread samples of the β-D-glucan.

- Kinetics in the formation and the identification of processes involved in the potential degradation of β-glucan are identified using analytical methods (restriction by carbohydrate-active enzymes) and by use of a candidate strain with molecular biological (heterologous protein expression) and bioinformatic studies (proteomics).

3 Materials and Methods

3.1 Microbiological techniques

3.1.1 Microorganisms

In this study, the β-glucan producing strains, *Levilactobacillus* (*L.*) *brevis* TMW 1.2112 and *Pediococcus* (*P.*) *claussenii* TMW 2.340 (isogenic with DSM 14800^T and ATCC BAA-344^T), were used, both isolated from spoiled beer (Fraunhofer et al., 2018a, Dobson et al., 2002). In addition, one of each strain's derived *Δgtf-2* mutant, named *L. brevis* TMW 1.2320 and *P. claussenii* TMW 2.2123, were characterized and used as negative controls for β-glucan-associated experiments (Table 2).
The α-amylase producing strain, *Lactiplantibacillus* (*La.*) *plantarum* TMW 1.2330, was utilized for co-cultivated sourdough fermentation with the β-glucan positive and negative LAB. *Escherichia* (*E.*) *coli* BL21 was used in experiments of heterologous protein expression.

Table 2. Microorganisms used in this thesis.

Species	Strain	Source	β-glucan formation	Accession No.	Reference
L. brevis	1.2112	Wheat beer	Yes	CP016797	(Fraunhofer et al., 2019)
P. claussenii	2.340 (DSM 14800^T, ATCC BAA-344^T)	Beer	Yes	CP003137	(Pittet et al., 2012)
L. brevis	1.2320	TMW	No	-	(Bockwoldt et al., 2020)
P. claussenii	2.2123	TMW	No	-	(Bockwoldt et al., 2020)
Lactiplantibacillus plantarum	1.2330	TMW	No	-	-
Escherichia coli BL21	2.2200	(StrataGene®)	No	CP060121	(Wood, 1966, Studier and Moffatt, 1986)

3.1.2 Media and cultivation conditions

The cultivation media were either autoclaved at 121 °C for 20 min or sterile filtered using a 0.2 µm sterile filter (Sarstedt, Darmstadt, Germany). Before autoclaving, the pH value of the medium was adjusted with NaOH or HCl (both Carl Roth GmbH & Co. KG, Karlsruhe, Germany) according to the respective specification. Sugar solutions were autoclaved separately from the medium. Agar plates were prepared by adding 15 g AgarAgar (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), before autoclaving.
LAB were cultivated using the modified MRS (mMRS) medium according to Schurr et al. (2013) adjusting the pH to 6.20 before autoclavation. Depending on the experiment, D-maltose was used as the sole carbon source with a quantity of 20 g/L (Table 3). The mMRS medium was used for the preparation of the LAB strain collection containing 34% (v/v) glycerol (Gerbu Biotechnik GmbH, Heidelberg, Germany) stored at – 80 °C. Precultures were prepared with 10 mL mMRS in 15 mL reaction tubes (Sarstedt AG & Co., Nürnbrecht, Germany) cultivated as static cultures for up to 48 h at 30 °C, then centrifuged (7,500× g, 15 min, 4 °C). The cell pellet was dissolved in saline solution (Ringer

tablets, Merck KGaA, Darmstadt, Germany) depending on the requirements of further experimental setup. Solid mMRS medium containing 15 g/L agar was used for cell count experiments and for cultivations of strains from the strain collection. The agar plates were cultivated at 30 °C for 48 h.

Table 3. Components of the mMRS medium for LAB cultivation

Compound	Concentration [g/L]	Supplier
D-maltose	10	GERBU Biotechnik GmbH, Heidelberg, Germany
D-fructose	5	Omni Life Science GmbH & Co. KG, Bremen, Germany
D-glucose monohydrate	5	Merck KGaA, Darmstadt, Germany
Peptone from casein	10	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Meat extract	5	Merck KGaA, Darmstadt, Germany
Yeast extract	5	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$K_2HPO_4 \cdot 3\ H_2O$	4	VWR International, Radnor, PA, USA
KH_2PO_4	2.6	VWR International, Radnor, PA, USA
NH_4Cl	3	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Tween® 80	1	Merck KGaA, Darmstadt, Germany
L-Cysteine	0.5	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$MgSo4 \cdot 7\ H_2O$	0.20	Sigma-Aldrich, St. Louis, MO, USA
$MnSo4 \cdot H_2O$	0.04	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

The semi-defined medium (SDM) is based on the medium of Dueñas-Chasco et al. (1997) but with modifications to improve bacterial growth and β-glucan formation. The compounds were dissolved in previously autoclaved sterile demineralized H_2O (dH_2O). Before sterile filtration, the final pH was adjusted to 5.5 (Table 4). The static cultures were cultivated at 30 °C for approx. 30 h.

Table 4. Components of the semi-defined medium

Compound	Concentration [g/L]	Supplier
D-maltose	20	GERBU Biotechnik GmbH, Heidelberg, Germany
Casamino acids	5	MP Biomedicals GmbH, Eschwege, Germany
Bacto yeast nitrogen base (Difco)	3.35	Becton, Dickinson & Company, Pont de Claix, France
$MnSO_4 \cdot H_2O$	0.05	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$K_2HPO_4 \cdot 3H_2O$	2	VWR International, Radnor, PA, USA
Sodium acetate	5	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Adenine sulfate	0.005	SERVA Electrophoresis GmbH, Heidelberg, Germany
Guanin	0.005	Sigma-Aldrich, St. Louis, USA
Xanthin	0.005	Sigma-Aldrich, St. Louis, US
Uracil	0.005	Fluka Chemie GmbH, Buchs, Switzerland

Malic acid	4	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Carbon Base (Difco)	3.35	Becton, Dickinson & Company, Pont de Claix, France
KH_2PO_4	3	VWR International, Radnor, PA, USA
$MgCl_2 \cdot 6H_2O$	0.05	Sigma-Aldrich, St. Louis, MO, USA
Tween® 80	1	Merck KGaA, Darmstadt, Germany

The chemically defined medium (CDM) has been previously described by Otto et al. (1983) and adapted by Sánchez et al. (2008) Further modifications were made e.g., by using D-maltose as the sole carbon source and casamino acids as the source of the amino acids (Table 5). Compounds of the respective solutions were dissolved in previously autoclaved sterile demineralized dH_2O. The pH values of the base medium and vitamin solution were adjusted to 6.2 and 7.0 respectively. The nucleic acid components were dissolved in a 0.1 M NaOH buffer. Each solution was then sterilized by passage through a membrane filter (0.2µm). The CDM composed of 970 mL base medium with 10 mL vitamin solution, 10 mL metal solution and 10 mL nucleic acid bases solution. Static cultures were incubated at 30 °C with varying periods depending on the experiment.

Table 5. Components of the chemically defined medium

Compound	Concentration [g/L]	Supplier
Base medium (pH 6.2)		
D-maltose	20	GERBU Biotechnik GmbH, Heidelberg, Germany
$K_2HPO_4 \cdot 3H_2O$	2.5	VWR International, Radnor, PA, USA
KH_2PO_4	3.0	VWR International, Radnor, PA, USA
di-Ammonium hydrogen citrate	0.6	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium acetate	1.0	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Cysteine-HCl	0.25	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Casamino acids	5.0	MP Biomedicals GmbH, Eschwege, Germany
Tween® 80	1	Merck KGaA, Darmstadt, Germany
Vitamin solution (pH 7.0)		
Aminobenzoic acid	1.0	Sigma-Aldrich, St. Louis, MO, USA
Thiamin-HCl (Vit. B_1)	0.1	Sigma-Aldrich, St. Louis, MO, USA
Riboflavin (Vit. B_2)	0.1	Sigma-Aldrich, St. Louis, MO, USA
Nicotinic acid (Vit. B_3)	0.1	Sigma-Aldrich, St. Louis, MO, USA
Pantothenic acid (Vit. B_5)	0.1	Sigma-Aldrich, St. Louis, MO, USA
Pyridoxine (Vit. B_6)	0.5	Sigma-Aldrich, St. Louis, MO, USA
D-Biotin (Vit. B_7)	1.0	Sigma-Aldrich, St. Louis, MO, USA
Folic acid (Vit. B_9)	0.1	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Cobalamin (Vit. B_{12})	0.1	AppliChem GmbH, Darmstadt, Germany
Orotic acid (Vit. B_{13})	0.5	Sigma-Aldrich, St. Louis, MO, USA
Thymidine	0.5	Sigma-Aldrich, St. Louis, MO, USA

Inosine	0.5	Sigma-Aldrich, St. Louis, MO, USA
Lipoic acid	0.25	Sigma-Aldrich, St. Louis, MO, USA
Metal solution		
$MgCl_2 \cdot 6\ H_20$	20	Sigma-Aldrich, St. Louis, MO, USA
$CaCl_2 \cdot 2\ H_20$	5	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$FeCl_2 \cdot 4\ H_20$	0.5	Fluka Chemie GmbH, Buchs, Switzerland
$CoCl_2 \cdot 6\ H_20$	0.25	Sigma-Aldrich, St. Louis, MO, USA
$ZnCl_2$	0.5	Merck KGaA, Darmstadt, Germany
Nucleic acid bases solution (0.1 M NaOH)		
Adenine sulphate	1	SERVA Electrophoresis GmbH, Heidelberg, Germany
Uracil	1	Sigma-Aldrich, St. Louis, MO, USA
Xanthine	1	Sigma-Aldrich, St. Louis, MO, USA
Guanine	1	Fluka Chemie GmbH, Buchs, Switzerland

The fermentability of 49 different carbohydrates by LAB was analyzed using API®50 CHL test strips (bioMérieux, Marcy-l´Étoile, France) in combination with the API®50 CHL medium (Table 6). The test stripes were incubated at 30 °C, then checked after 24 h and 48 h for color changes of the indicator bromocresol purple.

Table 6. Components of the API®50 CHL Medium

Compound	Concentration [g/L]	Supplier
Polypeptone	10	Becton, Dickinson & Company, Pont de Claix, France
Yeast extract	5	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Tween® 80	1	Merck KGaA, Darmstadt, Germany
$K_2HPO_4 \cdot 3H_2O$	2	VWR International, Radnor, PA, USA
Sodium acetate	5	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Diammonium citrate	2	Sigma-Aldrich, St. Louis, MO, USA
$MgSo4 \cdot 7\ H_2O$	0.20	Sigma-Aldrich, St. Louis, MO, USA
$MnSO_4 \cdot H_2O$	0.05	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Bromocresol purple	0,17	Merck KGaA, Darmstadt, Germany

The yeasts naturally occurring during sourdoughs fermentation were cultivated and analyzed using yeast peptone glucose (YPG) media (Table 7). Prior to autoclavation, the pH value of the medium was adjusted to 6.5. Inoculated YPG agar plates were grown under oxic conditions overnight at 30°C.

Table 7. YPG Medium

Compound	Concentration [g/L]	Supplier
Casein peptone	10	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Yeast extract	5	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
D-glucose monohydrate	20	Merck KGaA, Darmstadt, Germany
AgarAgar	15	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

E. coli strains were cultivated in lysogeny broth (LB) Lennox in shake flasks at 37 °C and 200 rpm (Table 8). Prior to autoclavation, the pH value of the medium was adjusted to 7.20. After autoclavation, ampicillin (Amp) (Gerbu Biotechnik GmbH, Heidelberg, Germany) was added to reach a final concentration of 100 µg/mL for the selection of transformed *E. coli* cells.

Table 8. Components of LB-Lennox

Compound	Concentration [g/L]	Supplier
Tryptone from casein	10	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
NaCl	5	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Yeast extract	5	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

3.1.3 Viable cell count and phenotype analysis

The viable cell counts of sourdoughs and culture broths were determined by colony forming units (CFU). The CFU of sourdough samples were prepared by dissolving 10 g sourdough in 90 g saline solution and homogenized for 2 min with a sterile blender (BagMixer R400, Interscience International, Saint Non, France). Dissolved sourdough and culture broth samples were diluted by decimal serial dilutions with saline solutions. mMRS agar plates were spread with the specific dilution and incubated at 30°C for 48 h. Phenotypes of β-glucan producing and non-producing LAB colonies were distinguished using sterile wooden toothpicks. Beta-glucan producing colonies have a ropy phenotype, while non-producing colonies (*Δgtf-2* mutant) do not (Jakob et al., 2021).

3.1.4 Strain identification using MALDI-TOF MS

Bacterial colonies from cell count plates, e.g., sourdough fermentation, were identified on a species level by Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). Single colonies were sampled from agar plates and smeared onto stainless steel targets (Bruker Daltonics GmbH, Bremen, Germany) using on-target extraction (Kern et al., 2013). Therefore, 1 µL of formic acid, 70 % (v/v) (Sigma-Aldrich, St. Louis, MO, USA) was applied on every spot and air dried. In the next step, 1 µL α-cyano-4-hydroxycinnamic acid matrix solution (Merck KGaA, Darmstadt, Germany) was overlaid on every bacterial colony spot. The mass spectrometry-based identification was performed with a Microflex LT MALDI-TOF MS (Bruker Daltonics GmbH, Bremen, Germany), equipped with a nitrogen laser (λ = 337 nm). For species identification, the resulting spectra were compared with spectra of the Bruker and a self-generated database.

3.1.5 Microscopic detection of EPS capsule formation

Bacterial cells from agar plates and sourdoughs were analyzed for the formation of capsules by negative staining and agglutination tests, using microscopy (Axiostar Plus, Carl Zeiss AG, Oberkochen, Germany) with a 1,000× magnification. The *Δgtf-2* mutant strains were used as negative controls for both analyses.

Colonies from agar plates could be easily picked, while sourdough samples were processed to reduce background noise. The sourdough samples were dissolved in saline solutions and after 15 min of settling time, the supernatant which contained the microbial cells was used for further analysis. Capsule staining was performed using cells either from agar plates or sourdough supernatant mixed with black ink (Ferreira et al., 2002). The mixture was thinly spread on a microscope slide, air dried, then the cells were counterstained with an aqueous safranin solution (1% w/v) (Fluka Chemie GmbH, Buchs, Switzerland). The samples were microscoped by light microscopy at 1000× magnification.

Beta-glucan capsules were identified by an agglutination test using *Streptococcus* (*S.*) *pneumoniae* type 37-specific antiserum (Axolab GmbH, Kulmbach, Germany) (Walling et al., 2005b). Colonies from agar plates were resuspended in saline solution, mixed 1:1 with the antiserum, and applied on a microscope slide with a cover-slip. After an incubation of 30 to 60 min at 4 °C, the samples were microscoped by light microscopy at 1000× magnification. If bacterial cells possessed β-glucan capsules, they agglutinated in the presence of the antiserum.

3.2 Analytical methods

3.2.1 High-performance liquid chromatography (HPLC)

3.2.1.1 Organic acids

The quantities of organic acids, such as lactic acid and acetic acid, in sourdough samples, were measured by following the protocol of Rühmkorf et al. (2012a). Therefore, the dough samples were dissolved in dH_2O 1:2 (w/v) and centrifuged at 8,000× g, 30 min at 10 °C. Subsequently, 1 mL of the supernatant was mixed with 50 µL perchloric acid (70%) (Sigma-Aldrich, St. Louis, MO, USA) and incubated overnight at 4 °C. An additional centrifugation step (10,000× g, 10 min at 10 °C) was applied after a perchloric acid treatment, before membrane filtration with 0.2 µm nylon filters (Phenomenex, Aschaffenburg, Germany). The organic acids were separated by HPLC (Dionex Ultimate 3000, ThermoFisher Scientific, Waltham, MA, USA) using a sulfonated styrene-divinylbenzene Rezex™ ROA column (Phenomenex Inc., Torrance, CA, USA) and 2.5 mM sulfuric acid (VWR International, Radnor, PA, USA) as the eluent. The flow rate was set to 0.6 mL/min at 85 °C and the injection volume was 20 µL. The column was connected to a refractive index (RI) detector (Refractomax ERC, Munich, Germany) tempered at 35 °C for the detection of the organic acids.

3.2.1.2 Glucose

The dough samples were processed as described in 3.2.1.1, except that samples mixed with perchloric acid were incubated at 100 °C for 7 h instead of at 4 °C overnight. The heat treatment was applied to induce polysaccharide hydrolysis. Samples were cooled down and centrifuged at 10,000× g, 10 min,

and 10 °C, filtered (0.2 µm nylon filters) and stored overnight at 4 °C with a second filtration step the next day. The hydrolyzed samples were analyzed for glucose amounts by HPLC using a Rezex™ RPM Pb2+ column (Phenomenex, Aschaffenburg, Germany) connected to a RI detector (35 °C). Filtered and degassed dH_2O was the eluent at a flow rate of 0.6 mL/min at 85 °C, and the sample injection volume was 20-µL. The total glucose concentration measured by HPLC analysis was used to calculate the theoretical β-glucan amount using the correction factor ($\frac{162}{180}$) to convert the free glucose to anhydroglucose, as it is present in β-glucan.

3.2.2 Analytical Profile Index (API®)

3.2.2.1 API®50 CHL

The analytical profile index (API) test API®50 CHL (bioMérieux, Marcy-l´Étoile, France) can be used to characterize the fermentation ability of LAB for 49 different carbohydrates. The test was performed according to the manufacturer's instructions. Precultures of the LAB in the mMRS medium were harvested by centrifugation at 7,500× g for 15 min at 4 °C, and the cell pellet was dissolved in a saline solution. The solution was used to inoculate the API®50 CHL medium with an initial $OD_{600\ nm}$ between 0.4 and 0.5. Subsequently, 100 µL of the inoculated medium was filled in the tubes of the test strips. The capsules of the tubes were covered by paraffin oil (Sigma-Aldrich, St. Louis, MO, USA). Incubated strips (30 °C) were checked after 24 h and 48 h for positive results indicated by color changes from purple to yellow.

3.2.2.2 API® ZYM

The API® ZYM (bioMérieux, Marcy-l´Étoile, France) test was used to characterize cell lysate samples enzymatic activity. The test was performed according to the manufacturer's instructions. The cell pellet of 5 mL culture volume was washed and resuspended with 2.5 mL PBS buffer (pH7). The cells were disrupted using glass beads (Ø 2.85–3.45 mm, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and a benchtop homogenizer (FastPrep®-24 MP, MP Biomedical Inc, Eschwege, Germany), in three 30 s cycles with 1 min cooling phases on ice. Subsequently, 65 µL of the cell lysate was filled in the cupules and the test stripes were incubated for 4 h at 37 °C (Martínez et al., 2016, Gulshan et al., 1990). Enzyme activity was determined by color changes using a scale from 0 to 5. With 0 representing no enzyme activity (0 nM substrate hydrolyzed) and 5 representing a strong enzyme activity (≥ 40 nM substrate hydrolyzed (Baldrian et al., 2011).

3.2.3 Enzyme-linked Immunosorbent Assay (ELISA)

A competitive enzyme-linked immunosorbent assay (ELISA) as previously described by Werning et al. (2014) was used for the quantification of bacterial β-glucan. The antibodies of the *S. pneumoniae* serotype 37, which produce branched β-1,3 β-1,2 glucan capsules, interact with *O2*-substituted (1,3)-β-glucan of LAB (Llull et al., 2001, Werning et al., 2014). If required, samples were diluted with a sterile PBS buffer (Table 9). Sourdough samples were dissolved 1:10 (w/v) in a PBS buffer and mixed with glass beads (Ø 2.85–3.45 mm, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) in a benchtop

homogenizer at 5 m/s, 30 s, 24x12, then centrifuged at 7,500× g, for 10 min at 4 °C. In the following step, the supernatant was filtered (0.2 µm nylon filters) and used for further analysis.

The ELISA specific F8 Maxisorp, Nunc Immuno Module plates (ThermoFisher Scientific, Waltham, MA, USA) were coated with a 200 µL β-glucan-PBS solution (32.5 µg/mL) and incubated for 16 h at 4 °C. Afterward, the plates were washed twice with a washing solution, then 300 µL of the blocking solution was added per well, and the plate was incubated at 21 °C for 3 h. The first antibody (*S. pneumoniae* serotype 37 antibody) was diluted 1:800 in a PBS buffer, and 150 µL of the dilution was mixed with 150 µL of the sample (or standard dilution series of β-glucan) then incubated for 30 min at 21 °C. In the next step, 200 µL of the antibody-sample mixture was added to the coated well of the plate and incubated at 21 °C for 90 min. The second antibody (polyclonal anti-rabbit IgG+alkaline phosphatase (Sigma-Aldrich, St. Louis, MO, USA) was diluted 1:25,000 in a blocking solution. The plate was washed three times before adding the second antibody which was incubated for another 2 h at 21 °C. The plate was then washed four times and 200 µL of the developing solution was added. This was followed by incubating the plate at 37 °C for 30 mins, before 50 µL of the stop solution was added to the developing solution. The absorption was read at 405 nm using a microplate reader (SPECTROstar Nano (BMG, Labtech, Ortenberg, Germany)). For sourdough analysis, non-fermented wheat and rye doughs were used as blanks and subtracted from each value.

Table 9. ELISA buffers and solution

Compound	Concentration	Supplier
PBS buffer		
Na_2HPO_4 2 · H_2O	0.0377 M	Merck KGaA, Darmstadt, Germany
NaH_2PO_4 · H_2O	0.0123 M	Merck KGaA, Darmstadt, Germany
Washing solution		
PBS buffer		
Tween® 20	0.05%	Merck KGaA, Darmstadt, Germany
Blocking solution		
Washing solution		
Gelatin	0.5%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Developing solution, pH 9.5		
Diethanolamine	0.2 M	Merck KGaA, Darmstadt, Germany
$MgCl_2$	0.005 M	MP Biomedicals GmbH, Eschwege, Germany
p-nitrophenylphosphate (ρ-Npp)	1 mg/mL	GERBU Biotechnik GmbH, Heidelberg, Germany
Stop solution		
NaOH	2 M	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

3.2.4 D-glucose quantification

The amount of D-glucose was determined using glucose oxidase/peroxidase assay (GOPOD, Megazyme Ltd., Bray, Ireland) according to the manufacturer's protocol. Samples were diluted with sterile dH_2O if appropriate. The assay was adapted to the microtiter plate (MTP) format using a 50 µL

sample volume (sufficiently diluted) and 150 µL of the GOPOD reagent per well. The sample-reagent mixture was incubated at 45 °C for 20 min, and the absorbance was read at 510 nm with a microplate reader (SPECTROstar Nano (BMG, Labtech, Ortenberg, Germany)). D-glucose as part of the assay kit was used to generate standard dilution series, and dH_2O mixed with GOPOD reagent at the same ratio as samples were used as a reagent blank.

3.2.5 Enzymatical β-glucan quantification

The determination of released D-glucose, after enzymatical digestion of β-glucan samples, was performed using the GOPOD reagent kit (3.2.4), therefore 3 mL of the GOPOD reagent was mixed with 0.1 mL of sample material and incubated for 20 min at 40 °C. The absorbance was measured at 510 nm against the blank. The β-glucan content was calculated using the following formula:

$$\beta - \text{Glucan}\ (\%\ \text{w}) = \Delta\text{E} \cdot \text{F} \cdot \text{VCF} \cdot \frac{1}{1000} \cdot \frac{100}{W} \cdot \frac{162}{180} \qquad \text{[Formula 1]}$$

$$\beta - \text{Glucan}\ (\%\ \text{w}) = \mu\text{g Glucose} \cdot \text{VCF} \cdot \frac{1}{1000} \cdot \frac{100}{W} \cdot \frac{162}{180} \qquad \text{[Formula 2]}$$

with ΔE for absorbance minus the absorbance of the blank, and *F* as a factor to convert absorbance values to µg glucose: 100 (µg glucose)/absorbance for 100 µg glucose. Alternatively, as in Formula 2, the slope of the linear equation of the glucose calibration curve can be used to calculate the amount of D-glucose. Furthermore, *VCF* corresponds to the volume correction factor (0.1 ml of x ml (total volume of sample) was analyzed), 1/100 to convert micrograms to milligrams, *W* corresponds to the weight of the analyzed sample, 100/W refers to a sample to 100 mg and $(\frac{162}{180})$ is used as a factor to convert free glucose to anhydroglucose as found in β-glucan (Cho and White, 1993, McCleary and Codd, 1991).

3.2.5.1 Endo-1,3-D-glucanase or exo-β-1,3-D-glucanase

The β-glucan with concentrations of 5 mg/mL and 10 mg/mL in 0.1 M sodium acetate buffer (pH 5) were dissolved for 1 h at either room temperature, 40 °C, and 40 °C with additional ultrasonic treatment (Sonorex Super, Bandelin GmbH & Co. KG, Berlin, Germany). In the next step, 0.1 mL of the suspension was mixed either with 0.9 mL endo-1,3-D-glucanase (*Trichoderma sp.*) or exo-β1,3-D-glucanase (*Aspergillus oryzae*), each with 0.2 U/mL, then incubated for 30 min at 40 °C (de la Cruz et al., 1995, Quéméner et al., 2007). Reaction samples were used for D-glucose concentration determination.

3.2.5.2 Beta-glucan enzyme assay (GEM assay)

The isolated β-glucan was solubilized by gelatinization; therefore 15-20 mg of β-glucan was mixed in 400 µL of 2 M potassium hydroxide solution and solubilized on ice for 20 min with repeated inversion of the reaction tubes. Next, 1.6 mL of 1.2 M sodium acetate buffer (pH 3.8) was added to the gelled β-glucan. The first enzymatic digestion step was performed using 10 U/µL lyticase also known as zymolyase (*Arthrobacter luteus*) at 50 °C for 18 h. The samples were cooled down to room temperature and centrifuged (11,000× g for 3 min). For the second enzymatic digest, an aliquot of 130 µL was incubated with 650 µL exo-1,3-D-glucanase (*Trichoderma sp.*) 12 U/ml and β-glucosidase (*Aspergillus*

niger) 2.4 U/ml for 1 h at 40 °C. Samples were then cooled to room temperature and centrifuged (11,000× g for 3 min) (Danielson et al., 2010). Reaction samples were used for D-glucose concentration determination.

3.2.5.3 Beta-glucosidase (BglB) activity of *L. brevis* TMW 1.2112

The isolated and purified bacterial β-glucan samples of *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 and the linear β-glucan curdlan (Megazyme Ltd., Ireland) were dissolved in PBS buffer (50 mM, pH 7) to a final concentration of 1 mg/mL. The recombinant β-glucosidase was added to the β-glucan samples and incubated at 37 °C for 4 h. The negative controls were basically dissolved β-glucan samples lacking the enzyme. Reaction samples were used for D-glucose concentration determination.

3.3 Sourdough fermentation and sourdough bread preparation

3.3.1 Sourdough preparation and fermentation

The doughs were prepared from two different flour types for fermentations: rye flour type 1150 (Roland Mills Nord GmbH & Co. KG, Bremen, Germany) and wheat flour type 550 (Eickernmühle GmbH, Lemgo, Germany) with a dough yield (DY) of 200. A preculture of the designated strain was cultivated overnight, centrifuged (7,500× g, 10 min at 4 °C) and resuspended in saline solution until an $OD_{600\ nm}$ of 1 was reached. The sourdough was prepared by mixing 98 g tap water with 100 g flour and 2 mL of the bacterial cell solution. In some experiments, either 1 or 4 mL bacterial cell solution was added instead of 2 mL. Fermentation time and temperature of the sourdough leavening process depended on the experiment, which was performed in air-tight containers (Lock & Lock, iSi Deutschland GmbH, Solingen, Germany). The back-slopping procedure was performed by mixing 5% of the ripe sourdough into a freshly prepared dough (DY 200); fermentation was performed under the same conditions as previously performed.

3.3.2 Rheology measurement of sourdoughs

Rheology measurements of fermented sourdough samples were performed in cooperation with Silvia Brandner of the Chair of Brewing and Beverage Technology (Technical University of Munich).

Viscosity measurements of wheat sourdough samples were performed by using an AR-G2 stress-controlled rheometer (TA Instruments, New Castle, DE, USA) for flow curve measurements. Wheat sourdough samples (20 g) inoculated by cell suspensions were placed into a conical concentric cylinder and analyzed at 20 °C. The head cylinder had a 5.917 mm gap width, and the lateral gap width was 1 mm. The equilibration time was 2 min before each measurement. Steady-shear conditions were applied for the flow curve experiments using a continuous ramp with a shear rate that ranged from 0.01 to 100 1/s in a 2 min test duration.

The Herschel-Bulkley model as a simple power law relation $\tau = K\dot{\gamma}^{n}$, with τ as shear stress, was plotted against γ representing the shear rate to establish a linear correlation between the two parameters (Rao, 2007). The proportional relative changes of the shear stress and shear rate were proven by evaluating

the coefficient of determination (r^2); K (Pa s^n) and n (−) were determined to be the consistency factor and flow behavior index, respectively. The statistical significance was analyzed by a one-way ANOVA model in combination with Tukey's multiple comparisons test with a significance level of 0.01 (V. 6.01 GraphPad Prism, GraphPad Software Inc., San Diego, CA, USA).

3.3.3 Sourdough bread preparation

The leavening and baking of the sourdough bread varied between the wheat and rye sourdough bread types and was performed for sensory analysis. The doughs of wheat sourdough breads were mixed using 72 g (12%) sourdough, 331.9 g wheat flour type 550 (Eickernmühle GmbH, Lemgo, Germany), 7.4 g NaCl, 11.1 g fresh yeast, and 177.5 g tap water. The dry components (flour and salt) were mixed; the wet components (sourdough, water, and yeast) were added; and mixed with a hand mixer (575WBosch MFQ 4885, Abstatt, Germany) for 5 min followed by a 10 min leavening period at room temperature. The dough was kneaded for another 10 s, and left for a further 10 min leavening period, before 500 g of the bread dough was distributed into an aluminum tray. Dough proofing was carried out at 32 °C and 80% humidity for 35 min. Wheat sourdough breads were baked at 230 °C for 30 min (Piccolo, Wachtel GmbH, Hilden, Germany) by injecting steam into the oven within the initial 20 s. The valve was closed for the first 20 min and opened during the final 10 min.

Rye sourdough breads were prepared by mixing 217.9 g of rye flour type 1150 (Roland Mills Nord GmbH & Co. KG, Bremen, Germany), and 6.7 g of NaCl, then adding, 244.7 g (40.6%) of fermented sourdough, 6.7 g of fresh yeast, and 134.1 g of tap water, and mixing again for 5 min using a hand mixer. This was followed by a 20 min leavening period at room temperature. A total of 500 g of the rye bread dough was distributed into an aluminum tray and proofed at 32 °C and 80% humidity for 120 min. Rye sourdough breads were baked under the same conditions as the wheat sourdough breads (230 °C for 30 min).

The bread loaves were cooled over-night before sensory analysis and sampling for further analyses.

3.3.4 Sensory analysis of sourdough breads

The loaves of the wheat and rye sourdough breads fermented by *L. brevis* and *P. claussenii* strains were sliced into cubes (2–3 cm side length). In addition to a quantity of control bread lacking sourdough, the coded samples were presented in a randomized order to an untrained panel (n = 14 for wheat bread and n = 16 for rye bread) for an affective test. The panel evaluated the breads according to five attributes: moisture, texture (two categories: airiness and texture), acidity, and overall acceptance of the fermented bread (Table 10). The rating of the attributes from one (dry, compact, chewy, mild, and not tasty) to five (moist, fluffy, crumbly, sour, and tasty) was performed by using a 5-point Hedonic scale.

Table 10. Sensory evaluation sheet for sourdough bread samples

Attribute	Sample type	5-point Hedonic scale						
Moisture	Control:	dry	1	2	3	4	5	moist
	Sample:	dry	1	2	3	4	5	moist
Airiness	Control:	compact	1	2	3	4	5	fluffy

	Sample:	compact	1	2	3	4	5	fluffy
Texture	Control:	chewy	1	2	3	4	5	crumbly
	Sample:	chewy	1	2	3	4	5	crumbly
Acidity	Control:	mild	1	2	3	4	5	sour
	Sample:	mild	1	2	3	4	5	sour
Overall acceptance	Control:	not tasty	1	2	3	4	5	tasty
	Sample:	not tasty	1	2	3	4	5	tasty

3.4 Beta-glucan isolation and characterization

3.4.1 EPS Production and isolation

Bacterial β-glucan was harvested by centrifugation, precipitation of the supernatant, dialysis, and freeze-drying. Culture broths of β-glucan forming LAB with high viscosities were centrifuged at 16,000× g, 4 °C for 30 min. The collected supernatant was precipitated overnight at 4 °C with three-fold ice-cold ethanol 99% (v/v) (VWR International, Radnor, PA, USA, and Carl Roth GmbH & Co. KG, Karlsruhe, Germany). The precipitate containing the β-glucan was collected by centrifugation (10,000× g, 4 C for 15 min), and subsequently dissolved in dH_2O mixed with glass beads (Ø 2.85–3.45 mm, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) in a benchtop homogenizer. The solution was treated in one of two ways: either directly dialyzed for three days against dH_2O (three times a day water change) at 4 °C, using a dialysis membrane with a cut-off of either 3.5 kDa or 50 kDa (SERVA Electrophoresis GmbH, Heidelberg, Germany); or, for further protein removal, mixed with perchloric acid to a final concentration of 0.5 M and rest for 5 min on ice, followed by centrifugation at 13,000× g at 4 °C for 2 min before dialysis. In the following the samples were freeze-dried (Freezone 2.5, Labconco Corporation, USA) after deep-freezing at -80 °C. Isolated β-glucan was used for the quantification analysis using ELISA.

3.4.2 Viscosity analysis of culture broths

Viscosity analyses of culture broths were performed in cooperation with Michael Kupetz of the Chair of Brewing and Beverage Technology (Technical University of Munich).

Changes in viscosity of culture broths due to EPS formation and degradation were analyzed by a rotational viscometer of the type Super 4 Rapid-Visco-Analyser (RVA) (Perten Instruments, PerkinElmer Company, Waltham, MA, USA) (Rittenauer et al., 2017a, Rittenauer et al., 2017b). In total 40 ± 0.01 g of the culture broth samples were weight in an RVA sample can (PerkinElmer LAS, Rodgau Germany) and analyzed at 20 °C and 160 rpm after a 2 min equilibration phase. Viscosity analyses ran for 5 min under stable temperature and speed conditions.

3.4.3 Quantification of nitrogen contaminations in β-glucan isolates

Protein contaminations of the isolated and purified β-glucan were analyzed by quantification of nitrogen compounds. The lyophilized β-glucan samples were analyzed by automated Dumas analysis (MAX N exceed analyzer (Elementar Analysensysteme GmbH, Langenselbold, Germany) using aspartic acid for calibration (Kupetz et al., 2018). Dumas analyses were conducted at the Chair of Brewing and Beverage Technology (Technical University of Munich).

3.5 Molecular biological techniques

3.5.1 Isolation and purification of DNA

Genomic and plasmid DNA was isolated from cells grown as previously described (3.1.2) by use of commercial kits according to the manufacturer's protocols. Culture broths were centrifuged (7,500× g, 10 min at 4 °C) and the supernatant was discarded. Genomic DNA of LAB was isolated using E.Z.N.A.® Bacterial DNA Kit (VWR International, Radnor, PA, USA). The cells were washed with TE buffer (1 mM Ethylenediaminetetraacetic acid (EDTA) (Gerbu Biotechnik GmbH, Heidelberg, Germany)) and 10 mM Tris (Gerbu Biotechnik GmbH, Heidelberg, Germany)), at a pH 8.0 reducing the EPS concentration. In the next steps, lysozyme (10 mg/mL TE buffer (SERVA Electrophoresis GmbH, Heidelberg, Germany)) was added and incubated at 37 °C for 1.5 h or at 4 °C overnight, then further steps were performed according to the manufacturer's protocol. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen, Hilden Germany), performed according to the manufacturer's protocol. DNA concentration was measured with the NanoDrop ND-1000 (Peqlab Biotechnologie GmbH, Erlangen, Germany) and the isolated DNA was stored at -20 °C until further use.

3.5.2 Polymerase chain reaction (PCR)

Primers used for PCR amplification are listed in Table 11.

Table 11. Primers

Name	Sequence [5'-3']	Aim	Reference
Gtf-2 fwd	GAATCCGAACTAGCAATACTCGC	Gtf-2	(Fraunhofer et al., 2018a)
Gtf-2 rev	ACTAGTGGAATGTGCAACACTGG	Gtf-2	
K_GTF2 fwd	GAAGGTAAACGCGGTTCTGA	Gtf-2	(Stahl, Master thesis, 2019)
K_GTF2 rev	GGATTAAAGCGCTGTGTCCTTC	Gtf-2	
16S fwd	AGAGTTTGATYMTGGCTCAG	16S	(Weisburg et al., 1991)
16S rev	ACTACYVGGGTATCTAAKCC	16S	(Dotzauer et al., 2002)
AZI09_02135 fwd	CGTTTAAACTCAATGATGATGATGA TGATGTTGGCGTAATAAGGTGTTTGCCCG	AZI09_02135	This study
AZI09_02135 rev	CGTTTTTTGGGCTAACAGGAGGAATTAACC ATGGACATCGAACGAACGCTTGCTGAACTC	AZI09_02135	

The amplification of the designated DNA sequences e.g., Gtf-2 or 16S-rDNA, by PCR was performed using Taq DNA polymerase (MP Biomedicals Inc., Eschwege, Germany) (Table 12). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen N. V., Venlo, Netherlands) or E.Z.N.A.® Cycle Pure Kit (VWR International, Radnor, PA, USA).

Table 12. PCR master mix and cycler program using Taq DNA polymerase

Ingredients	Volume [µL]	Step	Temperature	Time	
dH_2O	Fill up to 50 µL	Initial denaturation	94 °C	2 min	
$MgCl_2$ buffer	5	Denaturation	94 °C	30 s	32 x
10 mM dNTPs	1	Annealing	T_m	30 s	
10 µM fwd primer	2.5	Elongation	72 °C	1 min	
10 µM rev primer	2.5	Final Elongation	72 °C	5 min	
Template DNA	variable				
Taq DNA polymerase	0.5				

PCR for cloning approaches by Gibson assembly was performed with either Phusion® High-Fidelity (HF) DNA Polymerase or Q5® High-Fidelity (HF) DNA Polymerase (New England Biolabs GmbH, Frankfurt am Main, Germany) as listed in Table 13.

Table 13. PCR master mix and cycler program using HF DNA polymerases

Ingredients	Volume [µL]	Step	Temperature	Time	
dH_2O	Fill up to 50 µL	Initial denaturation	98 °C	30 s	
Reaction buffer	10	Denaturation	98 °C	10 s	30 x
10 mM dNTPs	1	Annealing	T_m	30 s	
10 µM fwd primer	2.5	Elongation	72 °C	30 s	
10 µM rev primer	2.5	Final Elongation	72 °C	10 min	
Template DNA	variable				
HF DNA polymerase	0.5				

3.5.3 Cloning

3.5.3.1 Restriction digest and Gibson assembly

Heterologous protein expression was performed by use of the pBAD/Myc-His A vector (Invitrogen, Carlsbad, CA, USA) with an ampicillin resistance cassette for the screening of positive clones and a C-terminal coded 6x histidine tag for affinity purification. The vector was digested using the two restriction enzymes NcoI and SalI (New England Biolabs GmbH, Frankfurt am Main, Germany), which simultaneously excised the Myc-region of the vector. The digested vector was purified using a kit and stored at -20 °C until use. The purified amplicons of the β-glucosidase (AZI09_02135) and linearized vector were ligated by use of the Gibson Assembly Master Mix (New England Biolabs GmbH, Frankfurt am Main, Germany) according to the manufacturer's protocol. Gibson assembly was based on an isothermal, single-reaction to assemble multiple DNA fragments with overlapping regions by use of a 5′ exonuclease, a DNA polymerase, and a DNA ligase (Gibson et al., 2009).

3.5.3.2 Heat shock transformation

Chemical competent cells, prepared by the rubidium chloride method, were thawed on ice (Kushner, 1978). In the next step, 2 µL of the Gibson assembly reaction mix (pBAD vector with insert) was added

to the *E. coli* BL21 cells and gently mixed. The mixture was kept on ice for 30 min until heat shock at 42 °C for 30 s. Subsequently, 1 mL of LB medium was added to the tube and incubated at 37 °C for 60 min at 200 rpm (Biometra TSC ThermoShaker, Analytik Jena AG, Jena, Germany). The regenerated cells were plated on LB agar plates containing 100 µg/mL ampicillin and incubated at 37 °C for 24 h to 48 h.

3.5.3.3 Screening

Agar plates from the heat shock transformation were screened for positive clones, first by growth of colonies and secondly by PCR. Cells able to grow on LB plates with ampicillin indicated the successful transformation of the pBAD vector. The insert (β-glucosidase sequence) within the multiple cloning site of pBAD named pBAD_bGLU, was amplified by PCR, and 20 µL of the purified DNA was sent to Eurofins Genomics Europe Sequencing GmbH (Konstanz, Germany), including 20 µL of custom primers (10 pmol/µL), for sequencing according to Sanger (SupremeRun Tube).

3.5.4 Expression and purification of heterologous protein

E. coli BL21 clones with pBAD_bGLU were cultivated at 37 °C and 200 rpm until $OD_{600\,nm}$ ~ 0.5 was reached. Subsequently, 0.25% L-arabinose (v/v) was added to induce heterologous protein expression and the cultures were further cultivated overnight at 15 °C and 200 rpm. In the following, the induced cells were harvested by centrifugation (3,000× g, 10 min at 4 °C) and resuspended in lysis buffer: 50 mM sodium phosphate, 300 mM NaCl, 5% glycerol, 1 mM phenylmethylsulphonyl fluoride (SERVA Electrophoresis GmbH, Heidelberg, Germany) and 10 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) with pH 7.5. The cells were disrupted using glass beads (Ø 2.85–3.45 mm, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and a benchtop homogenizer, in three 30 s cycles with 1 min cooling phases on ice. The cell debris was collected by centrifugation (17,000× g for 30 min at 4 °C) and discarded, while the supernatant, which contained the his-tagged recombinant protein, was further used for purification. Nickel-nitrilotriacetic acid (Ni-NTA) crosslinked agarose resins (SERVA Electrophoresis GmbH, Heidelberg, Germany) were used for the purification of the tagged proteins according to the manufacturer's protocol. The collected fractions were further analyzed as described in Chapter 3.6.

3.6 Protein methods

3.6.1 Separation and visualization of proteins

3.6.1.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein separation and visualization were performed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant dye Roti® Blue (Carl Roth GmbH+Co. KG, Germany) gel staining (Schägger and von Jagow, 1987, Laemmli, 1970). SDS-PAGE was carried out in a Mini-PROTEAN Tetra Cell Electrophoresis System (Bio-Rad Laboratories, Hercules, CA, USA). The gel compositions of the separation gel (12% (w/v)) and stacking gel (4% (w/v)) are listed in Table 14.

Table 14. Composition of two SDS gels (pH 8.45)

Compound	Separation gel [mL]	Stacking gel [mL]	Supplier
Acrylamide/Bis,37.5:1 30% (w/v)	4.00	0.68	SERVA Electrophoresis GmbH, Heidelberg, Germany
Tris buffer (3 M) pH 8.45	3.33	1.29	Gerbu Biotechnik GmbH, Heidelberg, Germany
SDS 25% (w/v)	0.04	0.016	SERVA Electrophoresis GmbH, Heidelberg, Germany
dH_2O	2.56	3.21	-
Tetramethylethylendiamine (TEMED)	0.007	0.007	Sigma-Aldrich, St. Louis, MO, USA
Ammonium persulfate (APS) 10% (w/v)	0.05	0,033	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

The separation gel was mixed by inversion and approx. 4.5 mL of the unpolymerized solution was poured between a spacer plate (1 mm) and a cover plate (Bio-Rad Laboratories, Hercules, CA, USA), and overlaid with isopropanol, 99.5% (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). The separation gel was polymerized for about 20 min until finished. Before the separation gel was overlaid with the stacking gel, the isopropanol was removed. A sample comb of 1 mm width (Bio-Rad Laboratories, Hercules, CA, USA) was inserted into the unpolymerized stacking gel to generate the later loading wells. The sample comb was carefully removed after polymerization and shortly before the experiment.
SDS-Page samples were mixed with a 2× Laemmli sample buffer (Sigma-Aldrich, St. Louis, MO, USA) and heated for 10 min at 70 °C. The electrophoresis system was prepared with cathode and anode buffers (Table 15) and the gel was loaded with a 12.5 µL sample volume and a 4 µL protein marker (PageRuler Plus Prestained Protein Ladder (ThermoFisher Scientific, Waltham, MA, USA)), with a size range from 10 to 250 kDa. After the initial voltage of 80 V was applied for the first 10 min, the voltage was increased to 120 V for another 60 min.

Table 15. Cathode and anode buffer solutions

Compound	Cathode buffer, pH 8.25	Anode buffer, pH 8.90	Supplier
Tris	0.1 M	0.2 M	Gerbu Biotechnik GmbH, Heidelberg, Germany
Tricine	0.1 M	-	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
SDS	0.1%	-	SERVA Electrophoresis GmbH, Heidelberg, Germany

The finished gel was stained by Coomassie staining using the Roti® Blue colloidal CBBG-250 staining solution (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) according to the manufacturer's protocol. All steps were carried out at room temperature; during incubation periods the gel was gently shaken (Uniequip Unitwist RT, Labstribute GmbH Labor-Auktionen, Munich, Germany) at room temperature.

3.6.1.2 Western blot

The electrophoretic transfer of proteins from SDS gels to membranes was previously described by Towbin et al. (1979) and further developed since. The transfer of protein bands from an SDS gel to an Immun-Blot™ PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA) was initiated by incubation

in a transfer buffer (Table 16) for 20 min, while the PVDF membrane was previously saturated with a ≥ 98.5% methanol (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). The semi-dry western blot transfer system (Owl™ HEP Serie, ThermoFisher Scientific, Waltham, MA, USA) was loaded with the SDS gel, PVDF membrane, and filter papers, and the transfer of proteins was performed at 50 mA for 1 h. His-tagged GFP was used as a positive control and applied on the membrane after blotting. The PVDF membrane was incubated in the blocking solution and shaken (Uniequip Unitwist RT, Labstribute GmbH Labor-Auktionen, Munich, Germany) overnight at 4 °C. The next day the membrane was washed three times with PBS-T buffer for 10 min at room temperature. The first antibody (heterologous protein expression: 6x-His Tag Monoclonal Antibody (HIS.H8) (Thermo Fisher Scientific Inc., Waltham, MA, USA) and β-glucan detection in sourdough samples: *S. pneumoniae* serotype 37 antibody) diluted 1:2000 in PBS-T buffer was added, and the membrane was shaken for 1.5 h, followed by another three wash steps (10 min each) with PBS-T buffer. The second antibody (heterologous protein expression: anti-mouse IgG+AP and β-glucan detection in sourdough samples: polyclonal anti-rabbit IgG+AP) was added, diluted 1:5000 in PBS-T buffer, and incubated for 1.5 h. After further washing steps with PBS-T, PBS buffers, and AP, staining with 7.5 µL NBT (nitro blue tetrazolium, SERVA Electrophoresis GmbH, Heidelberg, Germany) and 30 µL BCIP (5-bromo-4-chloro-3-indolyl phosphate, Gerbu Biotechnik GmbH, Heidelberg, Germany) in 15 mL AP buffer was performed for 2 to 10 min; the reaction was stopped with dH_2O.

Table 16. Solutions used for western blotting

Solution	Compound	Concentration	[g/L]
Transfer buffer	Tris	50 mM	6.06
	Glycerin	190 mM	14.26
	SDS	1 g/L	1
	Methanol	20 % (v/v)	200 mL
	dH_2O		800 mL
Blocking solution (pH 7.4 with HCl)	Tris	0,02 M	2.42
	NaCl	0,2 M	11.69
	BSA	3 % (w/v)	30
	dH_2O		1000 mL
PBS (pH 7.4 with NaOH)	KH_2PO_4	4 mM	0.54
	Na_2HPO_4	16 mM	2.85
	NaCl	115 mM	6.72
	dH_2O		1000 mL
PBS-T (pH 7.4 with NaOH)	KH_2PO_4	4 mM	0.54
	Na_2HPO_4	16 mM	2.85
	NaCl	115 mM	6.72
	Tween 20	0.1 % (v/v)	1 ml
	dH_2O		1000 mL
AP (100 mL) (pH 8.8 with NaOH)	Tris	100 mM	15.76
	NaCl	100 mM	0.58
	$MgCl_2$	5 mM	0.05
	dH_2O		100 mL
NBT (nitro blue tetrazolium)	NBT	75 mg/mL	0.08
		70 % (v/v)	0.7 mL

	DMF (di-methyl formamide) dH_2O		0.3 mL
BCIP (5-bromo-4-chloro-3-indolyl phosphate)	BCIP DMF	60 mg/mL 100 % (v/v)	0.06 1 mL
Methanol	Methanol	100%	--

3.6.2 Protein quantification

Coomassie (Bradford) protein assay (ThermoFisher Scientific, Waltham, MA, USA) was used for the quantification of protein amounts (Bradford, 1976) and BSA (ThermoFisher Scientific, Waltham, MA, USA) was used for standard dilution series; samples were diluted with sterile dH_2O if appropriate. The analysis was performed according to the manufacturer's protocol in a microtiter plate format, measured at 595 nm with a microplate reader (SPECTROstar Nano, BMG, Labtech, Ortenberg, Germany).

3.6.3 Screening for enzyme activity and stability

Seven different *p*-nitrophenyl phosphate (pNP) substrates were used to analyze the substrate specificity of recombinant enzymes: *p*NP α-D-glucopyranoside (*p*NPαGlc, Sigma-Aldrich, St. Louis, MO, USA), *p*NP β-D-glucopyranoside (*p*NPβGlc, Sigma-Aldrich, St. Louis, MO, USA), pNP α-L-fucopyranoside (*p*NPαFuc, Merck KGaA, Darmstadt, Germany), pNP β-D-fucopyranoside (*p*NPβFuc, Merck KGaA, Darmstadt, Germany), *p*NP α-D-galactopyranoside (*p*NPαGal, Carl Roth GmbH & Co. KG, Karlsruhe, Germany), *p*NP β-D-galactopyranoside (*p*NPβGal, Carl Roth GmbH & Co. KG, Karlsruhe, Germany), and *p*NP β-D-maltoside (Santa Cruz Biotechnology, Inc., Heidelberg, Germany).

Prior enzyme activity studies were conducted, heterologous enzymes were purified (Chapter 3.5.4) and dialyzed using 3.5 kDa dialysis tubing (SERVA Electrophoresis GmbH, Germany) in 50 mM PBS buffer (pH 6.8) at 4 °C overnight, to remove imidazole from the Ni-NTA purification. Protein concentration was determined according to Bradford (3.6.2). In the following, enzyme solutions were directly incubated with 2 mM pNP substrate in 50 mM PBS buffer (pH7) at 37 °C for 2 h, using a microtiter plate reader (SPECTROstar Nano, BMG Labtech GmbH, Germany). Enzyme stability was tested at different pH values (4-11) and temperatures (10-60 °C) in 50 mM PBS buffer with 2 mM *p*NPβGlc. Enzyme activity was detected by the release of pNP at 405 nm, resulting in a yellow coloration of the enzyme-substrate solution.

3.6.4 Enzyme kinetics

The isolated and purified enzyme, β-glucosidase, was analyzed for its Michaelis Menten constant (K_M) and maximum reaction rate (V_{max}) using a substrate with high affinity e.g., *p*NPβGlc with concentrations between 0.01 mM and 20 mM in 50 mM PBS buffer (pH 7) at 37 °C (Johnson and Goody, 2011). The release of *p*-nitrophenol was detected as an increase in the absorbance at 405 nm with a microtiter plate reader (SPECTROstar Nano, BMG, Labtech, Ortenberg, Germany). The Lineweaver–Burk plot was applied for the calculation of the kinetic constants (Lineweaver and Burk, 1934).

3.7 Proteomics

Proteomic analyses were performed in cooperation with Dr. Christina Ludwig and Dr. Chen Meng of the Bavarian Center for Biomolecular Mass Spectrometry (BayBioMS, Freising, Germany).

3.7.1 Sample preparation

L. brevis TMW 1.2112 culture broths were centrifuged at 17,000× g for 30 min at 4 °C. The supernatant was collected for the secretome analyses, and the cells were used for the cellular proteome analyses after further processing.

The bacterial cells were washed once with saline solution, centrifuged (17,000× g for 30 min at 4 °C), then shock-frozen with liquid nitrogen for storage at -80 °C until cell lysis. Depending on the previous $OD_{600\ nm}$ of the culture broths, the volume of the lysis buffer mixed from 8 M urea (Gerbu Biotechnik GmbH, Heidelberg, Germany), 100 mM NH_4HCO_3 (Sigma-Aldrich, St. Louis, MO, USA), 1 mM dithiothreitol (DTT, Gerbu Biotechnik GmbH, Heidelberg, Germany) dissolved in water with pH 8.0; was adapted i.e., the cell pellet of 50 mL culture broth with an $OD_{600\ nm}$ 1 was diluted with 1 mL lysis buffer. Cell disruption was performed by further adding acid-washed glass beads (Ø 2.85–3.45 mm) with a benchtop homogenizer in five cycles, each 45 s at 5 m/s with 1 min cooling phases in between the cycles. The collection of the cell lysate was done by centrifugation (17,000× g, 10 min, 4 °C) and the protein concentration was determined by Bradford assay (see 3.6.2). In the next step, 15 µg of protein were reduced with DTT (10 mM, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and chloroacetamide (CAA, Merck KGaA, Darmstadt, Germany) (55 mM). A twofold protein digest with 0.15 µg trypsin (Roche Deutschland Holding GmbH, Penzberg, Germany), first for 2 h and the second time overnight at 37 °C, was performed, and the next day the samples were desalted and resuspended in 2% acetonitrile (VWR International, Radnor, PA, USA), 98% dH_2O, and 0.1% formic acid (CARLO ERBA Reagents GmbH, Emmendingen, Germany), to a final concentration of 0.1 µg/µl protein. This sample was then analyzed by LC-MS/MS.

Secretome samples were generated by mixing the supernatant 2:1 (v/v) with an LDS Sample Buffer (4X) (ThermoFisher Scientific, Waltham, MA, USA), followed by heating at 70 °C for 10 min. The mixture was run on NuPAGE™ 4-12% Bis-Tris Protein Gel (ThermoFisher Scientific, Waltham, MA, USA) for 3 min; the single protein band was excised and reduced (50 mM DTT), alkylated (55 mM CAA), and digested with trypsin (trypsin-gold, Promega, Madison,WI ,USA) overnight according to Shevchenko et al. (2006). Before the MS measurement, the secretome sample was resuspended in 25 µl 2% acetonitrile, 0.1 formic acid. For the LC-MS/MS measurements, 3 µl were injected into the mass spectrometer per run.

3.7.2 Peptide measurements by mass spectrometry

The flow liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) measurements were performed by the Ultimate 3000 RSLCnano system coupled to a Q-Exactive HF-X mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA).

For proteome analyses (cellular and secretome) approx. 0.2 µg of peptide solution was injected into a trap column (ReproSil-pur C18-AQ, 5 µm, 20 mm × 75 µm, self-packed, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) with a flow rate of 5 µL/min (solvent A: HPLC grade H_2O with 0.1% formic acid). After a 10 min loading period, the peptide solution was transferred to an analytical column (ReproSil Gold C18-AQ, 3 µm, 450 mm × 75 µm, self-packed, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). The peptides were separated using a 50 min gradient from 4% to 32% of solvent B containing 0.1% formic acid in acetonitrile and 5% (v/v) Dimethyl sulfoxide (DMSO) with a 300 nL/min flow rate. Both nanoLC solvents (A and B) contained 5% DMSO to boost MS intensity.

The mass spectrometer (Q-Exactive HF-X) ran in data-dependent acquisition (DDA) and positive ionization mode. Recording of MS1 spectra (360–1300 m/z) was performed at a resolution of 60,000 under automatic gain control (AGC) with a target value of $3 \cdot 10^6$ at a maximum injection time (maxIT) of 45 ms. In the case of the full proteome analyses, up to 18 peptide precursors were selected regarding fragmentation. Precursors with charge state between 2 and 6 were exclusively selected and dynamic exclusion for 25 s was enabled. Higher energy collision-induced dissociation was used for peptide fragmentation and the normalized collision energy was 26%. The width of the precursor isolation window was 1.3 m/z, and that of the MS2 resolution was 15,000, with an AGC target value of $1 \cdot 10^5$ and maxIT of 25 ms.

3.7.3 Data and statistical analysis

The software MaxQuant (V1.6.3.4) with its built-in search engine Andromeda was used for peptide identification and quantification (Cox et al., 2011, Tyanova et al., 2016). The proteome of *L. brevis* TMW 1.2112 (GenBank accession No.: CP016797), comprising 2,537 coding sequences (with common contaminants, a built-in option in MaxQuant), was searched for the MS2 spectra. The following settings were applied: Trypsin/P as proteolytic enzyme, precursor tolerance of 4.5 ppm, and fragment ion tolerance of 20 ppm. The results were adjusted to a 1% false discovery rate (FDR) on peptide (spectrum match) and protein level implementing a target-decoy approach using reversed protein sequences. The minimal peptide length was specified to 7 amino acids, and the “match-between-run” function was enabled, applying a matching time window of 0.7 min and an alignment window of 20 min. The fixed modification was carbamidomethylated cysteine and the variable modifications were oxidation of methionine and N-terminal protein acetylation. Cellular proteome samples were analyzed by label-free quantification (LFQ) to compare relative protein abundances. LFQ assumes the comparability of the total protein abundance across analyzed samples. The secretome experiment clearly violated this assumption and therefore, intensity-based absolute quantification (iBAQ) was applied for this analysis (Ahrné et al., 2013).

First, the proteins identified as “only identified by site”, “reversed”, and “potential contaminants” were removed in the downstream process. The iBAQ intensities (as applied for the secretome experiment) were centered across samples based on the proteins shared between the time points 8 hours and 7 days. LFQ intensities (applied for cellular proteome) are already well normalized. The intensity values were logarithmized on base 10. The threshold for protein identification was set to 3 replicates out of 4; fewer identifications were excluded from statistical analysis. The remaining missing values were imputed

using the lower detection limit method of Holland and McElroy (1986), which means that missing protein expression values are replaced by constants half of the lowest detected values. Since missing values are more likely a result of low abundant proteins, imputed values higher than 15% quantile of all detected values, were replaced by the 15% quantile.

Significant differentially expressed proteins of the secretome experiment (8 hours and 7 days) were identified by the Student t-test. For the cellular proteome experiment, the Pearson correlation analysis was used to identify proteins that correlated positively and negatively with viscosity, β-glucan, and D-glucose abundance. Fisher's exact test was used in the enrichment analysis and the functional annotation of the proteins was done by PANNZER2 (Törönen et al., 2018) and InterProScan (Quevillon et al., 2005, Jones et al., 2014). R statistical environments (V3.6.3) were used for all statistical analyses (Rizzo, 2019, Ramachandran and Tsokos, 2020). Furthermore, the sequence of *L. brevis* TMW 1.2112 was analyzed by RAST (rapid annotations using subsystems technology) (Overbeek et al., 2014) and eggNOG-Mapper (Cantalapiedra et al., 2021, Huerta-Cepas et al., 2017), for functional annotation and clustering of orthologous groups (COG). Putative secretory proteins were identified by SignalP-5.0 (Armenteros et al., 2019) by signal peptide sequence.

3.7.4 Proteomic data availability

All data of the mass spectrometry proteomic analyses are deposited online (http://proteomecentral.proteomexchange.org) with the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD031809 (Perez-Riverol et al., 2021).

3.8 Bioinformatic tools

In the course of this work, various databases and software were used to process data for statistical analyses, characterization of, e.g., DNA or protein sequences, or visualization of the results (Table 17).

Table 17. Software and databases used for analyses mentioned in this study

Software/ Database	Application	Source
Bionumerics® software package V7.62	Construction of phylogenetic trees	Applied Maths, Sint-Martens-Latem, Belgium (Vranckx et al., 2017)
BRENDA	Enzyme database	(Schomburg et al., 2002)
CAZy (Carbohydrate-Active enZYmes)	Database of carbohydrate-active enzymes	(Lombard et al., 2014)
Clone Manager 9	DNA sequence analysis and primer design	Sci Ed Software, Westminster, CO, USA
Chromeleon™ V6.8	HPLC software	(Harned, 2002)
eggNOG-Mapper V2	Functional annotation of genomes	(Cantalapiedra et al., 2021)

GraphPad Prism V6.01	Statistical analyses and scientific graphing	(Swift, 1997)
MaxQuant Cruncher	Proteomic analysis	BayBioMS in-house software
NCBI BLAST	DNA and protein sequence similarity identifier	(Altschul et al., 1990)
PANNZER2	Functional annotation of genomes	(Törönen et al., 2018)
PredictProtein	Protein characterization	(Bernhofer et al., 2021)
R statistical environment V3.6.3	Statistical analyses	(Rizzo, 2019)
RAST (rapid annotations using subsystems technology)	Genome annotation	(Overbeek et al., 2013)
SignaP 5.0	Signal peptide prediction	(Armenteros et al., 2019)
SWISS-MODEL	Protein structure prediction	(Waterhouse et al., 2018)
UniProt	Protein characterization	(Consortium, 2020)

4 Results (Thesis publication)

4.1 Persistence and β-glucan formation of beer-spoiling lactic acid bacteria in wheat and rye sourdoughs

Research for high-quality and healthy food products has been increasing continuously in recent years. The incorporation of health-promoting cereals rich in β-glucan, like oat and barley, in the processing of baked goods has nonetheless some disadvantages on product quality. Here, the enrichment of low β-glucan flour types like wheat and rye using β-glucan forming lactic acid bacteria (LAB) could add both technological and nutritional values to the baked goods. And unlike other homopolysaccharides (HoPS) requiring sucrose addition for *in situ* enrichment, the bacterial β-glucan could be formed from sugars naturally present in the flour and dough such as maltose or glucose. However, the applicability of β-glucan positive LAB isolated from spoiled beer in sourdough fermentations was so far not analyzed.
On this account, the persistence of the β-glucan-forming beer isolates *Levilactobacillus* (*L.*) *brevis* TMW 1.2112 and *Pediococcus* (*P.*) *claussenii* TMW 2.340 (isogenic with DSM 14800^T and ATCC BAA-344^T) in addition to isogenic, β-glucan-deficient (*Δgtf-2*) strains *L. brevis* TMW 1.2320 and *P. claussenii* TMW 2.2123 was analyzed in sourdough fermentation using wheat and rye flour. For this purpose, the LAB were grown in pre-cultures to inoculate the flour-water mixtures which were incubated for 24 hours. Fermented sourdoughs were back-slopped twice to inoculate fresh flour-water mixtures with ripe sourdough samples. The sourdough samples were analyzed considering three features: the microbiota using cell counts and MALDI-TOF MS; technological properties via pH values, sourdough viscosity, and organic acid production; and the presence of β-glucan formation counting the number of ropy colonies, capsule-staining, and agglutination test. It was shown that both the β-glucan-positive and -negative LAB were dominant in wheat and rye sourdoughs. The strains acidified the flour-water mixtures reaching sourdough characteristic pH ranges. As a heterofermentative LAB, *L. brevis* produced acetic acid and lactic acid during dough fermentation. On the other hand, sourdoughs containing the homofermentative *P. claussenii* resulted in only traces of acetic acid probably by endogenous LAB of the doughs. The β-glucan formation by *L. brevis* TMW 1.2112 in both sourdough types was stable, in contrast to *P. claussenii* TMW 2.340, which showed a decreasing trend of ropy colonies with each back-slopping step. In addition, *L. brevis* TMW 1.2112 in wheat sourdoughs produced significantly higher viscosities than *P. claussenii* TMW 2.340 or the mutant strains. The detection of β-glucan capsules by capsule-staining or an agglutination test was also only effective with rod-shaped *L. brevis* cells, which could be attributed to the cell size since *P. claussenii* sp. exhibit coccoid cells. The β-glucan-deficient strains used as negative controls showed neither capsule formation nor rheological effects but showed growth and acidification behavior like the β-glucan-positive strains. Consequently, the beer isolates and isogenic EPS-deficient strains were persistent in wheat and rye sourdough fermentation. Regarding *in situ* β-glucan enrichment of doughs by LAB, the avoidance of sugar additions and the associated residual sweetness are particularly positive. These properties suggest well-suited sourdough starters. In particular, *L. brevis* TMW 1.2112 has the potential to act as a functional starter culture due to their additional rheological properties.

The supplementary files (Figure S 1 and Figure S 2) of the publication are attached to the appendix in Chapter IV.

Author contributions: Julia A. Bockwoldt was responsible for the conceptualization, formal analysis, methodology, and visualization and wrote the original draft of the manuscript. Leonie Stahl supported Julia A. Bockwoldt in the investigations. Silvia Brandner was involved in the rheology experiments. Frank Jakob conducted the conceptual setup and methodology and was the project leader responsible for supervision. Frank Jakob, Matthias A. Ehrmann, and Rudi F. Vogel were in charge of project administration and supervision and further conducted the editing of the final manuscript.

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Persistence and β-glucan formation of beer-spoiling lactic acid bacteria in wheat and rye sourdoughs

Julia A. Bockwoldt, Leonie Stahl, Matthias A. Ehrmann, Rudi F. Vogel, Frank Jakob*

Lehrstuhl für Technische Mikrobiologie, Technische Universität München, Freising, Germany

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ABSTRACT

Some beverage-spoiling lactic acid bacteria (LAB) produce capsular β-glucans from UDP-glucose, which is accompanied by cell network formation causing viscosity increases of liquids. This feature of certain LAB is feared in breweries but could be useful for structural and nutritional improvement of baked goods, provided that these LAB are suited for the manufacture of sourdoughs. The aim of this study was to investigate the persistence and β-glucan formation of the brewery isolates *Levilactobacillus* (*L.*) brevis TMW 1.2112 and *Pediococcus* (*P.*) *claussenii* TMW 2.340 in wheat and rye sourdoughs. Both the wild-type strains and the respective β-glucan-deficient mutants were dominant in wheat and rye sourdoughs and acidified them to characteristic pH ranges. The formation of β-glucan capsules during sourdough fermentations was stable in *L. brevis* TMW 1.2112 in contrast to *P. claussenii* TMW 2.340. Wheat sourdoughs fermented with the β-glucan producing *L. brevis* TMW 1.2112 cells were significantly more viscous than doughs fermented by the *P. claussenii* TMW 2.340 cells and the applied mutant strains. In conclusion, *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 were suited and persistent wheat and rye sourdough starters, while the *in situ* β-glucan formation in sourdoughs was hardly detectable in case of *P. claussenii*.

1. Introduction

Baked goods made with wheat or rye sourdough have a long tradition in the human diet. The use of sourdoughs, which are mainly fermented by lactic acid bacteria (LAB) and yeasts, has beneficial effects on the shelf life, texture, and taste of breads. Many of these positive effects are due to the metabolic activity of LAB, especially the production of small metabolites like lactic and acetic acid. Moreover, the formation of high molecular weight exopolysaccharides (EPSs) positively affects the staling process and improves the water binding capacity of the doughs (Arendt et al., 2007; Brandt, 2007; Galle et al., 2011; Poutanen et al., 2009). The most frequently studied EPSs produced in sourdoughs are α-glucans (e.g. dextrans and reuterans) (Chen et al., 2016; Galle et al., 2012; Kaditzky et al., 2008; Katina et al., 2009; Kothari and Goyal, 2016; Wolter et al., 2014) and β-fructans (e.g. levans and inulins) that contain a single type of monosaccharide and are thus termed homopolysaccharides (Galle et al., 2010; Jakob et al., 2013; Rühmkorf et al., 2012b; Tieking et al., 2005a; Tieking and Gänzle, 2005b; Tinzl-Malang et al., 2015; Ua-Arak et al., 2016). For *in situ* α-glucan and β-fructan production, sucrose has to be added to water-flour mixtures, as it is the substrate of the secreted glucan- and fructansucrases (Bounaix et al., 2009; Korakli et al., 2001; Korakli and Vogel, 2006).

Despite the beneficial effects of these EPSs in terms of structural and shelf-life improvements, the addition of sucrose may lead to excessive acetate formation in sourdoughs (Kaditzky et al., 2007). Therefore, and also because of their possible otherwise superior properties, further LAB derived EPSs like heteropolysaccharides or β-glucans, both of which are produced from intracellularly activated sugar nucleotides precursors, are promising alternatives for improvement of bread properties despite being usually produced in lower quantities compared to sucrose-derived homopolysaccharides (De Vuyst and Degeest, 1999; De Vuyst and Neysens, 2005; Fraunhofer et al., 2018b; Galle et al., 2011; Prechtl et al., 2018). Beta-glucans, whose backbone consists of β-(1 → 3) glyosidic bonds, have gained much interest in the food industry due to their health-promoting properties (Wood, 2007). Stack et al. (2010) showed increased probiotic effects on LAB through β-glucan encapsulation (Stack et al., 2010). Furthermore, prebiotic and immune system modulating effects were described for β-glucan produced by *Pediococcus parvulus* 2.6 (Notararigo et al., 2014; Russo et al., 2012). The majority of studies dealing with the health-promoting effects of β-glucans were focused on β-glucans from oat and barely (Behall et al., 2006; Bell et al., 1999; Panahi et al., 2007; Smith et al., 2008; Wood, 2007). However, β-glucan-rich cereals like barley (2.5–11.3%) and oat

* Corresponding author.
E-mail address: frank.jakob@tum.de (F. Jakob).

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(2.2–7.8%) are not suited for the majority of baking applications, where wheat (0.4–1.4%) and rye (1–3%) based flours are predominantly used (Gill et al., 2002; Lazaridou and Biliaderis, 2007; Rieder et al., 2012; Vinkx and Delcour, 1996).

As such, the use of β-glucan forming LAB as sourdough starters may offer a new solution for naturally enriching baked goods with structure forming bacterial EPS and concomitant further exploitation for added value functions for the consumer. Bacterial β-glucan biosynthesis occurs in wine- and beer-spoiling strains of *Levilactobacillus* (*L.*) *brevis* and *Pediococcus* (*P.*) *claussenii* and is predominantly plasmid-encoded, with intracellularly pre-synthesized UDP-glucose serving as the substrate for the capsular β-glucan formation. The structure-forming, or viscosity increasing, effect can be strong due to the concomitant bacterial network formation rather than by the secretion of polysaccharides (Fraunhofer et al., 2018a,b). Hence, the cause of β-glucan-triggered, structure-forming effects essentially differ from that triggered by secreted EPS, which only exhibit hydrocolloid function. Fraunhofer (2018) described the strong network-like cell structure due to capsular β-glucans for *L. brevis* TMW 1.2112 in liquid cultivation media. They observed a multifold increase in viscosity based on its diffuse growth.

In the present study, the persistence of β-glucan producing LAB *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 in wheat and rye sourdoughs was investigated. The respective *gtf-2* deficient mutants of these strains were used as controls in separate sourdough fermentations to reveal the viscosity increasing effects of the *in situ* formed β-glucan. In this way, the applicability of β-glucan forming LAB in wheat and rye sourdoughs should be demonstrated.

2. Material and methods

2.1. Microorganisms and cultivation

In this manuscript we use the new nomenclature of the genus *Lactobacillus* as previously published by (Zheng et al., 2020), according to which *Lactobacillus brevis* is now called *Levilactobacillus brevis*. *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 (isogenic with DSM 14800^{T} and ATCC BAA-344^{t}) isolated from breweries (Fraunhofer et al., 2017; Suzuki, 2011) were used in this study. Additionally, the two Δ*gtf-2* mutants *L. brevis* TMW 1.2320 and *P. claussenii* TMW 2.2123 derived from *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 were used as negative control strains. Both Δ*gtf-2* mutants were not able to form ropy colonies (Fig. S1) or increase the viscosity of culture broths. *L. brevis* TMW 1.2320 resulted from a spontaneous IS30 transposon (locus tag: AZI09_RS12480) insertion (total length of the insertion: 1049 bp) at position 1148 bp of the *gtf-2* gene located on plasmid pl12112-4 (accession: NZ_CP016801). The deficiency of *P. claussenii* TMW 2.2123 to produce β-glucan is based on the loss of the *gtf-2* coding plasmid pPECL-7 (accession: CP003144). The presence or absence of the plasmid was determined by PCR analysis using primers that flanked the *gtf-2* gene (Fig. S2). The strains were cultivated as static cultures at 30 °C in modified Man, Rogosa and Sharpe medium (mMRS) at pH 6.20. The mMRS medium was composed of (quantities per liter): 10 g of peptone, 5 g of yeast extract, 5 g of meat extract, 4 g of K_2HPO_4, 2.6 g of $KH_2PO_4{\cdot}3H2O$, 3 g of NH_4Cl, 1 g of Tween80, 0.5 g of cysteine-HCl, 10 g of maltose, 5 g of glucose, 5 g of fructose, 0.2 g of $MgSO_4{\cdot}7H_2O$ and 0.038 g of $MnSO_4{\cdot}H_2O$. This modified mMRS medium is typically used for the cultivation of beer-spoiling bacteria (Fraunhofer et al., 2017; Schurr et al., 2013).

2.2. Sourdough preparation and leavening process

The doughs were prepared with either rye flour type 1150 (Roland Mills Nord GmbH & Co. KG, Germany) or wheat flour type 550 (Eickernmühle GmbH, Germany) with a dough yield (DY) of 200. For inoculation of the doughs, an overnight culture of the respective strain was first prepared. The pre-culture was then centrifuged (7500 × *g*, 10 min at 4 °C), cells were resuspended in a saline solution, and 2 ml of the cell suspension with an adjusted $OD_{600\ nm}$ of 1 was added to a mixture of 100 g flour and 98 g tap water (performed in duplicates). Fermentation was conducted at 28 °C inside closed containers. After 24 h of fermentation, a 5% ripe sourdough was used to inoculate a fresh dough mixture with the same DY. The refreshed dough was incubated under the same conditions as described above. The back-slopping procedure was performed twice, and pH values were measured for the ripe and fresh back-slopped doughs.

2.3. Cell count and analysis of sourdough microbiota

Before each refreshment step (24 h) the pH values and colony forming units (CFUs) were recorded. The CFUs per g sourdough were counted for each sample in duplicate after incubation at 30 °C for 48 h. Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) was used to identify the sourdough isolates at the species level. Viable single colonies (48 colonies per replicate) were picked from the cell count plates and smeared onto stainless steel targets using on-target extraction (Kern et al., 2013). The resulting spectra were compared with the Bruker database (Bruker Daltonics, Germany) and a self-generated database.

2.4. Analysis of organic acids

Organic acids were quantified after 24 h of dough fermentation by following the protocol of Rühmkorf et al. (2012a). Here, an additional centrifugation step (10,000 × *g*, 10 min at 10 °C) was used after the perchloric acid (70%) treatment to remove the insoluble particles. The acids were separated by high performance liquid chromatography (HPLC). This was done by using a sulfonated styrene-divinylbenzene Rezex™ ROA column (Phenomenox, USA), 2.5 mM sulfuric acid as eluent, a flow rate of 0.6 ml min^{-1} at 85 °C, and a 20 μL injection volume. A refractive index (RI) detector (tempered at 35 °C) was connected to the column for the detection of acids. The produced amounts of lactic and acetic acid in wheat and rye sourdoughs, respectively, were measured after the initial 24 h of fermentation (non-back-slopped doughs).

2.5. Detection of capsules and β-glucan formation

Cells from mMRS plates and sourdoughs were analyzed for capsule formation by a negative staining approach and agglutination test, respectively. In contrast to picked colonies from the mMRS plates, the sourdough samples needed additional preparation to reduce the background noise. This was accomplished by dissolving approx. 1 g of dough in 2–3 ml of saline solution. After 15 min of settling time, the supernatant containing the microbial cells was used for further analysis.

Capsule staining was performed with cells from either the inoculation loop or sourdough supernatant, which were mixed with black ink (Ferreira et al., 2002). After air drying, an aqueous safranin solution (1% w/v) was used to stain the cells. The presence of β-glucan capsules was additionally identified by an agglutination test using *Streptococcus pneumoniae* type 37-specific antiserum (Fraunhofer, 2018; Walling et al., 2005). In the presence of the antiserum, the cells agglutinated if β-glucan capsules were present. The *gtf-2* deficient mutant strains were used as the negative controls. In both methods the cells were observed by light microscopy at a 1000 × magnification setting.

2.6. Rheology

The sourdough viscosity was analyzed by flow curve measurements using an AR-G2 stress-controlled rheometer (TA Instruments, USA). Non-back-slopped wheat sourdoughs (20 g, 24 h of fermentation) were analyzed at 20 °C by applying a conical concentric cylinder. The cylinder had a 5.917 mm gap width at the head and a lateral 1 mm gap

width. An equilibration time of 2 min was set before each measurement. Flow curve experiments were conducted under steady-shear conditions using a continuous ramp with a shear rate that ranged from 0.01 to 100 1/s in a 2 min period. Sourdoughs prepared in duplicates (2.2) were analyzed twice (each 20 g), resulting in four measurements per strain. According to the Herschel-Bukley model of the power law ($\tau = K\dot{\gamma}^n$), the shear stress, τ, was plotted against the shear rate, $\dot{\gamma}$ to establish a linear correlation between the two parameters (Rao, 2007). The proportional relative changes in the two parameters were proven by evaluating the coefficient of determination (r^2). In this way, K (Pa s^n) and n (−) were determined to be the flow behavior index. For proof of statistical significance, the flow behavior index results were analyzed by a one-way ANOVA model in combination with Tukey's multiple comparisons test with significance level of 0.01 (V. 6.01 GraphPad Prism, GraphPad Software Inc., USA).

3. Results

3.1. Growth and persistence of β-glucan forming LAB in wheat and rye sourdoughs

L. brevis TMW 1.2112 (wt), *L. brevis* TMW 1.2320 (Δ*gtf-2* mutant), *P. claussenii* TMW 2.340 (wt) and *P. claussenii* TMW 2.2123 (Δ*gtf-2* mutant) were checked and compared to each other for their persistence in wheat and rye sourdoughs. The initial cell counts after inoculation of the wheat and rye doughs ranged between 10^6 and 10^7 CFU/g. The maximum cell counts after 72 h of growth in wheat sourdoughs fermented with *L. brevis* TMW 1.2112 (wt) and TMW 1.2320 (Δ*gtf-2*) were approx. 2×10^9 CFU/g. In rye sourdoughs the cell counts were approx. 5×10^9 CFU/g (Fig. 1A and B). The maximum cell counts for the used *P. claussenii* strains were approx. 1×10^9 CFU/g in wheat sourdough and 2×10^9 CFU/g in rye sourdough (Fig. 1C and D).

After inoculation of the doughs, the initial pH values were pH 6.17 ± 0.05 and decreased within 24 h to pH 3.78 ± 0.05 in wheat and rye sourdoughs. After back-slopping, the pH values decreased within 24 h to pH 3.69 ± 0.02.

MALDI-TOF MS was used to analyze the microbiota of the sourdoughs at the species level over a 72-h fermentation period (Fig. 2). The agar plates that were initially used for the CFU studies were used for these studies as well. After the application of *L. brevis* TMW 1.2112 (wt), all picked colonies were identified as *L. brevis* in wheat and rye sourdoughs (Fig. 2A). The same results were obtained for *L. brevis* TMW 1.2320 (Δ*gtf-2*). The microbiota of doughs inoculated with *P. claussenii* TMW 2.340 (wt) changed over the fermentation time of 72 h and resulted in 96.9% identified *P. claussenii* in wheat sourdoughs and 84.1% in rye sourdoughs. Similar results as those found for the wild-type strain were obtained for *P. claussenii* TMW 2.2123 (Δ*gtf-2*). In sourdoughs inoculated with *P. claussenii*, heterofermentative LAB were identified, such as *Weissella confusa* (up to 2.2%) and *Leuconostoc citreum* (up to 2.2%) and the yeasts *Candida kefyr* (7%–11.1%) and *Candida glabrata* (up to 4.3%).

Lactic and acetic acid formation were analyzed by HPLC. After 24 h of fermentation, the lactic and acetic acid concentration in wheat sourdoughs fermented by *L. brevis* TMW 1.2112 (wt) were 37.1 ± 2.8 and 5.5 ± 0.4 mM and by *L. brevis* TMW 1.2320 (Δ*gtf-2*) 37.3 ± 0.5 and 5.9 ± 0.2 mM, respectively. The lactic and acetic acid concentrations in rye sourdoughs by the same strains were 46.3 ± 2.2 and 14.9 ± 0.4 mM (wt) and 44.2 ± 2.6 and 14.5 ± 1.1 mM (Δ*gtf-2*), respectively. Homofermentative *P. claussenii* samples contained trace amounts of acetic acid (0.4 ± 0.4 mM) compared to the amounts of lactic acid formed (32.5 ± 3.5 mM) in wheat and rye sourdoughs.

Furthermore, the presence of ropy colonies, which is characteristic for the β-glucan producing wild-type strains, was confirmed after every 24 h fermentation period and selected using a wooden stick. The identity of the picked colonies was further verified by MALDI-TOF MS. All analyzed *L. brevis* TMW 1.2112 (wt) colonies were ropy over the whole fermentation period in wheat and rye sourdoughs. *L. brevis* TMW 1.2320 (Δ*gtf-2*) showed no reversion to the ropy wild-type phenotype. By contrast, a significantly lower number of ropy colonies was counted for *P. claussenii* TMW 2.340 (wt). At the beginning of the fermentation, approx. 52.1 and 37.5% of colonies were ropy in wheat and rye doughs, respectively. During fermentation, the percentage of ropy colonies declined to 27.1% in both wheat and rye sourdough fermentations. *P. claussenii* TMW 2.2123 (Δ*gtf-2*) showed no reversion to the wild-type phenotype.

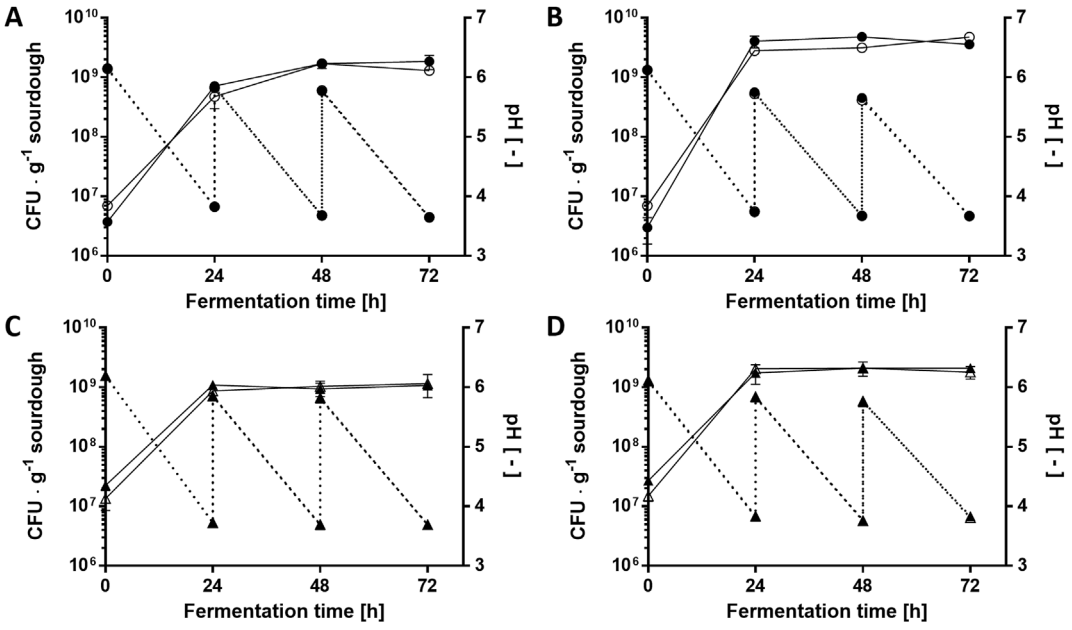

Fig. 1. Changes in cell count and pH values during wheat and rye sourdough fermentations. A) *L. brevis* TMW 1.2112 (●) and *L. brevis* TMW 1.2320 (○) in wheat sourdough; B) *L. brevis* TMW 1.2112 and *L. brevis* TMW 1.2320 in rye sourdough; C) *P. claussenii* TMW 2.340 (▲) and *P. claussenii* TMW 2.2123 (△) in wheat sourdough; and D) *P. claussenii* TMW 2.340 and *P. claussenii* TMW 2.2123 in rye sourdough. Cell counts (solid lines) and pH (dashed lines) values are means of biological duplicates with standard deviations. Very similar values resulted in overlapping graphs.

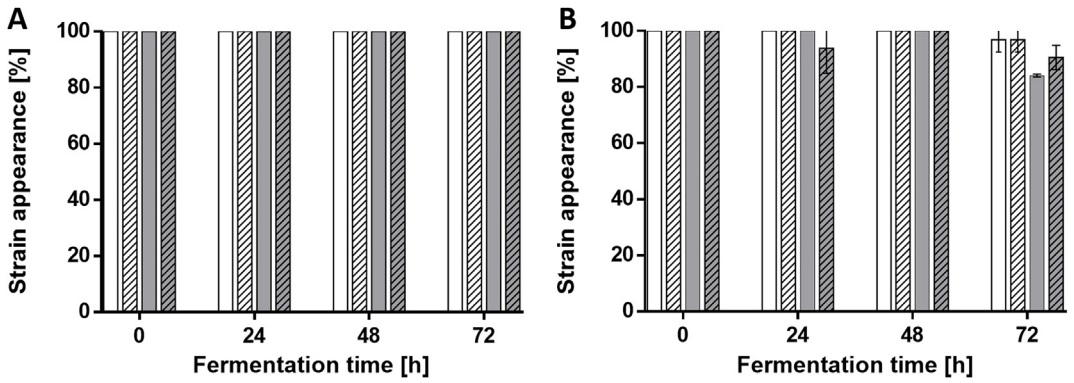

Fig. 2. Strain distributions in fermented wheat and rye sourdoughs. A) *L. brevis* TMW 1.2112 (wt) and TMW 1.2320 (Δ*gtf-2*) (dashed bars); and B) *P. claussenii* TMW 2.340 (wt) and TMW 2.2123 (Δ*gtf-2*) (dashed bars) in wheat sourdough (▯) and rye sourdough (▯). Values are means of biological duplicates with standard deviations.

3.2. Investigation of β-glucan forming cells in wheat and rye sourdoughs

The presence of β-glucan capsules was analyzed by applying microscopic techniques. *L. brevis* TMW 1.2112 (wt) and TMW 1.2320 (Δ*gtf-2*) isolates from sourdoughs and nutrient media were used for capsule staining and agglutination tests after a 24 h fermentation period (Fig. 3). Stained wheat sourdough cells of *L. brevis* TMW 1.2112 exhibited a capsule around the cells, which appeared as a halo zone (Fig. 3 A, encircled). On the contrary, the respective *gtf-2* deficient mutant cells lacked the halo zone (Fig. 3 B, encircled). Furthermore, *L. brevis* TMW 1.2112 (wt) cells agglutinated to cell aggregates. In the case of *L. brevis* TMW 1.2320 (Δ*gtf-2*), no aggregation was observed (Fig. 3C and D) after the application of the 37-specific antiserum. Capsule staining for both *P. claussenii* strains was not applicable, which is likely due to the loss of the ability to form capsules (mutant cells) or too small capsules (wild-type), which could not be visualized by light microscopy. Accordingly, the aggregates of *P. claussenii* TMW 2.340 (wt) obtained after treatment with the antiserum were composed of a lower number of cells in comparison to *L. brevis* TMW 1.2112 (wt) (Fig. S3). Capsule formation was additionally checked using colony-forming cells picked from agar plates. L. brevis TMW 1.2112 (wt) showed a diffused, network-like structure with a clear, uneven ink background between the cells (Fig. 3 E). However, this structure was not observed for the *L. brevis* TMW 1.2320 (Δ*gtf-2*) samples (Fig. 3 F).

3.3. Flow properties of wheat sourdoughs prepared with β-glucan producing LAB and their gtf2-deficient mutants

Wheat doughs fermented by β-glucan producing wild-type strains and their Δ*gtf-2* mutants were further used for rheological measurements (Fig. 4). The flow curves of the fermented rye sourdoughs (DY 200) could not be recorded due to the firmness of these sourdoughs.

The viscosity [Pa s] was plotted against the applied shear rate [1/s] (Fig. 4). All fermented doughs exhibited shear-thinning properties. The highest viscosities were detected for *L. brevis* TMW 1.2112 (wt). Power law calculations enabled the comparison of the consistency and flow behavior indices of the fermented doughs (Table 1). The flow behavior indices of wild-type *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340

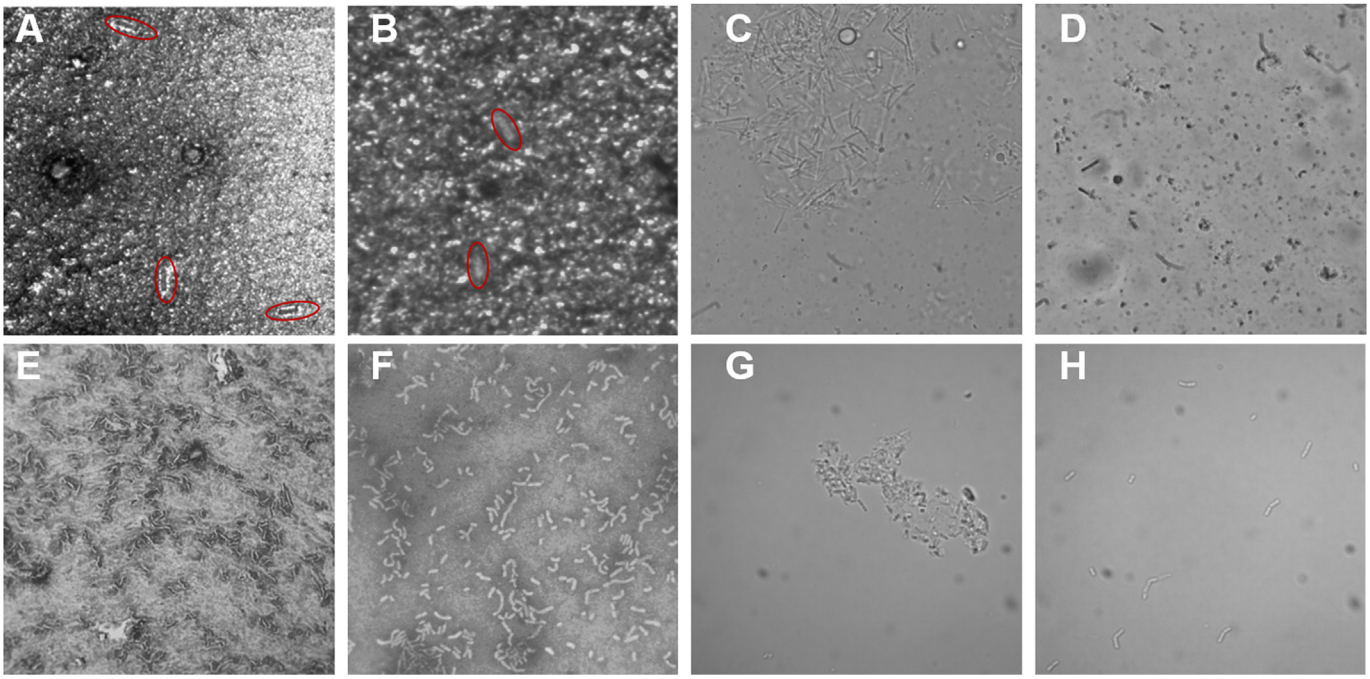

Fig. 3. Capsule staining and agglutination tests of *L. brevis* TMW 1.2112 (wt) and *L. brevis* TMW 1.2320 (Δ*gtf-2*). A-D are *in situ* stained cells extracted from wheat sourdoughs: A) capsule staining of *L. brevis* wt; B) capsule staining *L. brevis* Δ*gtf-2*; C) agglutination testing of *L. brevis* wt; and D) agglutination testing of *L. brevis* Δ*gtf-2*. *E*-H are picked colonies from agar plates: E) capsule staining of *L. brevis* wt; F) capsule staining of *L. brevis* Δ*gtf-2*; G) agglutination testing of *L. brevis* wt; and H) agglutination testing of *L. brevis* Δ*gtf-2*.

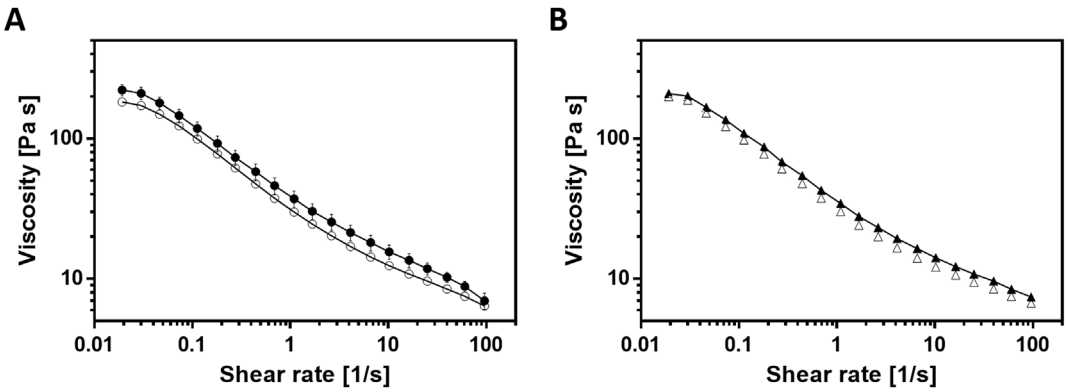

Fig. 4. Flow curves obtained for wheat sourdough inoculated with A) *L. brevis* TMW 1.2112 (wt) (●) and *L. brevis* TMW 1.2320 (Δ*gtf-2*) (○); and B) *P. claussenii* TMW 2.340 (wt) (▲) and *P. claussenii* TMW 2.2123 (Δ*gtf-2*) (△). Values are means of quadruplicates with standard deviations.

Table 1
Power law constants of wheat sourdough samples recorded at 20 °C, obtained with a conical concentric cylinder at 5.917 mm gap width.

Strain	K (Pa s^n)	N	r^2
L. brevis TMW 1.2112 (wt)	35.398	0.650	0.999
L. brevis TMW 1.2320 (Δ*gtf-2*)	26.143	0.693	0.999
P. claussenii TMW 2.340 (wt)	25.728	0.681	0.998
P. claussenii TMW 2.2123 (Δ*gtf-2*)	21.125	0.700	0.998

were 0.650 and 0.681, respectively. Statistical analysis using one-way ANOVA (2.6), demonstrated a significantly higher viscosity in wheat sourdoughs fermented by *L. brevis* TMW 1.2112 (wt) as compared to *L. brevis* TMW 1.2320 (Δ*gtf-2*), and both *P. claussenii* strains with p-values ranging between 0.0003 and 0.0038.

4. Discussion

4.1. Persistence, growth, and acid formation of β-glucan producing beer-spoilers in wheat and rye sourdoughs

The effects of cereal β-glucans in foods have been extensively studied (Zhu et al., 2016). By contrast, *in situ* β-glucan production by LAB during wheat and rye sourdough fermentations has not been described so far. In this study, the ability of *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 to produce capsular β-glucan and grow in wheat and rye doughs was investigated for the first time. The selected strains were persistent upon several back-slopping trials and acidified water-flour mixtures via lactic and acetic acid production. However, *P. claussenii* by tendency were displaced by other organisms (e.g. *W. confusa* and *L. citreum*) after a few propagation steps, which has already been observed for pediococci in traditional sourdough fermentations (Van der Meulen et al., 2007). The final cell counts of the β-glucan producing LAB strains used in this study were in the range of 10^9 CFU per g of wheat or rye sourdoughs. Even after back-slopping of the doughs, similar cell densities were reached after 24 h of fermentation. These results correspond well to typically described LAB cell densities ($\geq 10^8$ CFU/g) in traditional sourdoughs (De Vuyst and Neysens, 2005). Microbiota identification by MALDI-TOF MS revealed a dominance of the inoculated strains by an appearance between 84% and 100% over a fermentation period of 72 h in doughs of both flour types (Fig. 2).

The ability of *L. brevis* and *P. claussenii* to grow in wheat and rye sourdoughs up to 10^9 CFU/g could be explained by their isolation sources, namely spoiled beer (Fraunhofer et al., 2017; Suzuki, 2011). Both doughs and beer are cereal-based foods that provide comparatively similar nutrient sources. During sourdough fermentation, the pH decreases and, under anaerobic conditions, the bacteria deal with nutrient availability that is similar to the conditions in beer (Hammes and Gänzle, 1998). Beer is generally unfavorable for microbial growth due to hop bitter compounds, ethanol, and carbon dioxide presence as well as low maltose and glucose concentrations (Gobbetti, 1998; Sakamoto and Konings, 2003). Both *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 can grow even under the restrictive conditions present in beer. As indicated in the present work, water-flour mixtures may represent a less harmful and well-suited substrate for their growth.

Strains of *L. brevis* are reported to be a part of the diverse traditional sourdoughs (Vogel et al., 1994), while *P. claussenii* is solely known to spoil beer (Pederson, 1949; Pittet et al., 2012). However, some species of the genus *Pediococcus* (*P. pentosaceus* and *P. acidilactici*) are also known as part of the microbiota of spontaneously fermented sourdoughs, which implied that members of this genus are suited for sourdough manufacturing (De Vuyst and Neysens, 2005). Both the wild-type and *gtf-2* deficient mutants of *L. brevis* and *P. claussenii* were persistent in wheat and rye sourdoughs. This strongly suggests that β-glucan formation is not essential for the growth of the producing strains in sourdoughs. Furthermore, the formation of β-glucan capsules, which is an energy-demanding process, could be a competitive disadvantage that does not provide any detectable selective advantage for survival and persistence under these growth conditions.

Accordingly, parts of the growing populations of *P. claussenii* TMW 2.340 tended to lose the *gtf-2* encoding plasmid upon growth in sourdoughs, indicating a low plasmid stability in this strain. On the contrary, *L. brevis* TMW 1.2112 was stable with respect to β-glucan formation, which could be due to the presence of the actively expressed RelE-RelB toxin/antitoxin-system encoded on the *gtf-2* encoding plasmid (Fraunhofer, 2018). In this work, RelE was predicted to be the toxin and RelB the corresponding antitoxin by genomic annotations. In theory, the spontaneous loss of this plasmid leads to cell death, as RelE is more stable than its antitoxin and accumulates within the cells. (Christensen et al., 2001; Gerdes et al., 2005; Gotfredsen and Gerdes, 1998). According to the toxin/antitoxin-system, the rarely detected loss of β-glucan biosynthesis via the transposon insertion in the *gtf-2* gene in *L. brevis* TMW 1.2112 grown in mMRS medium supports the hypothesis of the plasmid stability. This observation is based on a random event detected in our lab upon serial propagation of this strain (data is not shown). Moreover, the phenotypes of the wild-type and mutant strain of *L. brevis* were stable and dominant in wheat and rye sourdough fermentations. This coincides with the observations made for *P. claussenii* wildtype and its *gtf2*-deficient mutant and further indicates that β-glucan formation by wine-/beer-spoiling LAB is likely not essential for growth in sourdoughs.

However, β-glucan encapsulation can efficiently increase the viability of cells in an acidic, gastrointestinal environment as compared to those lacking the β-glucan capsules (Stack et al., 2010). The absence of acid stress in type I sourdough fermentations may select against β-glucan formation of LAB, while longer and more acidified fermentations

(e.g. Type II sourdough) might benefit the *gtf-2* encoding plasmid maintenance and β-glucan formation (Gobbetti et al., 2008).

The quantification of *in situ* formed acids revealed that the wheat sourdoughs fermented by *L. brevis* TMW 1.2112 contained a 6.5 times higher concentration of lactic acid compared to acetic acid. By contrast, the lactate concentration in wheat sourdough (DY 200) fermented by levan-producing *Fructilactobacillus* (*(F.)F.*) *sanfranciscensis* TMW 1.392 was solely 2.5 times higher than that of acetate. Moreover, application of the levansucrase deletion mutant of *F. sanfranciscensis* TMW 1.392 resulted in a 1.6-fold decrease in acetate formation, implying excessive acetic acid formation by sucrose feeding in sourdough (Kaditzky et al., 2007). According to the latest revised taxonomy of the genus *Lactobacillus* the species *Lactobacillus sanfranciscensis* is now called *Fructilactobacillus sanfranciscensis* (Zheng et al., 2020). In doughs prepared with *P. claussenii*, trace amounts of acetic acid were detected, which could be explained by spontaneously growing LAB (*W. confusa* and *L. citreum*) and yeasts (*C. kefyr* and *C. glabrata*) colonies in the sourdoughs.

4.2. In situ β-glucan formation by beer-spoiling LAB and its impact on sourdough viscosity

Beta-glucan formation by *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 was investigated by detecting the produced capsules (capsule staining and agglutination test) and comparison with the corresponding mutant strains that served as the respective controls. Negative staining is a long-established method for capsule analysis using electron or light microscopy (Bayer, 1990; Ferreira et al., 2002; James and Swanson, 1977). Stained *L. brevis* wild-type cells exhibited capsules as visible halos surrounding the cells, whereas these effects were not observed for the *gtf-2* deficient mutant. The depicted cells in Fig. 3A were most likely the designated strains, as revealed by MALDI-TOF MS analysis (Fig. 2). In case of *P. claussenii*, no halo could be visualized which indicated the lack of capsules or the presence of smaller capsules that could not be detected. However, by applying the *S. pneumoniae* type 37-specific antiserum (Walling et al., 2005), the presence of capsular β-glucan was detected in *P. claussenii*. In cases where *L. brevis* strains (wt and Δ*gtf-2*) were used, the agglutination test clearly corresponded to the capsule staining results. Moreover, the number of agglutinated cells was significantly higher for *L. brevis* than *P. claussenii*. This finding could be due to the decreasing number of ropy *P. claussenii* TMW 2.340 colonies, which is most likely caused by plasmid losses during fermentation (≤40% of ropy colonies were still present after 24 h).

Rheological experiments emphasized the impact of capsular β-glucans on the viscosity of wheat sourdoughs fermented by wild-type *L. brevis* TMW 1.2112 ($n = 0.650$). In such studies, a flow behavior index of 1.0 describes Newtonian fluids. Materials with indices < 1.0 include viscoplastic fluids (Herschel-Bukley model), like polymer solutions that exhibit time-independent viscous shear-thinning behavior (Hernández-Ortiz, 2004; Sofou et al., 2008). Viscoplastic materials (e.g. sourdough, clay, or pastes) resist flowing until the shear stress level exceeds the yield stress limit (Bingham model). With increasing polymer concentration, the consistency index increases and the flow behavior index decreases gradually (Song et al., 2006). This suggests that sourdoughs fermented by wild-type *L. brevis* TMW 1.2112 contained a higher polymer concentration as compared to those fermented by the other investigated strains. An increased viscosity of the EPS-enriched sourdoughs by the use of the appropriate LAB was also reported in other works focusing on secreted dextrans (Coda et al., 2018; Katina et al., 2009). However, different LAB species and morphologies (cocci vs. rods) were applied in the present study. Their different morphologies and physiology may have influenced the extent of β-glucan-mediated capsule and cell-network formation. Therefore, the comparatively small differences in viscosity between wheat sourdoughs fermented by *P. claussenii* wildtype and its mutant (Fig. 4B) could be due to the loss of the *gtf2*-containing plasmid or the possibly downregulated, non-efficient β-glucan formation by *P. claussenii* in wheat sourdoughs. Fraunhofer et al., 2018a described that β-glucan producing cells of *L. brevis* TMW 1.2112 form sticky networks in mMRS media, whose viscosities can drastically increase during fermentation. These viscosity effects are rather attributed to the network between cells and β-glucans than the released β-glucan itself (Fraunhofer, 2018; Fraunhofer et al., 2018a,b). Hence, the significant impact of β-glucan producing *L. brevis* TMW 1.2112 on the viscosity of wheat sourdough (Fig. 4) is likely due to *in situ* cell network formation. By contrast, other ropy LAB (producing capsular HePS) had no significant impact on the viscosity of sourdoughs, despite increasing the viscosity of the nutrient media (Galle et al., 2011). Brandt et al., (2003) and Tieking et al. (2003) described that *in situ* produced EPS by LAB is more effective than artificially added EPS in dough systems. These results may indeed support the value of *in situ* formed β-glucan networks in dough matrices initiated by capsular cross-linking of bacterial cells. Since the isolation and quantification of β-glucan is hampered by the capsules tightly bound to the cell wall (Fraunhofer, 2018; Fraunhofer, 2018; Hutzler et al., 2013), an efficient method for the quantification of capsular β-glucan still needs to be established to enable a more precise interpretation of the structural effects initiated by the formed β-glucan-cell-networks in complex dough matrices.

5. Conclusion

Our study reveals that β-glucan forming LAB strains isolated from spoiled beer are persistent in wheat and rye doughs even after several back-slopping steps. *L. brevis* TMW 1.2112 was determined to be a suitable starter culture for stable β-glucan formation in wheat and rye doughs. As compared with baked goods enriched by sucrose derived homopolysaccharides, β-glucan-based structure formation avoids issues of excessive acetate formation by heterofermentative LAB. This is because β-glucan-formation does not require sucrose addition, but is preferably formed upon the consumption of maltose (Fraunhofer et al., 2018b) and other sugars intrinsically available in the dough. Several parameters can be optimized to increase β-glucan levels, such as DY, flour type, carbon source availability, and cell counts. These factors, and the use of other β-glucan producing LAB, may be applied for further food biotechnological development of β-glucan enriched sourdough breads.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2020.103539.

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4.2 β-Glucan Production by *Levilactobacillus brevis* and *Pediococcus claussenii* for In Situ-Enriched Rye and Wheat Sourdough Breads

The persistence and β-glucan formation of *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340, both isolated from alcoholic beverages, was previously demonstrated in wheat and rye sourdoughs. Even rheological effects due to β-glucan formation were detected in wheat sourdough fermented by *L. brevis.* However, the quantification of the β-glucan in dough matrices have been lacking previously. The impact of certain parameters, e.g., fermentation temperature or time, is relevant for determining the optimum fermentation conditions for the maximum β-glucan concentration. Furthermore, for the development of sourdough starters, the consumer's taste experience and overall acceptance of the resulting breads need to be analyzed.

To identify the optimum process conditions, different parameters in sourdough fermentation were modified and compared with each other. The changes in the β-glucan content of the doughs were determined by HPLC and an immunological assay. The latter proved to be the more appropriate method for β-glucan quantification, especially because of its specificity. *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 and their isogenic β-glucan negative (*Δgtf-2*) strains were used for dough inoculation by i) varying the initial cell count or ii) co-cultivating with the α-amylase producing strain, *Lactiplantibacillus* (*La.*) *plantarum* TMW 1.2330. A co-cultivation was assumed to increase the availability of substrate for increased EPS production. However, neither approach proved to be effective. Additionally, the iii) fermentation temperature and iv) fermentation time were analyzed. As the fermentation time progressed, the EPS concentration decreased significantly, indicating the degradation of the polymer. However, the greatest influence on increasing the β-glucan concentration was achieved via the fermentation temperature which altered between flour types and LAB species. While the optimum of *P. claussenii* TMW 2.340 was at 25 °C regardless of flour type, the optimum of *L. brevis* TMW 1.2112 was at 35 °C in wheat and 25 °C in rye. Moreover, the results demonstrated that cell count and EPS formation are independent of each other, implying that external factors influence the biosynthesis. Since EPS producers with higher yields are more attractive for industrial processes regarding techno-functional or health beneficial effects, it is necessary to identify and characterize enzymes involved in the synthesis and degradation of β-glucan. Analyses of the sensory properties of sourdough breads of both LAB species using the optimized process conditions with maximum β-glucan concentration resulted in an overall acceptance of the wheat and rye sourdough breads. In addition, although wheat breads with *L. brevis* were preferred over *P. claussenii* and the blank control, the rye breads were found to be predominantly sour, but this can be considered characteristic of rye breads The supplementary files (Table S 1 and Table S 2) of the publication are attached to the appendix in Chapter IV.

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β-Glucan Production by *Levilactobacillus brevis* and *Pediococcus claussenii* for In Situ Enriched Rye and Wheat Sourdough Breads

Julia A. Bockwoldt, Johanna Fellermeier, Emma Steffens, Rudi F. Vogel and Matthias A. Ehrmann *

Lehrstuhl für Technische Mikrobiologie, Technische Universität München, 85354 Freising, Germany; julia.bockwoldt@tum.de (J.A.B.); johanna.fellermeier@tum.de (J.F.); emma.steffens@tum.de (E.S.); rudi.vogel@tum.de (R.F.V.)

* Correspondence: matthias.ehrmann@tum.de; Tel.: +49-(0)-8161-71-3301

Abstract: Sourdough fermentation is a common practice spread across the globe due to quality and shelf life improvement of baked goods. Above the widely studied exopolysaccharide (EPS) formation, which is exploited for structural improvements of foods including baked goods, β-glucan formation, by using lactic acid bacteria (LAB), offers additional values. Through renunciation of sucrose addition for bacterial β-D-glucan formation, which is required for the production of other homopolysaccharides, residual sweetness of baked goods can be avoided, and predicted prebiotic properties can be exploited. As promising starter cultures *Levilactobacillus* (*L.*) *brevis* TMW (Technische Mikrobiologie Weihenstephan) 1.2112 and *Pediococcus* (*P.*) *claussenii* TMW 2.340 produce *O*2-substituted (1,3)-β-D-glucan upon fermenting wheat and rye doughs. In this study, we have evaluated methods for bacterial β-glucan quantification, identified parameters influencing the β-glucan yield in fermented sourdoughs, and evaluated the sourdough breads by an untrained sensory panel. An immunological method for the specific detection of β-glucan proved to be suitable for its quantification, and changes in the fermentation temperature were related to higher β-glucan yields in sourdoughs. The sensory analysis resulted in an overall acceptance of the wheat and rye sourdough breads fermented by *L. brevis* and *P. claussenii* with a preference of the *L. brevis* fermented wheat sourdough bread and tart-flavored rye sourdough bread.

Keywords: EPS; β-glucan; sourdough; LAB; temperature effects; high performance liquid chromatography (HPLC); ELISA; sourdough bread; sensory evaluation

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1. Introduction

Food fermentation by bacteria and yeasts is longer practiced than we know about the existence of microbes. Various lactic acid bacteria (LAB), including lactobacilli, lactococci, and pediococci, are exploited and deliberately applied as starter cultures in a broad range of safe fermented food, e.g., in the dairy and meat industry, for vegetables, baked goods, and alcoholic beverages [1,2]. Sourdough fermentation as one of the oldest biotechnological processes offers beneficial effects for the bread producers and consumers as the products have improved sensory qualities and shelf life [3–5]. The formation of exopolysaccharides (EPS) by LAB during sourdough fermentation offers additional values due to improved water binding capacity and the associated prebiotic potential [6–12]. Moreover, EPSs are discussed to positively influence human's health, e.g., as prebiotics acting as fermentable substrates for the intestinal microbiota, immunoregulatory effects, reducing serum cholesterol levels, and lowering postprandial blood glucose and insulin response [13–17].

EPSs produced by LAB are either homopolysaccharides (HoPS) such as β-D-glucans, α-D-glucans, and β-fructans, formed by the same monosaccharide units or heteropolysaccharides (HePS), which are mainly composed of D-glucose, D-galactose, and L-rhamnose. Most HoPS are polymerized by extracellular glucansucrases (alteran, dextran, reuteran, or mutan) or

fructansucrases (levan and inulin) from sucrose as the substrate [9,14,18–20]. In contrast, β-D-glucans and HePS formation proceeds by intracellularly synthesis of nucleotide-activated sugar moieties and subsequent polymerization by glycosyltransferases (*gtf*) [21–25]. By comparing both EPS types produced by LAB, extracellularly produced EPSs reach much higher yields (≤16 g/kg dough) than intracellular produced EPSs with yields below 0.6 g/L medium under optimal culture conditions [6,26]. It is previously reported that EPS formed by glucansucrase activity from sucrose during sourdough fermentation beneficially affects the viscoelastic properties, the texture, and shelf life of the dough [6].

In this case, however, sucrose addition is mandatory for HoPS synthesis and may lead to surplus acetate in the sourdough upon formation of glucan and utilization of the remaining fructose as an electron acceptor. In addition, residual sucrose from such sweet sourdoughs frequently results in sweet baked goods. In contrast, for β-D-glucan formation, the available soluble carbohydrates in wheat (≤1.85% maltose, sucrose, glucose, and fructose) and rye (≤2.0% maltose) flour are sufficient and, therefore, meet the demands of clean-label products [27–34]. Even low quantities of β-glucan have effects due to a network formed by EPS-encapsulated cells increasing the viscosity of liquid media, wheat sourdough, oat ferment, or spoiled beer [12,22,30]. Besides techno-functional properties of β-glucan from LAB, its health beneficial effects are gaining more attention, e.g., regulations of the blood cholesterol level, anti-inflammatory effects, and the stimulation of probiotic microorganisms [11,35,36].

The brewery isolates *Levilactobacillus* (*L.*) *brevis* (formerly *Lactobacillus brevis*) TMW (Technische Mikrobiologie Weihenstephan) 1.2112 [37] and *Pediococcus* (*P.*) *claussenii* TMW 2.340 synthesize β-glucan as a capsule around the cells by generating ropy colonies. Both strains carry a plasmid encoded glycosyltransferase-2 gene (*gtf-2*) enabling β-glucan synthesis. A lack of the gene upon plasmid loss (*P. claussenii*) or disruption of the gene by a mobile genetic element (*L. brevis*) leads to the loss of this function, generating non-ropy mutant colonies. Studies about the effects of sourdoughs enriched with bacterial β-glucan including the mutant strains profit from a direct comparison [12,30,38]. In a previous study, we have demonstrated the persistence of beer-spoiling *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 in wheat and rye sourdough fermentation, which contained in situ β-glucan [30]. However, the accurate quantification of β-glucan produced in situ, especially when it is present as a cell-bound network, is still a major challenge.

The aim of the present study was to evaluate two methods (high performance liquid chromatography (HPLC)-based and enzyme-linked immuno-sorbent assay (ELISA)-based for the β-glucan quantification in sourdoughs using the respective *gtf-2* deficient strains as controls. Moreover, various parameters (temperature, inoculum size, providing precursor by cocultivation) potentially influencing the β-glucan content during fermentation were analyzed. With sensory evaluation, the potential acceptance of β-glucan enriched wheat and rye sourdough breads by consumers was investigated.

2. Materials and Methods

2.1. Strains and Materials

Five different LAB strains were used in this study: β-glucan-forming wild-type (wt) *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 (isogenic with DSM 14800^T, and ATCC BAA-344^T) isolated from breweries. Furthermore, two mutant strains were used, which are derived naturally from the β-glucan-forming wild-type strains. *L. brevis* TMW 1.2320 resulted from a spontaneous transposon insertion (*gtf-2-1148*::IS30) and *P. claussenii* TMW 2.2123 resulted from loss of the *gtf*-2-coding plasmid (Δ*gtf-2*) [30,39,40]. The mutants were used as negative control strains since they lack the β-glucan formation and form non-ropy colonies. Furthermore, the α-amylase producing strain *Lactiplantibacillus* (*La.*) *plantarum* TMW 1.2330 [37] was incorporated within sourdough fermentation. The α-amylase activity was tested using the API 50 CHL system and performed according to the instruction manuals.

The LAB were cultivated in modified Man, Rogosa, and Sharpe medium (mMRS) with pH 6.20 at 30 °C as static cultures. The mMRS medium contained (quantities per liter): 10 g

of peptone, 5 g of yeast extract, 5 g of meat extract, 4 g of K_2HPO_4, 2.6 g of $KH_2PO_4 \cdot 3\ H_2O$, 3 g of NH_4Cl, 1 g of Tween 80, 0.5 g of cysteine-HCl, 10 g of maltose, 5 g of glucose, 5 g of fructose, 0.2 g of $MgSO_4 \cdot 7\ H_2O$, and 0.038 g of $MnSO_4 \cdot H_2O$ [39,41]. Two different flour types were used for sourdough fermentations: rye flour type 1150 (Roland Mills Nord GmbH & Co. KG, Bremen, Germany) and wheat flour type 550 (Eickernmühle GmbH, Lemgo, Germany).

For β-glucan isolation and purification, a semi-defined medium (pH 5.5) as previously described by Dueñas-Chasco et al. with modifications for improved growth and β-glucan formation was used (quantities per liter): 20 g of maltose, 5 g of casamino acids, 3.5 g of a bacto-yeast nitrogen base (Difco), 3.5 g of yeast carbon base (Difco), 0.05 g of $MnSO_4 \cdot H_2O$, 0.05 g of $MgCl_2 \cdot 6\ H_2O$, 2 g of $K_2HPO_4 \cdot 3\ H_2O$, 1 g of KH_2PO_4, 5 g of sodium acetate, 0.005 g of adenine sulfate, 0.005 g of guanine, 0.005 g of xanthine, 0.005 g of uracil, 4 g of DL-malic acid, and 1 g of Tween80 [22,42]. The cultivation was performed at 30 °C for 30 h before β-glucan isolation.

2.2. Sourdough Fermentation

The conditions for the cultivation of the LAB in mMRS medium and sourdough fermentation were according to those reported by Bockwoldt et al. [30]. Fermentations of rye and wheat sourdoughs with a dough yield (DY) of 200 were performed in triplicates upon variation of the conditions. The fermentation temperature of the wheat and rye doughs was once set to 25 °C and, when raised to 35 °C, compared to the initial publication. Furthermore, wheat and rye doughs were inoculated with LAB solution of 1 and 4 mL (adjusted OD_{600nm} 1) per 200 g dough and fermented at 28 °C. The cocultivation in a 1:1 ratio (2 mL adjusted OD_{600nm} 1 per 200 g dough) with the α-amylase producing *La. plantarum* TMW 1.2330, and *L. brevis* TMW 1.2112, or *P. claussenii* TMW 2.340 was performed at 28 °C. The incorporation of the α-amylase positive strain based on the results of the study from Fraunhofer et al., which described an increased maltose availability supported the β-D-glucan production by LAB [22]. Samples for β-glucan quantification were taken every 4 h over 24 h and after 48 and 72 h in the initial experiment to identify the relevant period of β-glucan formation. Consequently, samples were taken after 16, 20, and 24 h from sourdoughs of experiments with changing conditions.

2.3. Cell Count, pH, and Analysis of Sourdough Microbiota

The colony forming units (CFU) per g of sourdough were analyzed in triplicate before fermentation, and after inoculation of the doughs and at the end of fermentation. Inoculated mMRS agar plates were incubated at 30 °C for 48 h before counting. Species identification of 96 colonies per sourdough triplicate was performed with matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) [43]. Ropy colonies are a phenotypical characteristic of β-glucan forming strains (wt). This characteristic was analyzed by using a wooden stick to test if the colonies form ropy strands. The ratio of ropy to non-ropy colonies was determined for 100 colonies per sourdough triplicate after 24 h and the results were verified by the results of the MALDI-TOF MS [30]. Additionally, the pH values of the ripe doughs were measured.

2.4. Sourdough Bread Preparation and Baking

The preparation of sourdough breads was done by using the optimized fermentation temperature and fermentation time analyzed in this study. Wheat sourdoughs with *L. brevis* TMW 1.2112 were fermented at 35 °C and rye sourdoughs at 25 °C while wheat and rye sourdoughs with *P. claussenii* TMW 2.340 were fermented at 25 °C. A 24-h fermentation period was selected for all sourdoughs. Wheat sourdough breads were mixed from 72-g (12%) sourdough, 331.9-g wheat flour, 7.4-g salt, 11.1-g fresh yeast, and 177.5-g tap water. The dry components were mixed, sourdough, water, and yeast were added, and everything was mixed with a hand mixer (575 W Bosch MFQ 4885, Abstatt, Germany) for 5 min, which was followed by 10 min when left at room temperature, kneaded for 10 s, and a further

10 min leaving before distributing 500 g into aluminum trays. The doughs were proofed at 32 °C, 80% humidity for 35 min, and baked at 230 °C for 30 min in the oven (Piccolo, Wachtel GmbH, Hilden, Germany) with an initial 20-s steam injection. The valve was closed for the first 20 min and opened for the last 10 min. For rye sourdough breads, 217.9 g of rye flour, and 6.7 g of salt were mixed, and 244.7 g (40.6%) of fermented sourdough, 6.7 g of fresh yeast, and 134.1 g of tap water were added before everything was mixed with the hand mixer for 5 min, which was followed by 20 min leaving at room temperature. A total of 500 g of the dough was distributed into aluminum trays. The doughs were proofed at 32 °C and 80% humidity for 120 min and baked under the previously described wheat bread conditions. The bread loaves were cooled over night before sensory analysis and sampling.

2.5. Sensory Analysis

Wheat and rye sourdough breads were sliced, and cubes of the crumb with sides of 2–3 cm were coded and presented to an untrained panel (n = 14 for wheat breads and n = 16 for rye breads) in a randomized order. To determine the acceptance of the breads by consumers, an affective test was performed by evaluating five attributes: moisture, texture (two categories: airiness and texture), acidity, and overall acceptance. The panel analyzed wheat and rye sourdough breads of *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340, and the control bread without sourdough. A 5-point Hedonic scale was used for the rating of the attributes from one attribute (dry, compact, chewy, mild, and not tasty) to five attributes (moist, fluffy, crumbly, sour, and tasty).

2.6. Isolation and Purification of β-Glucan

Beta-glucan of *L. brevis* TMW 1.2112 was harvested by precipitating the culture supernatant, which was followed by dialysis and freeze-drying of the samples. *L. brevis* TMW 1.2112 was cultivated in a semi-defined medium [42] after cultivation for 30 h at 30 °C. The supernatant was collected by centrifugation at 16,000× g, 4 °C for 30 min, and precipitated with ice cold ethanol (three-fold) overnight at 4 °C [18]. The precipitate was collected by centrifugation 10,000× g, 4 °C for 15 min, and dissolved in distilled water using glass beads (Ø 2.85–3.45 mm) and a benchtop homogenizer (FastPrep®-24 MP, MP Biomedical Inc, Eschwege, Germany). The solution was mixed with a final concentration of 0.5 M perchloric acid for protein precipitation for 5 min on ice, which was followed by centrifugation at 13,000× g at 4 °C for 2 min. The resulting supernatant was dialyzed for three days against distilled water changed three times a day, using a dialysis membrane with a cut-off of 50 kDa (SERVA Electrophoresis GmbH, Heidelberg, Germany). After freezing the solution at −80 °C, the samples were freeze-dried (Freezone 2.5, Labconco Corporation, USA). Residues of nitrogen compounds in lyophilized samples were quantified by automated Dumas analysis (MAX N exceed analyzer (Elementar Analysensysteme GmbH, Langenselbold, Germany) by using a calibration with aspartic acid [44]. The isolated and purified samples were used for bacterial β-glucan quantification in wheat and rye doughs.

2.7. Quantification of the Bacterial β-Glucan

2.7.1. Quantification by HPLC

Hydrolysis of EPS such as β-glucan in fermented doughs was performed according to Rühmkorf et al. and Ua-Arak et al. with modifications listed in the following [45,46]. The doughs were dissolved in deionized water (1:2 w/v). This is followed by 30 min centrifugation at 8000× g and 10 °C. The supernatant was then mixed with 5% perchloric acid (70%) and hydrolyzed at 100 °C for 7 h. The cooled samples were centrifuged at 10,000× g, 10 min, and 10 °C. The supernatant was filtered (0.2 µm nylon filters) and stored overnight at 4 °C. This was followed by a second filtration step. The samples were stored at −20 °C until analysis. The hydrolyzed glucose concentration was analyzed by high performance liquid chromatography (HPLC) using a Rezex™ RPM Pb2+ column (Phenomenex, Aschaffenburg, Germany) with degassed and filtered distilled water as

eluent with a flow rate of 0.6 mL/min at 85 °C, and 20-µL injection volume. The column was connected to a refractive index (RI) detector, tempered at 35 °C. The total glucose concentration was calculated into theoretical β-glucan, using a correction factor ($\frac{162}{180}$) converting free glucose to anhydroglucose, as it occurs in β-glucan. Sourdough samples of the wild-type strains able to produce bacterial β-glucan were compared with sourdoughs fermented by the Δ*gtf-2* mutants [47].

2.7.2. Quantification by ELISA

Werning et al. developed a competitive enzyme-linked immuno-sorbent assay (ELISA) based on the *Streptococcus* (*S.*) *pneumoniae* serotype 37 antibodies for the quantification of the bacterial β-glucan [48]. In previous studies, these antibodies were used for agglutination tests of β-glucan forming LAB [25,30,49]. In this study, the ELISA assay was used for the quantification of *O*2-substituted (1,3)-β-D-glucan in wheat and rye sourdoughs and baked breads. The β-glucan of *L. brevis* TMW 1.2112 was used for the coating (32.5 µg/mL) of 96-well F8 Maxisorp microtiter plates (Nunc Immuno Module, Thermo Fisher Scientific, Darmstadt, Germany) and the preparation of standards (500, 1000, 5000, 10,000, 50,000, und 100,000 ng/mL) in phosphate buffered saline (PBS) pH of 7.0. The dough samples were dissolved in PBS (1:10 *w*/*v*) using glass beads (Ø 2.85–3.45 mm) and the benchtop homogenizer, which was followed by 10 min centrifugation at 7500× *g* and 4 °C. The supernatant was filtered (0.2-µm nylon filters) and the samples were stored at −20 °C until analysis. The following steps of this assay were performed as previously described by Werning et al. except that the absorbance was measured at 405 nm in a microtiter plate reader (SPECTROstar Nano, BMG Labtech GmbH, Ortenberg, Germany) [48]. Analysis of non-fermented material resulted in very limited cross-reactions. Therefore, non-fermented samples were used as a blank and subtracted from each value.

2.8. Statistical Analysis

A one-way ANOVA model combined with the Tukey's multiple comparisons test (significance level of 0.01) by use of the V. 6.01 GraphPad Prism, GraphPad Software Inc., San Diego, CA, USA, calculated the statistical significance of the β-glucan quantities and the sensory analysis.

3. Results

3.1. Growth Characteristics of Strains in Wheat and Rye Sourdoughs

The microbiota of the wheat and rye sourdoughs, especially for *L. brevis* TMW 1.2112 (wt), *L. brevis* TMW 1.2320 (*gtf-2-1148*::IS30), *P. claussenii* TMW 2.340 (wt), and *P. claussenii* TMW 2.2123 (Δ*gtf-2*), was analyzed by confirming their persistence. Wheat and rye doughs were inoculated with initial cell counts between 10^5 and 10^6 CFU/g and reached cell counts between 10^8 and 10^9 CFU/g within a 24-h fermentation period. Comparisons of the cell counts between rye and wheat sourdoughs resulted in three-fold to six-fold higher CFUs in rye fermentations with the *L. brevis* strains and two-fold to three-fold higher CFUs with *P. claussenii* strains. MALDI-TOF MS analysis was used to identify inoculated strains at a species level and to confirm their persistence after 24 h of fermentation and revealed ratios with a minimum of 93% except cocultivations. The pH values of the fermented doughs decreased within 16 h between 3.88 and 4.10 and, within 24 h, the pH values ranged from 3.72 ± 0.22 and 3.75 ± 0.17 in wheat and rye sourdoughs, respectively. By increasing the fermentation temperature from 25 °C to 35 °C, the pH values of the doughs decreased more rapidly. Analyses of the strain appearance by MALDI-TOF MS resulted in 98% to 100% during fermentations with different temperatures (25 °C and 35 °C) and dough inoculation sizes with LAB solution (1-mL and 4-mL cell suspension).

3.1.1. Growth Characteristics of Strains Co-Cultivated with *La. plantarum* TMW 1.2330

The microbiota of the cocultivation of β-glucan formers with α-amylase positive *La. plantarum* TMW 1.2330 to provide additional glucose was analyzed after 0 and 24 h. The

cocultivation with *La. plantarum* TMW 1.2330 resulted in one-fold to two-fold higher total cell counts in rye sourdoughs after 24 h compared with the other approaches. Total cell counts of the wild-type strains were higher when compared to the mutant strains when co-cultivated with *La. plantarum* TMW 1.2330. Wheat and rye doughs with *L. brevis* TMW 1.2112 consisted of 45%–48% after 0 h and 26%–48% after 24 h, while *P. claussenii* TMW 2.340 appearance ranged between 50%–66% after 0 h and 61%–70% after 24 h of the respective β-glucan forming strain. The co-cultivation with *La. plantarum* TMW 1.2330 resulted in 25% and 48% EPS positive colonies for *L. brevis* TMW 1.2112 and 8% and 5% ropy colonies for *P. claussenii* TMW 2.340 in wheat and rye sourdough, respectively.

3.1.2. Determining Plasmid Stability

The location of the *gtf-2* gene on a plasmid and the ropy phenotype of colonies allows determination of plasmid stability. The ratio of ropy to non-ropy colonies is important regarding the interpretation of quantified β-glucan. In all experiments, *L. brevis* TMW 1.2112 (wt) colonies were ropy at the beginning and the end of wheat and rye sourdough fermentations. In contrast, the ratio of ropy to non-ropy *P. claussenii* TMW 2.340 (wt) colonies was 16% to 95% straight after inoculation (0 h) and 34% to 98% within 24 h, respectively. The mutant strain *L. brevis* TMW 1.2320 (*gtf-2-1148*::IS30) showed no reversion to the wild-type phenotype in any of the experiments and, therefore, the transposon insertion within the *gtf-2* gene was highly stable. Since *P. claussenii* TMW 2.2123 (Δ*gtf-2*) resulted from losing the *gtf-2*-coding plasmid, a reversion to the ropy phenotype was not expected and was not observed (Tables S1 and S2).

3.2. Quantification of β-Glucan in Fermented Sourdoughs

3.2.1. Quantification by HPLC

To monitor the time course of β-glucan formation, the sourdoughs were fermented at 28 °C with sampling every 4 h over 24 h as well as after 48 and 72 h. After 24 h, sampling of *P. claussenii* TMW 2.340 (wt) and *P. claussenii* TMW 2.2123 (Δ*gtf-2*) was terminated. HPLC was used to quantify bacterial β-glucan after perchloric acid hydrolyzation and calculation of the β-glucan concentrations from the total glucose concentration. The wild-type strains and mutants were compared for these differences. The initial calculated β-glucan concentrations of the wheat and rye doughs ranged between 47.9 and 51.8 g/kg as well as 31.3 and 47.5 g/kg, respectively (Figure 1).

Within the first 12 h, the calculated β-glucan concentrations of wheat sourdoughs fermented by *L. brevis* TMW 1.2112 (wt) increased to 84.5 ± 3.4 g/kg and *L. brevis* TMW 1.2320 (*gtf-2-1148*::IS30) to 81.4 ± 3.0 g/kg. A similar trend was detected after 12 h for rye sourdoughs (Figure 1A,C). After another 12 h, the β-glucan concentrations in wheat and rye sourdoughs subsequently decreased close to the initial values. The concentration declined further and, after 72 h, the β-glucan concentration increased again for *L. brevis* TMW 1.2320 (*gtf-2-1148*::IS30) in wheat sourdough and for *L. brevis* TMW 1.2112 (wt) in rye sourdough.

The β-glucan concentration of the wheat and rye sourdoughs with *P. claussenii* TMW 2.340 (wt) and *P. claussenii* TMW 2.2123 (Δ*gtf-2*) increased constantly (Figure 1B,D) during 24 h of fermentation. After 16 h, the β-glucan concentrations in the sourdoughs with the Δ*gtf-2* mutant dropped and were below the concentration values of the wild-type. The maximal β-glucan concentrations of the wild-type was 71.6 ± 1.4 g/kg in wheat sourdoughs after 20 h and 81.3 ± 2.1 g/kg in rye sourdoughs after 16 h. During the 24-h fermentation, similar trends in β-glucan release from all *P. claussenii* sourdoughs were observed.

Changes in calculated β-glucan concentrations of the sourdoughs were almost identical between the same species strains with no significant differences between the wild-type strains, which are able to produce bacterial β-glucan and the Δ*gtf-2* mutants. An actual quantification by comparing calculated β-glucan concentrations of the wt and respective Δ*gtf-2* strain was not effective.

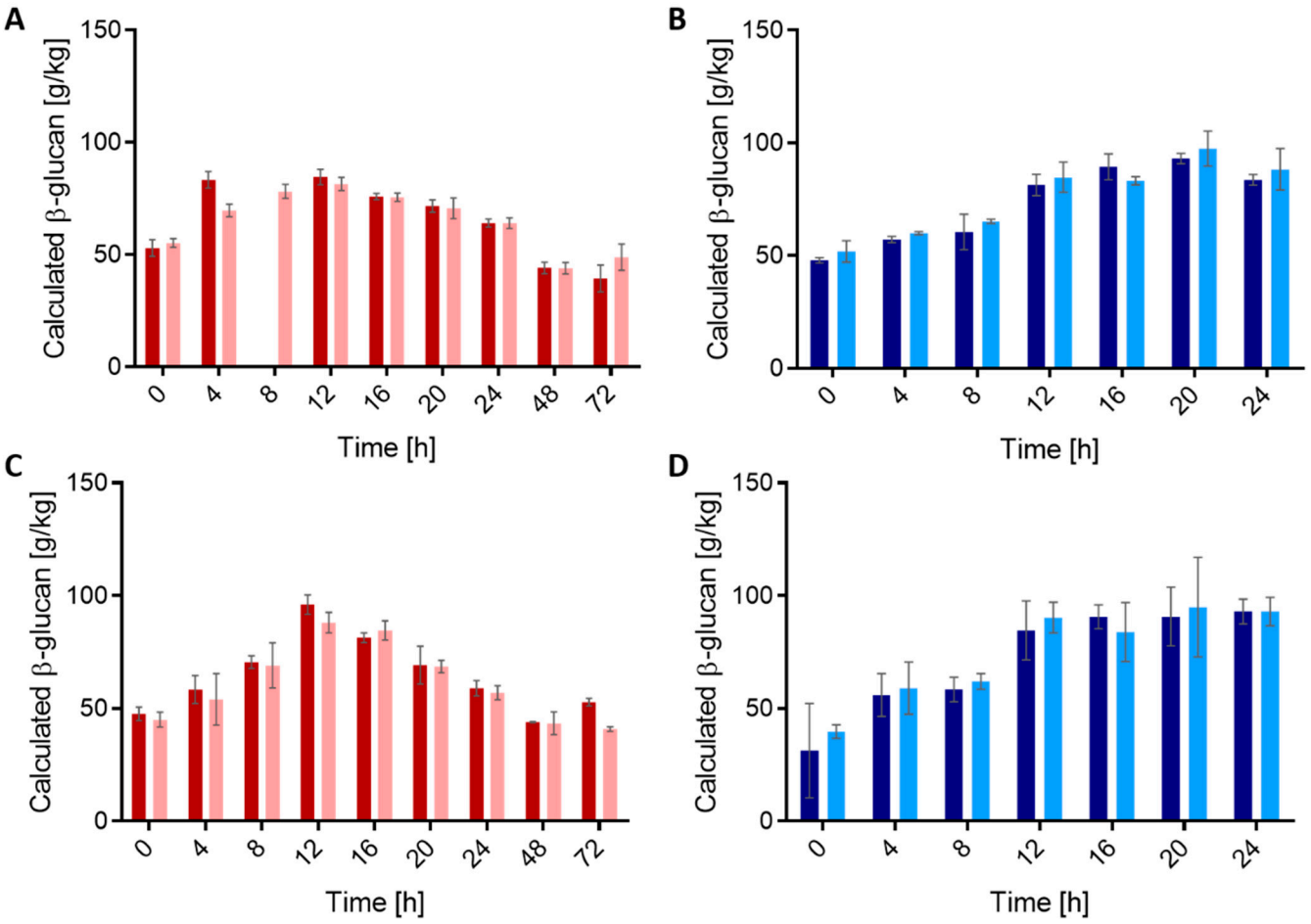

Figure 1. Calculated β-glucan concentration of the total glucose content in wheat and rye sourdoughs measured by HPLC. (**A**) *L. brevis* TMW 1.2112 (▮) and *L. brevis* TMW 1.2320 (▮) in wheat sourdough, (**B**) *P. claussenii* TMW 2.340 (▮) and *P. claussenii* TMW 2.2123 (▮) in wheat sourdough, (**C**) *L. brevis* TMW 1.2112 and *L. brevis* TMW 1.2320 in rye sourdough, and (**D**) *P. claussenii* TMW 2.340 and *P. claussenii* TMW 2.2123 in rye sourdough. The calculated β-glucan amounts were analyzed from biological triplicates with standard deviations.

3.2.2. Quantification by ELISA

In addition to the HPLC analysis, the same samples were analyzed by ELISA to quantify the bacterial β-glucan in wheat and rye sourdoughs. The initial β-glucan concentrations of the wheat and rye doughs were 14.4 ± 9.0 and 21 ± 18.5 mg/kg, respectively (Figure 2).

The β-glucan concentration increased within 20 h to a maximal β-glucan concentration for *L. brevis* TMW 1.2112 (wt) with 279 ± 73.3 mg/kg in wheat sourdoughs. This was significantly higher when compared to *L. brevis* TMW 1.2320 (*gtf-2-1148*::IS30) with 19.1 ± 5.9 mg/kg. After the maximum was reached, a decrease in the β-glucan concentration to 88.7 ± 60.6 mg/kg after 48 h and 61.2 ± 46.7 mg/kg after 72 h (Figure 2A) was detected. The maximal β-glucan concentration in rye sourdoughs fermented by *L. brevis* TMW 1.2112 was measured after 24 h with 412.3 ± 47.4 mg/kg and, therefore, significantly higher when compared to the isogenic β-glucan non-producing *L. brevis* TMW 1.2320 (48.7 ± 27.4 mg/kg). Within a 72-h fermentation period, the β-glucan concentration of *L. brevis* TMW 1.2112 decreased again to 60.8 ± 5.4 mg/kg.

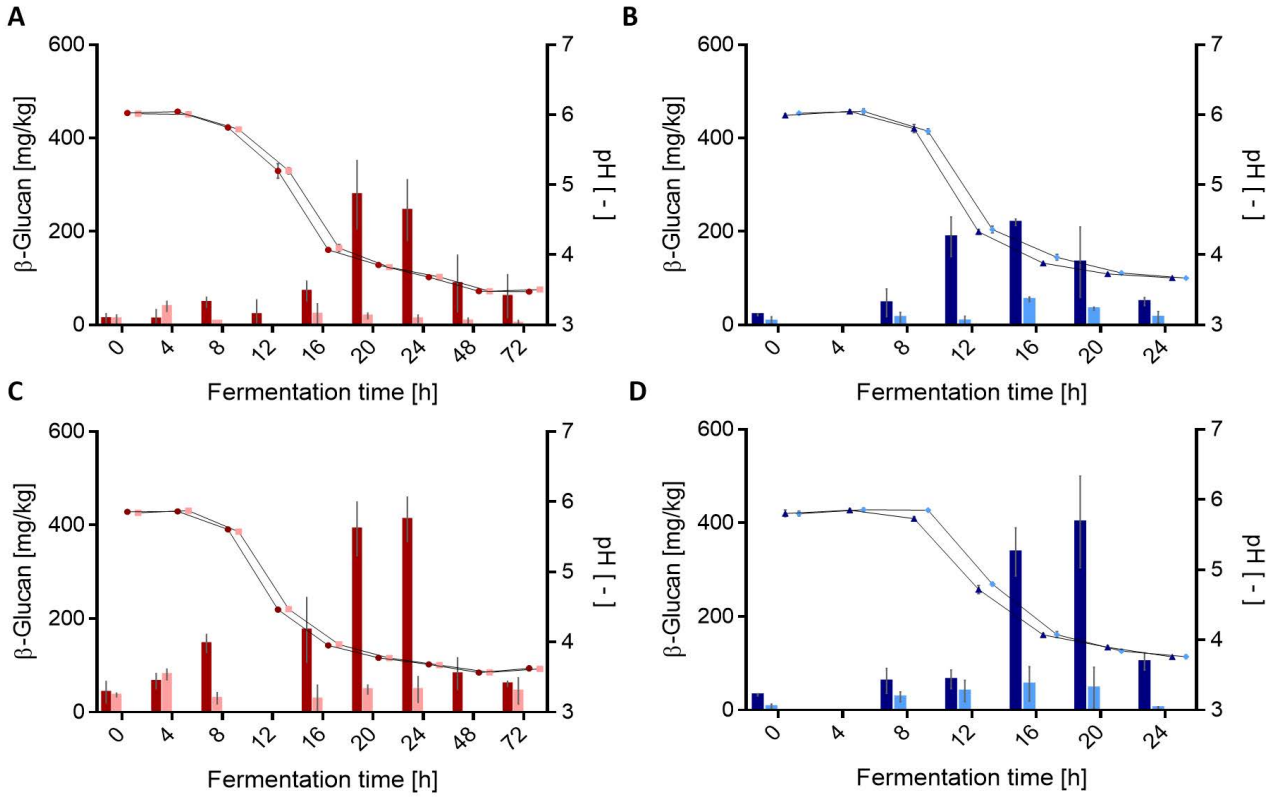

Figure 2. Changes in β-glucan concentration measured by ELISA and pH values during wheat and rye sourdough fermentations. (**A**) *L. brevis* TMW 1.2112 (●) and *L. brevis* TMW 1.2320 (■) in wheat sourdough. (**B**) *P. claussenii* TMW 2.340 (▲) and *P. claussenii* TMW 2.2123 (♦) in wheat sourdough, (**C**) *L. brevis* TMW 1.2112 and *L. brevis* TMW 1.2320 in rye sourdough, and (**D**) *P. claussenii* TMW 2.340 and *P. claussenii* TMW 2.2123 in rye sourdough. The β-glucan amounts and pH values were analyzed by biological triplicates with standard deviations.

Furthermore, the formation of the bacterial β-glucan by *P. claussenii* TMW 2.340 (wt) was observed and resulted in maximal β-glucan concentrations after 16 h in wheat sourdoughs (219.9 ± 7 mg/kg) and after 20 h in rye sourdough with 402.3 ± 69.4 mg/kg (Figure 2C,D). After the maximal values were reached, the β-glucan concentrations decreased down to ≤110 mg/kg within 24 h of fermentation. The measured β-glucan concentrations of the Δ*gtf*-2 mutants were at a steady low level during wheat and rye sourdough fermentation. During fermentation, the pH values of the wild-type sourdough decreased faster than the respective doughs with the *gtf*-2 deficient mutant until merging after 20 h. Using ELISA, the differences between the wild-type strains able to produce β-glucan and Δ*gtf*-2 mutants were significant. Therefore, this method was used for β-glucan quantification in future experiments.

3.3. Parameters Influencing In Situ Formation of β-Glucan in Wheat and Rye Sourdoughs

In the following, the impact of changing several sourdough fermentation parameters to increase the β-glucan formation by LAB were analyzed. The fermentation temperature was decreased to 25 °C and increased to 35 °C. The inoculation amount was halved and doubled and a cocultivation adding *La. plantarum* TMW 1.2330 was performed. The previous results of fermentations at 28 °C using 2 mL of cell suspension adjusted to OD_{600nm} 1 per 200 g dough were used as a base for changes in β-glucan concentration and the period for maximum β-glucan production (between 16 and 24 h), which was identified in Section 3.2. The β-glucan produced by *L. brevis* TMW 1.2112 (wt) and *P. claussenii* TMW 2.340 (wt) were quantified by ELISA.

Temperature effects on β-D-glucans production in wheat sourdoughs was observed at 25, 28, and 35 °C (Table 1). An increase of the fermentation temperature up to 35 °C resulted in a significantly higher β-glucan concentration at 24 h with *L. brevis* (506.2 ± 101.6

5 mg/kg) compared to 25 and 28 °C. In contrast, this led to a decrease of the fermentation temperature to 25 °C from a significant maximal concentration (521.1 ± 57.4 mg/kg) after 16 h for *P. claussenii.* It must be considered that, within the temperature experiment, the ratio of ropy *P. claussenii* colonies was 96% and, therefore, the highest in all wheat experiments. Furthermore, with higher fermentation temperatures, the pH values of both strains decreased faster. In a 24-h fermentation period at 25 °C, the pH value was 3.85 ± 0.09 and, at 35 °C, the pH was 3.56 ± 0.06.

Table 1. β-glucan [mg/kg] production by varying parameters in wheat fermentation by *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340.

Strain	Temperature	Inoculum Size	Fermentation Time [h]		
			16	20	24
L. brevis TMW 1.2112	25 °C	1×	35 ± 29.7	104.5 ± 15	208.5 ± 11.2
	28 °C	1×	72.2 ± 21.5	279 ± 73.3	245.9 ± 65
	35 °C	1×	131.1 ± 43.7	91.6 ± 48.7	506.2 ± 101.6
	28 °C	1/2×	60.6 ± 6	172.3 ± 12.6	188.7 ± 36.5
	28 °C	2×	21.1 ± 2.2	223.3 ± 47.7	111.1 ± 10.1
	28 °C	1/2× *L. brevis* 1/2× *La. plantarum*	46.5 ± 8.2	14.1 ± 3.5	43.8 ± 6
P. claussenii TMW 2.340	25 °C	1×	521.1 ± 57.4	222 ± 52.8	220.4 ±28.2
	28 °C	1×	219.9 ± 7	134.5 ± 75.4	50.4 ± 8.5
	35 °C	1×	142.8 ± 18.	224.9 ± 32.9	235.6 ± 25.4
	28 °C	1/2×	53.5 ± 27.9	64.5 ± 36.2	123.5 ± 56.4
	28 °C	2×	218.9 ± 31.1	178.4 ± 13.7	313.2 ± 24.9
	28 °C	1/2× *P. claussenii* 1/2× *La. plantarum*	12.5 ± 4.9	11.4 ± 11.9	1.7 ± 5.2

Variations of the inoculation rate with *L. brevis* resulted in similar concentrations compared to the base value (28 °C) except changes in fermentation time and higher maximal concentrations within doubled inoculated doughs. The doubling of the inoculation rate resulted in a 4 h earlier concentration maximum and concentration values of halving the inocula suggested that the maximum is reached. After 24 h, similar trends were observed with *P. claussenii*. In addition, a plasmid loss was observed for 66% and 59% of pediococci cells, respectively.

Wheat sourdoughs co-fermented by *L. brevis* or *P. claussenii* and the α-amylase producing *La. plantarum* TMW 1.2330 resulted in the lowest maximum β-D-glucans concentrations after 24 h. The concentrations ranged between 1.7 ± 5.2 and 43.8 ± 6 mg/kg for *P. claussenii* and *L. brevis*, respectively. Co-cultures of *P. claussenii* with *La. plantarum* contained 61% of *P. claussenii* with a ratio of only 8% ropy colonies while co-cultures with *L. brevis* contained 26% *L. brevis* colonies and all were ropy. The remaining percent in both co-cultures was *La. plantarum*. These results are more comprehensible as lower numbers of EPS positive cells might have led to lower β-glucan concentrations. The cocultivations in wheat and in rye doughs were generally less productive in β-glucan formation, which might be the result of up to 70% of *P. claussenii* cells with only 5% ropy colonies and 48% of *L. brevis* cells while the remaining percent was *La. plantarum*.

Compared to the results of the wheat sourdough fermentations, the β-glucan concentrations in rye sourdoughs were slightly higher (Table 2). The CFUs of both strains were up to six-fold higher in rye fermentation than in wheat fermentation. *L. brevis* reached its maximum β-glucan concentration of 573.6 ± 78.7 mg/kg in rye sourdoughs when fermented at 25 °C for 24 h. Within 20 to 24 h, *P. claussenii* maximum was ≤624.7 ± 62.8 mg/kg at 25 °C with a ratio of ropy colonies of 98%, which was the maximum within all rye fermentations.

Table 2. β-glucan [mg/kg] production by varying parameters in rye fermentation by *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340.

Strain	Temperature	Inoculum Size	Fermentation Time [h]		
			16	20	24
L. brevis TMW 1.2112	25 °C	1×	399.1 ± 39.2	422.4 ±56.4	573.6 ± 78.7
	28 °C	1×	175.9 ± 69.4	391.7 ± 57.5	412.3 ± 47.4
	35 °C	1×	195.7 ± 40.9	230 ± 37.5	220.6 ±52.9
	28 °C	1/2×	191.3 ± 27.2	314.4 ± 34.6	363.6 ± 66.4
	28 °C	2×	41.2 ± 10.7	477 ± + 91.7	468.7 ± 90.2
	28 °C	1/2× *L. brevis* 1/2× *La. plantarum*	111.4 ± 8.8	99.3 ± 38.1	138.7 ± 37.1
P. claussenii TMW 2.340	25 °C	1×	493.6 ± 52.7	624.7 ± 62.8	623 ± 11.4
	28 °C	1×	338.5 ± 51.5	402.2 ± 98.1	104 ± 18.1
	35 °C	1×	346 ± 36.3	514.1 ± 17.8	448.7 ± 5.2
	28 °C	1/2×	146.3 ± 78.1	200.8 ± 74.8	195.2 ± 121.1
	28 °C	2×	400.4 ± 117.3	328.8 ±71.9	429.5 ±129.8
	28 °C	1/2× *P. claussenii* 1/2× *La. plantarum*	29.8 ± 10.5	19.2 ± 21.1	26.4 ± 15.7

The pH values were again lower with a higher fermentation temperature: 3.66 ± 0.08 (35 °C) and 3.85 ± 0.06 (25 °C). The decrease of the pH values seemed not to influence the β-glucan formation. Increasing the inoculum resulted in similar trends, as observed during wheat fermentation (lower initial cell numbers and lower β-glucan concentrations).

Figure 3 shows the maximal β-glucan concentrations with different fermentation conditions (temperature, inoculation size, and co-cultivation). Generally, the maximal β-glucan concentration was reached earlier with *P. claussenii* than with *L. brevis*. In wheat sourdough fermentations, the fermentation temperature had the most significant influence for β-glucan formation by *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340. While *L. brevis* seemed to prefer 35 °C for increased β-glucan formation, *P. claussenii* preferred 25 °C (Figure 3A). The measured β-glucan concentration in rye sourdoughs were, in general, higher when compared to wheat sourdoughs, which might be attributed to higher cell counts. Changes in the fermentation temperature and amount of inoculum influenced the β-glucan formation in rye sourdoughs the most. However, these effects were interfered with random extensive loss of the ability to form EPS by *P. claussenii*. On the contrary, the EPS formation of *L. brevis* TMW 1.2112 colonies were positive in all experiments. Both strains formed the highest β-glucan levels in rye doughs when fermented at 25 °C.

3.4. Analysis of β-Glucan-Enriched Bread Characterisics

Sensory analysis was performed, using an affective test on wheat and rye sourdough breads of *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 compared to the control. The untrained panel used a 5-points Hedonic scale to rate five attributes (moisture, airiness, texture, acidity, and overall acceptance) of the breads. Mean values of the results are presented using spider diagrams including statistical significance values (Figure 4). Significant differences in the sensory quality of the wheat sourdough breads, especially for the moisture, airiness, and overall acceptance were observed. Wheat sourdough breads of *L. brevis* ($p < 0.0001$) and *P. claussenii* ($p = 0.0001$) were significantly fluffier than the control, while the bread of *L. brevis* was significantly moister ($p = 0.0022$) than *P. claussenii* ($p = 0.0266$) and the control breads. Within the two categories of texture and acidity, the panel detected no significant differences between the three samples. In the category of overall acceptance, the panel had significantly higher preference for the wheat breads of *L. brevis* with an average of 3.7 ($p < 0.0001$) and 3.2 for *P. claussenii* ($p = 0.0027$) than the control with an average value of 2.6 (dislike slightly).

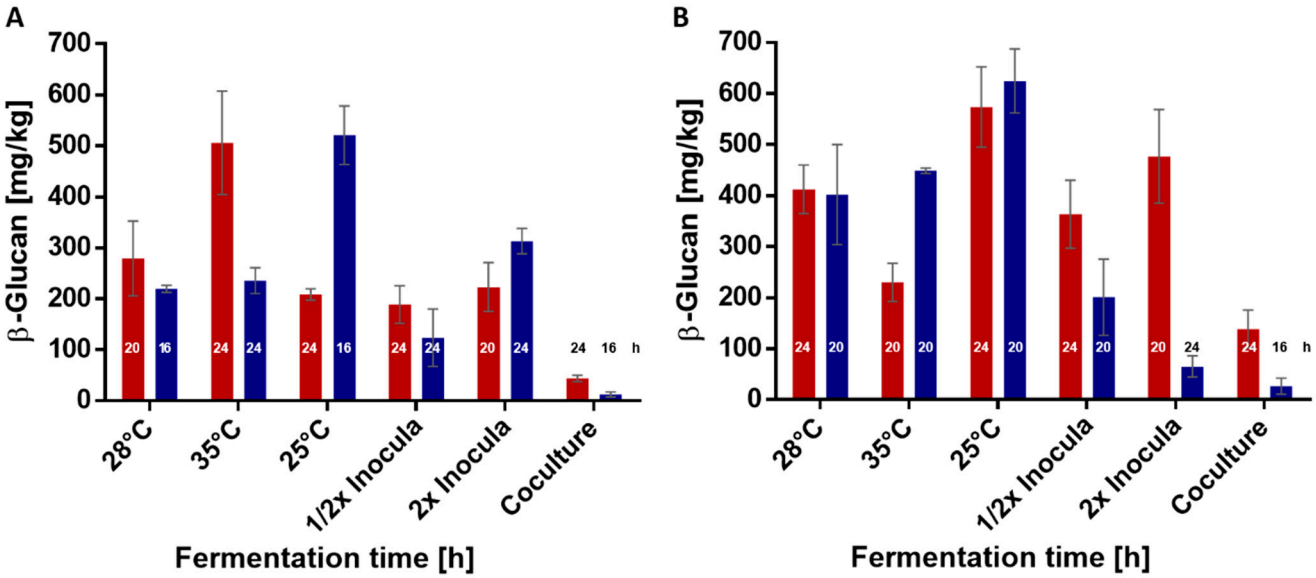

Figure 3. Maximal β-glucan concentrations in fermented wheat and rye sourdoughs under different conditions. (**A**) *L. brevis* TMW 1.2112 (▮) and *P. claussenii* TMW 2.340 (▮) in wheat sourdough and (**B**) *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 in rye sourdough with the respective fermentation times in h. Values are means of biological triplicates including standard deviations.

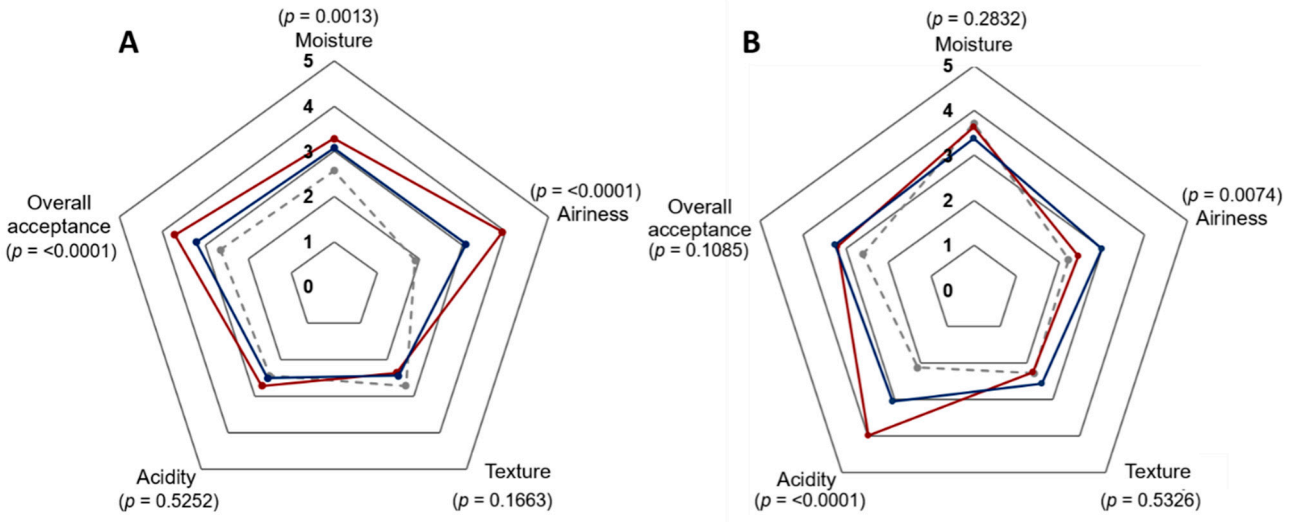

Figure 4. Consumer acceptability of β-glucan enriched wheat and rye breads. (**A**) Wheat bread with *L. brevis* TMW 1.2112 (red), *P. claussenii* TMW 2.340 (blue) compared to the control (dashed line, grey). (**B**) Rye bread with *L. brevis* TMW 1.2112, *P. claussenii* TMW 2.340 compared to the control. Data are presented in a 5-point hedonic scale. Sensory attributes with $p \leq 0.01$ indicate a significant difference among the samples.

In addition, the same sensory analysis was performed with rye sourdough breads (Figure 4B). The acidity of the breads resulted in high ratings of *L. brevis* with an average of four compared to *P. claussenii* (3) and the control bread (2.1). The statistical analysis resulted in significant differences between the three samples with $p < 0.0001$ for *L. brevis* and the control, $p = 0.0080$ for *L. brevis* and *P. claussenii*, and $p = 0.0019$ for *P. claussenii* and the control bread. The airiness of *P. claussenii* rye sourdough breads were rated with 3 while *L. brevis* was rated with 2.4 and the control bread was rated with 2.2. Despite the higher rated acidity and the lower rated fluffiness, the overall acceptance of *L. brevis* was rated with 3.2 and, similarly to the rating of *P. claussenii*, it was rated with 3.3. Within

the attributes of moisture, texture, and overall acceptance, no significant differences were observed between the samples.

4. Discussion

In the present study, we provide insights into the formation of β-D-glucan produced by LAB during wheat and rye sourdough fermentation. The competitiveness of two brewery isolates *L. brevis* and *P. claussenii* in sourdough fermentations against the endogenous microbiota was demonstrated in our previous study [30]. In the same study, a significant effect of β-glucan-forming *L. brevis* TMW 1.2112 on the viscosity of wheat sourdoughs compared to *P. claussenii* TMW 2.340 and the mutants was described but missed quantity information.

A common method to quantify β-glucans is the determination of glucose by HPLC after acidic hydrolysis of the β-glucan with perchloric acid. Ua-Arak et al. successfully used a similar method for the quantification of fructose monomers of in situ produced levan in buckwheat sourdoughs [46]. However, in addition to bacterial β-glucan, further polysaccharides, e.g., starch and cereal β-glucan, are sources, which release glucose upon acid hydrolysis [50]. The β-glucan calculation of hydrolyzed samples inevitably leads to an overestimation of the β-glucan concentration. In this study, HPLC analysis revealed that differences between β-glucan producing wt strains and non-producer strains were not significant and gave contradictory results with higher calculated β-glucan concentrations with non-producers than with EPS-forming wild-type strains. As the comparison of the β-glucan concentration between the wild-types and mutants was crucial for a quantification by HPLC, this method might not be specific enough. The initial increase of glucose (later calculated β-glucan) observed could be explained by the phosphorolytic cleavage of the preferred maltose by maltose phosphorylase (MP) of *L. brevis* [51]. Within the genome sequence of *L. brevis* TMW 1.2112, a putative MP (AZI09_01010) was detected while no such genes were found for *P. claussenii* TMW 2.340 [39,52].

The formation of the type 37 capsule of *S. pneumoniae* is driven by the single gene *tts*, which shows similar sequence characteristics of other β-glycosyltransferase genes as the *gtf-2* gene of *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 [53]. Due to this similarity, the quantification of the β-glucan using serotype 37 antibodies was possible. In our study, the β-glucan quantification by ELISA applied to β-glucan produced during growth in medium demonstrated a high sensitivity by detecting even slight traces in the doughs. In contrast to barley, oat, and rice fermentations, cross-reactions between the antibodies with wheat and rye samples were observed, and, therefore, non-fermented material was used as a blank and subtracted from fermented material [12]. Nevertheless, even in samples with the *gtf-2* deficient strains, β-glucan concentrations up to 80 mg/kg sourdough were measured after blank correction. Fermented sourdoughs contain yeast β-glucan (mixed linked (1,3) and (1,6)-β-D-glucan) and other polysaccharides, which potentially cross-react with the assay [54–57]. Werning et al. analyzed the binding of the antibodies to curdlan (linear (1,3)-β-D-glucan), laminarin (6-substituted (1,3)-β-D-glucan), and xanthan a HePS [48]. While xanthan was unable to bind and curdlan had only a weak affinity, laminarin was able to compete almost equally with the *O*2-substituted (1,3)-β-D-glucan [48]. However, since laminarin is a marine-based polysaccharide from algae, in our study, this aspect was insignificant [58]. Despite these minor drawbacks, the immunological assay proved to be a suitable quantification method of β-glucan in cereal matrices since significant increases of produced β-glucan followed by decreases were observable. Within 20 to 24 h, the β-glucan concentrations of rye sourdoughs fermented by *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 increased up to 412 mg/kg and 402 mg/kg, respectively, and corresponded with the results of the oat flour fermentation by *Pediococcus* (*P.*) *parvulus* 2.6, which produced 139.7 ± 40.8 mg/L β-D-glucan within 24 h [12].

Since the artificial addition of EPS in doughs is less effective as in situ-formed EPS, an increase of β-D-glucan formation by LAB in sourdoughs was tested by changing the fermentation conditions [23,59]. One approach was the addition of α-amylase producing *La. plantarum* TMW 1.2330 to increase available glucose and maltose by degrading starch

to promote growth and production of β-D-glucan by *L. brevis* TMW 1.2112 or *P. claussenii* TMW 2.340. However, it resulted in the lowest β-D-glucan amounts of this study. Notably, under these competitive conditions, lower cell counts of the β-D-glucan mutant strains compared to the wild-type strains were observed (Table S1 and Table S2). *P. claussenii* TMW 2.2123 was almost displaced by *La. plantarum*. EPS encapsulated cells might be better protected from inhibitory compounds as more vulnerable mutant strains [60]. Finally, the starch degradation was not sufficient for increasing the β-D-glucan formation and compensating substrate utilization by *La. plantarum* at the same time. The addition of 10% maltose to wheat and rye sourdoughs fermented by *L. brevis* TMW 1.2112 did not lead to increased β-D-glucan, and unveiled the β-D-glucan formation is not restricted by the constituents in flour and dough systems (unpublished data). Furthermore, the high cell counts (10^8 to 10^9 CFU/g), characteristically for traditional fermented sourdoughs, excluded substrate limitation [61].

Sourdoughs inoculated with different amounts of cells featured similar cell counts and pH values at the end of fermentation, but the course of β-D-glucan formation differed as higher inoculated doughs ($\leq 6.6 \times 10^6$ CFU/g) reached earlier and higher maximum glucan concentrations. While lower inoculated doughs ($\leq 1.6 \times 10^6$ CFU/g) seemed to reach their maximum at a later stage in fermentation. Thus, β-D-glucan concentrations in dough fermentation seems not to be linked to the cell counts of the producer cells.

In our previous study, we described the loss of EPS formation by *P. claussenii* TMW 2.340 while formation in *L. brevis* TMW 1.2112 was perfectly stable [30]. The same effects were demonstrated in this study. The β-glycosyltransferases genes are plasmid-encoded traits in both strains. Plasmid instability and the resulting loss of β-D-glucan formation might be prevented by a toxin/antitoxin-system in *L. brevis* but not in *P. claussenii* [30,62,63]. Additionally, the relatively high β-D-glucan concentration at high cell counts with low percentages of EPS-forming colonies may be explained by an additional regulation of the plasmid copy number, regulation of the amount of enzyme at a transcriptional level, or the regulation of the enzyme activity [64–66].

The most significant impact on β-D-glucan formation was observed by changing the fermentation temperature (Tables 1 and 2). This approach obtained concentrations of 624.7 ± 62.8 mg/kg in rye and 521.1 ± 57.4 mg/kg in wheat sourdoughs fermented with *P. claussenii* TMW 2.340 at an optimal temperature of 25 °C. The comparably highly stable β-D-glucan formation in *P. claussenii* TMW 2.340, with 95% to 99% EPS-forming colonies could be a reason for such high concentrations. Furthermore, the preferred sourdough fermentation temperature of *P. claussenii* seemed to be 25 °C, and the previous 28 °C might have been unfavorable for this strain. *L. brevis* produced the highest β-D-glucan concentrations at 35 °C and 25 °C in wheat and rye sourdoughs, respectively. The selected fermentation temperatures (25 and 35 °C) corresponded with the optimum growth temperature ranges of *P. claussenii* and *L. brevis* sp. [67–69]. Upon cultivation in media of *L. brevis* TMW 1.2112, Fraunhofer reported that β-D-glucan formation was increased at lower pH values [25]. This impact was not observed for sourdough fermentations. Instead, the opposite occurred, as more β-D-glucan was quantified upon a slower pH decrease.

Pérez-Ramos et al. used *P. parvulus* 2.6 to ferment oats and produced 659.4 ± 45.18 mg/L β-D-glucan within 64 h. A decreasing trend of the β-D-glucan concentration as observed in our study was not described. The oat matrices were initially fermented by *La. plantarum* and heated before adding *P. parvulus* 2.6 [12,70]. The pre-fermentation may have accelerated starch breakdown, released soluble monosaccharides and disaccharides, which can be used as a substrate from *P. parvulus* 2.6 while the heating inactivated largely cereal microbiota and endogenous enzymes able to degrade formed bacterial β-D-glucan [57,71]. In contrast, in our study, raw materials were used and bacterial β-D-glucan degradation could be expedited by cereal-based enzyme activities, e.g., endo-β-glucanase [71]. In addition, cell-own glycosyl hydrolases might decrease the β-glucan amount, e.g., putative endoglucanase (AZI09_02135) or 1,3-β-D-glucanase (AZI09_02170) of *L. brevis* TMW 1.2112 to maintain the substrate supply after depletion of environmental sources [25,72–74]. Since the bacterial β-glucan is mainly

formed as capsular EPS, a relatively large fraction of the EPS gets lost during sample preparation for ELISA due to centrifugation and filtration steps [30,38]. Therefore, it can be reasonably assumed that the concentration of the β-D-glucan is much higher within the fermented wheat and rye sourdoughs.

The sensory quality of wheat and rye sourdough breads fermented with *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 was analyzed by an untrained panel. Wheat bread fermented with *L. brevis* was, overall, higher scored than wheat bread with *P. claussenii.* Whereas rye bread with *L. brevis* was graded predominantly sour and *P. claussenii* not. Nevertheless, the overall acceptance of both rye breads was similar and supposedly is related to the unique flavor of sourdough breads produced by heterofermentative LAB compared to homofermentative LAB. Banu et al. described that a trained panel rated the overall flavor intensity of rye bread crumbs of heterofermentative LAB (including *L. brevis sp.*) higher as homofermentative produced rye breads [75,76]. In our previous study, we demonstrated that homofermentative *P. claussenii* produced only lactic acid while heterofermentative *L. brevis* produced lactic and acetic acid during wheat and rye sourdough fermentation [30]. Acetic acid can improve bread quality, such as the lactate and acetate ratio contributing to the flavor [1,77,78]. The sensory analysis demonstrated the overall acceptance of sourdough breads fermented by *L. brevis* or *P. claussenii* and resulted in a significant contribution to bread quality.

5. Conclusions

Taken together, this study compares homofermentative *P. claussenii* and heterofermentative *L. brevis* in β-glucan formation during wheat and rye sourdough fermentation. In our hands, quantification of glucan with an ELISA approach was superior to the indirect determination of deliberated glucose after hydrolysis. It was demonstrated that the fermentation process can significantly influence the β-D-glucan concentration of the sourdoughs. The cell count and EPS level seemed to be independent of each other, implying external factors trigger the EPS production. Temperature control proved to mainly influence EPS formation depending on flour type and microbiota. Since high EPS concentrations are favorable with respect to techno-functional properties and health beneficial effects, an increase of the EPS amount is an important factor. This applies in particular to HePS and β-D-glucans, which are formed in low quantities compared to HoPS. However, the β-D-glucan concentration declined during fermentation and, therefore, further investigation should be performed to determine the factors responsible for the decrease of the β-D-glucan during fermentation, which is most likely cereal and cell-own glycosyl hydrolases. Compared with sucrose-fed LAB for in situ HoPS formation, the β-D-glucan forming LAB can be used for clean label products without residual sweetness or excessive acetic acid formation by utilizing naturally contained monosaccharides and disaccharides of the flour for optimum growth.

Supplementary Materials: The following are available online at https://www.mdpi.com/2304-8158/10/3/547/s1. Table S1: Results of wheat sourdough analyses: cell count, pH values, MALDI-TOF MS, and ratio of EPS positive colonies and Table S2: Results of rye sourdough analyses: cell count, pH values, MALDI-TOF MS, and ratio of EPS positive colonies.

Author Contributions: Conceptualization, J.A.B., M.A.E., and R.F.V. Methodology, J.A.B., J.F., and E.S. Formal analysis, J.A.B. Investigation, J.F., E.S., and J.A.B. Resources, R.F.V. Writing—original draft preparation, J.A.B. Writing—review and editing, J.A.B., J.F., E.S., R.F.V., and M.A.E. Visualization, J.A.B. Supervision, M.A.E. Project administration, M.A.E. and R.F.V. Funding acquisition, R.V.F. All authors have read and agreed to the published version of the manuscript.

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4.3 Characterisation of recombinant GH 3 β-glucosidase from β-glucan-producing *Levilactobacillus brevis* TMW 1.2112

So far, it was presented that Gtf-2 of *L. brevis* TMW 1.2112 acts as the key enzyme in the biosynthesis of capsular *O2*-substituted (1,3)-β-D-glucan from activated sugar nucleotide precursors. Beta-glucans are HoPS consisting exclusively of D-glucose monomers with varying linkages and are further discussed as storage compounds. The degradation of β-glucan during sourdough and culture broth fermentations with *L. brevis* TMW 1.2112 was already observed, however, *O2*-substituted (1,3)-β-D-glucan degrading enzymes are so far not described.

Glycohydrolases also called glycoside hydrolases (GH) are carbohydrate active enzymes able to hydrolyze glycosidic bonds, therefore, they could also be considered as antagonists of the Gtf-2. The genome sequence of *L. brevis* TMW 1.2112 was screened for GH sequences potentially able to hydrolyze β-glycosidic bonds. The β-glucosidase BglB (AZI09_02170) was identified and heterologous expressed using *Escherichia coli* BL21. The enzyme was assigned to GH family 3 which further has a monomer structure with the molecular weight of 83.5 kDa and most likely is an intracellular exo-active enzyme. The preferred substrate was determined to be β-glycosidic bonds such as *p*NP-β-D-glucopyranoside, among others, which was also used to determine the Km value (0.22 mM). However, the enzyme exhibited only marginal hydrolytic activity toward the isolated bacterial (1,3)-β-D-glucan from *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340. Several β-glucosidases from even partly EPS-forming LAB species were so far described. The gene sequences were used for phylogenetic studies, to obtain an indication of the relationship between this enzyme and the respective strains, also with regard to EPS degradation. However, the analysis demonstrated that the clustering of the LAB was independent to EPS formation characteristics rather than to the species level. This suggests that this enzyme in particular is not necessarily related to β-glucan or EPS formation in general to enable the utilization of the high-molecular compounds for energy generation of the LAB.

The challenge in this study was to obtain an adequate resolution of the polymer in the buffer solution. Furthermore, since the enzyme is expressed intracellularly, its participation in polymer degradation is more likely to occur at a later time, e.g., when oligomers are already present. This makes the involvement of several enzymes in the potential degradation of the polymer even more likely.

Author contributions: Julia A. Bockwoldt was responsible for the conceptualization, formal analysis, investigation, and visualization and wrote the original draft of the manuscript. Matthias A. Ehrmann conducted the conceptual setup, project supervision, and editing of the manuscript.

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ORIGINAL PAPER

Characterisation of recombinant GH 3 β-glucosidase from β-glucan producing *Levilactobacillus brevis* TMW 1.2112

Julia A. Bockwoldt · Matthias A. Ehrmann

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Abstract *Levilactobacillus* (*L.*) *brevis* TMW 1.2112 is an isolate from wheat beer that produces *O2*-substituted (1,3)-β-D-glucan, a capsular exopolysaccharide (EPS) from activated sugar nucleotide precursors by use of a glycosyltransferase. Within the genome sequence of *L. brevis* TMW 1.2112 enzymes of the glycoside hydrolases families were identified. Glycoside hydrolases (GH) are carbohydrate-active enzymes, able to hydrolyse glycosidic bonds. The enzyme β-glucosidase BglB (AZI09_02170) was heterologous expressed in *Escherichia coli* BL21. BglB has a monomeric structure of 83.5 kDa and is a member of the glycoside hydrolase family 3 (GH 3) which strongly favoured substrates with β-glycosidic bonds. K_m was 0.22 mM for pNP β-D-glucopyranoside demonstrating a high affinity of the recombinant enzyme for the substrate. Enzymes able to degrade the (1,3)-β-D-glucan of *L. brevis* TMW 1.2112 have not yet been described. However, BglB showed only a low hydrolytic activity towards the EPS, which was measured by means of the D-glucose releases. Besides, characterised GH 3 β-glucosidases from various lactic acid bacteria (LAB) were phylogenetically analysed to identify connections in terms of enzymatic activity and β-glucan formation. This revealed that the family of GH 3 β-glucosidases of LABs comprises most likely exo-active enzymes which are not directly associated with the ability of these LAB to produce EPS.

Keywords β-glucosidase · β-glucan · Exopolysaccharide · Glycoside hydrolase 3 · Heterologous expression · *Levilactobacillus brevis*

J. A. Bockwoldt · M. A. Ehrmann (✉)
Chair of Microbiology, Technical University of Munich, Freising, Germany
e-mail: matthias.ehrmann@tum.de

Introduction

The exopolysaccharide (EPS) formation by lactic acid bacteria (LAB) gained increased interest by the food industry in the past decades due to health-promoting effects and their application as natural viscosifier and thickening agents (Goh et al. 2005; Korcz et al. 2021; Moradi et al. 2021; Ruas-Madiedo et al. 2005; Zannini et al. 2016). The major advantages are the generally recognised as safe (GRAS) status of EPS forming LAB and further an in situ EPS enrichment of food products makes the use of additives (e.g., guar gum or pectin) redundant (Freitas et al. 2011; Velasco et al. 2009; Zannini et al. 2016). EPSs formed by LABs are either homopolysaccharides (HoPS) or heteropolysaccharides (HePS) (Badel et al. 2011; Fraunhofer et al. 2018b; Freitas et al. 2011; Notararigo et al. 2013). β-glucans (consisting solely of glucose monomers) are produced intracellularly by activated sugar nucleotide precursors and compared to HoPS have lower yields (Mozzi et al. 2006; Notararigo et al. 2013). Regarding the fermentation of foods, low yields and

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the degradation of in situ synthesized EPS are critical parameters for industrial applications (De Vuyst et al. 2001). Previous studies described the decrease of EPS concentrations with increasing fermentation periods of LAB either through enzymatic activity or physical parameters (Cerning et al. 1992; Degeest et al. 2002; Dierksen et al. 1995; Vuyst et al. 1998; Zannini et al. 2016). Degeest et al. (2002) and Pham et al. (2000) reported EPS degradation by cell extract of *Streptococcus thermophilus* LY03 and *Lacticaseibacillus rhamnosus* R. Glycohydrolases or glycoside hydrolases (GH), such as α-D-glucosidase, β-D-glucosidase, α-D-galactosidase or β-D-galactosidase, were found to be involved in EPS degradation, thus reducing the viscosity of the LAB culture broths. The GHs are grouped in more than 170 families which are classified based on their amino acid sequences. These enzyme families possess hydrolytic activities towards glycosidic bonds of carbohydrates and non-carbohydrate fractions. Furthermore, GHs can be classified into retaining and inverting enzymes depending on their catalytic mechanism. Inverting enzymes perform nucleophilic substitution and retaining enzymes form and hydrolyse covalent intermediates (Ardèvol et al. 2015; Koshland Jr. 1953; Naumoff 2011). The β-glucosidases of the GH 3 family, for example, retains the anomeric configuration of substrates and have a frequently occurring $(\beta/\alpha)_8$-barrel structure (Naumoff 2006; Rigden et al. 2003). GH 3 β-glucosidases could act as exo enzymes, able to hydrolyse terminal, non-reducing β-D-glycosyl residues including β-1,2-: β-1,3-; β-1,4-; β-1,6-linkages and/or aryl-β-glucosides with subsequent β-D-glucose release (Cournoyer et al. 2003; Harvey et al. 2000). It was demonstrated that in general the GH 3 family is one of the more abundant GH families in bacterial genomes. Moreover, the bacterial genome size correlated with the presence of this family, which means that smaller genomes (1066 ± 294 open reading frames (orf)) lacked the presence of GH 3 enzymes (Cournoyer et al. 2003).

Levilactobacillus (*L.*) *brevis* TMW 1.2112 is a wheat beer isolate which produces *O2*-substituted (1,3)-β-D-glucan, a HoPS. In this study, the genome sequence of *L. brevis* TMW 1.2112 was screened for GHs by in silico genome mining. One orf (AZI09_02170) was identified as putative β-glucosidase BglB (GH 3). BglB was heterologously expressed, characterised, and analysed for its ability to degrade isolated and purified β-glucan. Since β-glucosidases of LAB were previously described to be involved in EPS degradation (Degeest et al. 2002; Pham et al. 2000), BglB was of interest in this study also considering the β-linked EPS. Furthermore, the enzyme was compared with previously characterized lactic acid bacterial GH 3 β-glucosidases from the literature for a brief overview and to infer relations between the EPS forming and non-forming LAB.

Material and methods

Bacterial strains, plasmids, and cultivation

The EPS forming wheat beer isolate *L. brevis* TMW 1.2112 was cultivated in modified Man, Rogosa, and Sharpe medium (mMRS) with pH 6.2 at 30 °C as static cultures as previously described by (Fraunhofer et al. 2017; Schurr et al. 2013). *L. brevis* TMW 1.2112 and *Pediococcus claussenii* TMW 2.340 (isogenic with DSM 14800 ^{T}, and ATCC BAA-344 ^{T}) were cultivated in a modified semi-defined (SDM) at pH 5.5 with 20 g L^{-1} maltose as sole carbon source for EPS isolation. The isolation was performed according to Bockwoldt et al. (2021) except perchloric acid treatment (Dueñas-Chasco et al. 1997).

Escherichia (*E.*) *coli* BL21 (StrataGene®) cells and pBAD/*Myc*-His A (Invitrogen) were used for cloning and expression of the enzyme. Recombinant *E. coli* cells were grown in lysogeny broth (LB) Lennox medium (pH 7.2) at 37 °C with and 200 rpm or on solid LB medium with 1.5% (w/v) agar. Transformed cells were selected by adding 100 µg ampicillin mL^{-1} to the LB medium. The pBAD vector was constructed by introducing the appropriate DNA fragment of the β-glucosidase (AZI09_02170) into the NcoI and SalI sites of pBAD/myc-His by Gibson Assembly.

Bioinformatic analysis

The previously sequenced genome of *L. brevis* TMW 1.2112 (Fraunhofer et al. 2018a) was used for similarity analysis of GH 3 by genome mining (Ziemert et al. 2016). The DNA and protein sequences were analysed by BLASTn and BLASTx, respectively (Altschul et al. 1990). Further characterizations

of the enzymes and the GH family affiliation were performed by using CAZy (Lombard et al. 2014), functional information of the enzymes by UniProt (Consortium, 2020), and homology modelling was performed by SWISS-MODEL (Waterhouse et al. 2018). Prediction of a putative signal peptide was performed by using SignalP-5.0 (Armenteros et al. 2019).

Construction of heterologous expression vector

The GH 3 β-1,3-glucosidase (AZI09_02170) gene was identified from the genome sequence of *L. brevis* TMW 1.2112 (GenBank accession No.: CP016797). The appropriate DNA sequence was amplified by PCR with Q5 High Fidelity DNA-Polymerase (NEB, Germany) using forward and reverse primers with pBAD overlaps 5′- CGTTTAAACTCAATGATGATG ATGATGATGTTGGCGTAATAAGGTGTTTGC CCG-3′ and 5′- CGTTTTTTGGGCTAACAGGAG GAATTAACCATGGACATCGAACGAACGCTT GCTGAACTC-3′, respectively. Amplicons were generated by the PCR program as follows: initial denaturation at 98 °C for 30 s, followed by 30 cycles of 10 s at 98 °C, 20 s at 71 °C and 90 s at 72 °C with a final extension at 72 °C for 2 min. The PCR product was purified and integrated into the previously digested pBAD/*Myc*-His A vector by Gibson assembly (Gibson Assembly® Master Mix, NEB, Germany). The vector was digested using the enzymes *NcoI* and *SalI* (NEB, Germany) which simultaneously excised the *Myc*-region. The recombinant plasmid pBAD_bGLU was transformed into *E. coli* BL21 by the heat-shock method (Froger et al. 2007).

Expression and purification

Positive clones of *E. coli* BL21 carrying the vector pBAD_bGLU were screened and selected for enzyme expression. LB medium containing 100 µg ampicillin ml^{-1} was inoculated with *E. coli* pBAD_bGLU and incubated at 37 °C and 200 rpm until $OD_{600\,nm} \approx 0.5$. The cells were induced with 0.25% L-arabinose (v/v) overnight at 15 °C and 200 rpm. In the next step, the cells were harvested by centrifugation at 3,000 × g for 10 min at 4 °C and resuspended in lysis buffer: 50 mM sodium phosphate, 300 mM NaCl, 5% glycerol, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 mM β-mercaptoethanol, pH 7.5. Cell disruption was performed using glass beads (Ø 2.85–3.45 mm) and a benchtop homogenizer (FastPrep®-24 MP, MP Biomedical Inc, Germany) in three cycles each 30 s. The cell debris was harvested by centrifugation 17,000 × g for 30 min at 4 °C and discarded. The supernatant including the his-tagged recombinant protein was added to nickel-nitrilotriacetic acid (Ni–NTA) crosslinked agarose resins (SERVA Electrophoresis GmbH, Germany) and purified according to the manufacturer's protocol. The purified fractions were analysed and visualised on 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) gels by staining with Coomassie Brilliant dye Roti® Blue (Carl Roth GmbH + Co. KG, Germany). The protein concentration within the several fractions was determined by Coomassie (Bradford) protein assay kit using bovine serum albumin (BSA) as the standard (Thermo Fisher Scientific, Germany). Imidazole was removed by dialysis against 50 mM PBS buffer pH 6.8 overnight at 4 °C using 3.5 kDa dialysis tubing (SERVA Electrophoresis GmbH, Germany).

Screening for the substrate specificity

The substrate specificity of the recombinant BglB and the cell lysate of *E. coli* BL21 was analysed using six different *p*-nitrophenyl phosphate (pNP) substrates: pNP β-D-glucopyranoside (pNPβGlc), pNP α-L-fucopyranoside (pNPαFuc), pNP β-D-fucopyranoside (pNPβFuc), pNP α-D-galactopyranoside (pNPαGal), pNP β-D-galactopyranoside (pNPβGal), and pNP β-D-maltoside (Carl Roth GmbH + Co. KG, Germany, Santa Cruz Biotechnology, Inc., USA and Merck, Germany). The purified and dialysed enzyme solution was incubated in 100 µL of 50 mM PBS buffer (pH7) with 2 mM pNP substrate at 37 °C for 2 h using a microtiter plate reader at 405 nm (SPECTROstar Nano, BMG Labtech GmbH, Germany). Determinations were done using biological duplicates each with at least technical duplicates.

In addition, API® ZYM (bioMérieux, Marcy-l'Étoile, France) test stripes were used for enzyme characterisation of the cell lysate samples from *E. coli* BL21 and induced *E. coli* pBAD_bGLU. The cell pellet of 5 mL culture volume was washed and resuspended with 2.5 mL PBS buffer (pH7). Cell disruption was done as previously described. The analysis was performed by inoculating each cupule of the test stripe with 65 µL of cell lysate and subsequently

incubated for 4 h at 37 °C (Gulshan et al. 1990; Martínez et al. 2016). Water was added into the plastic trays to creating a humid atmosphere, preventing the enzymes from drying out. The reaction was terminated according to the manufacturer's protocol. Colour changes were read after 5 min using a range from 0 to 5. While 0 represented no changes in the colour (0 nM substrate hydrolysed), represented a 5 a clear and strong colour change (≥ 40 nM substrate hydrolysed) and therefore a positive enzyme reaction (Baldrian et al. 2011). Determinations were done using biological duplicates.

Influence of temperature and pH on β-glucosidase activity and stability

The optimal pH range of the recombinant BglB was measured at 37 °C in 50 mM PBS buffer containing 2 mM pNPβGlc with pH values ranging between 4 to 11 for 20 min. The temperature optimum was determined using 50 mM PBS buffer containing 2 mM pNPβGlc with the optimal pH incubated for 20 min at temperatures between 10 and 60 °C. The pH stability of the enzyme was determined in 50 mM PBS buffer with pH 4 to pH 11 for 2 h at 37 °C. The effect of the temperature on enzyme stability was tested by incubating the enzyme in 50 mM PBS (pH 7) for 2 h at various temperatures from 10 to 60 °C. The relative activities were calculated by released pNP from 2 mM pNPβGlc measured at 405 nm with a microtiter plate reader. Determinations were done using biological duplicates.

Kinetic parameters of β-glucosidase

The Michaelis Menten constants (K_M) and maximum reaction rate (V_{max}) of the enzyme were determined in 50 mM PBS buffer (pH 7) at 37 °C using pNPβGlc concentrations between 0.01 and 20 mM (Johnson et al. 2011). An increase in absorbance by released *p*-nitrophenol was recorded at 405 nm with a microtiter plate reader. The recorded absorbance values of the first 4 min directly after adding the enzyme to buffers containing different pNPβGlc concentrations were used for the claculations. The kinetic constants of the β-glucosidase were calculated using Lineweaver–Burk plots (Lineweaver et al. 1934). Determinations were done using biological duplicates.

Hydrolytic activity against isolated β-glucans

Isolated and purified bacterial β-glucan of *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 and curdlan (Megazyme Ltd., Ireland) were dissolved in 50 mM PBS buffer (pH 7) with a final concentration of 1 mg β-glucan mL^{-1}. The β-glucan samples were inoculated with the recombinant β-glucosidase and incubated at 37 °C for 4 h. In addition, negative controls of the dissolved β-glucans were incubated without enzyme addition. Released D-glucose was enzymatically determined by glucose oxidase/peroxidase assay (GOPOD, Megazyme Ltd., Ireland) according to the manufacturer's protocol, except adjustments of sample and reagent volumes. The assay was adapted to microtiter plate volumes with 50 µL sample volume and 150 µL of the GOPOD reagent. A standard curve using D-glucose was used to determine hydrolytic enzyme activity. Determinations were done using biological triplicates.

Neighbour-joining tree of characterized GH 3 β-glucosidases of LAB

The visualization of the relationship of the GH 3 β-glucosidases was performed by reconstruction a phylogenetic tree.). A phylogenetic tree-based similarity matrix of amino acid sequences was constructed by the neighbour-joining method (Saitou and Nei 1987) using the Bionumerics^R software package V7.62 (Applied Maths, Belgium). Bootstrapping analysis was undertaken to test the statistical reliability of the topology of the tree using 1000 bootstrap resampling of the data.

Results and discussion

In silico *characterization of L. brevis TMW 1.2112 glycoside hydrolases*

Several glycoside hydrolases were identified within the genome sequence of *L. brevis* TMW 1.2112. The bioinformatic analyses revealed i.a. the GH 3 a β-glucosidase (bglB), GH 30 a glycosylceramidase, GH 65 a maltose phosphorylase and GH 88 a d-4,5 unsaturated β-glucuronyl hydrolase. The enzymatic

activity of the β-glucosidase (BglB) was characterised. In addition, the putative hydrolytic activity towards bacterial β-glucan was tested.

Characterisation of the GH 3 β-glucosidase gene and its ubiquity in other Lactobacillus strains

The BglB encoding gene AZI09_02170 (GenBank accession No.: ARN89439) of the beer spoiling and β-glucan forming *L. brevis* TMW 1.2112 which consists of 2256 bp was annotated as a putative intracellular glycoside hydrolase. The hydrolase with homology to the glycoside hydrolase family 3 encodes 751 amino acids with a molecular mass of 83.5 kDa. Sequence analysis with the BLAST program resulted similarities to several *L. brevis* glycoside hydrolases e.g., *L. brevis* ZLB004 (GenBank accession No.: AWP47268) with a 98% identity, a β-glucosidase-related glycosidase of *L. brevis* ATCC 367 (GenBank accession No.: ABJ65020) with 96% identity and two described thermostable β-glucosidases of *L. brevis* LH8 Bgy1 (GenBank accession No.: BAN07577) and Bgy2 (GenBank accession No.: BAN05876) isolated from Kimchi with 96% similarity. The thermostable β-glucosidases were analysed for the ability to form compound K from ginsenosides (Quan et al. 2008; Zhong et al. 2016a, 2016b). Michlmayr et al. (2010a) described a β-glucosidase of *L. brevis* SK3 isolated from a starter culture preparation for malolactic fermentation related to aroma compounds formation. Further sequence analysis resulted in a 67% identity with a thermostable β-glucosidase B (GenBank accession No.: VDC15331) of the (1,3)-β-D-glucan producing strain *Oenococcus* (*O.*) *oeni* IOEB 0205 (UBOCC-A-315001) (Ciezack et al. 2010; Dols-Lafargue et al. 2008; Gagné et al. 2011).

Phylogenetic analysis of the GH 3 β-glucosidases from LAB using *Bifidobacterium* (*B.*) *longum* H-1 as an outgroup resulted in four distinct groups: Bifidobacteria, *L. brevis* strains, *O. oeni* strains, and *Limosilactobacillus* (*Li.*) antri DSM 16,041 (Fig. 1). *O. oeni* IOEB 0205 and *O. oeni* ATCC BAA-1163 were both isolated from fermented wine and while *O. oeni* IOEB 0205 dispose of the glycosyltransferase family 2 gene (*gtf2*) resulting in β-glucan formation was *O. oeni* ATCC BAA-1163 lacking this gene (Ciezack et al. 2010). The *L. brevis* strains were isolated from faeces (ATCC 14,869 = DSM 20,054 and ZLB004),

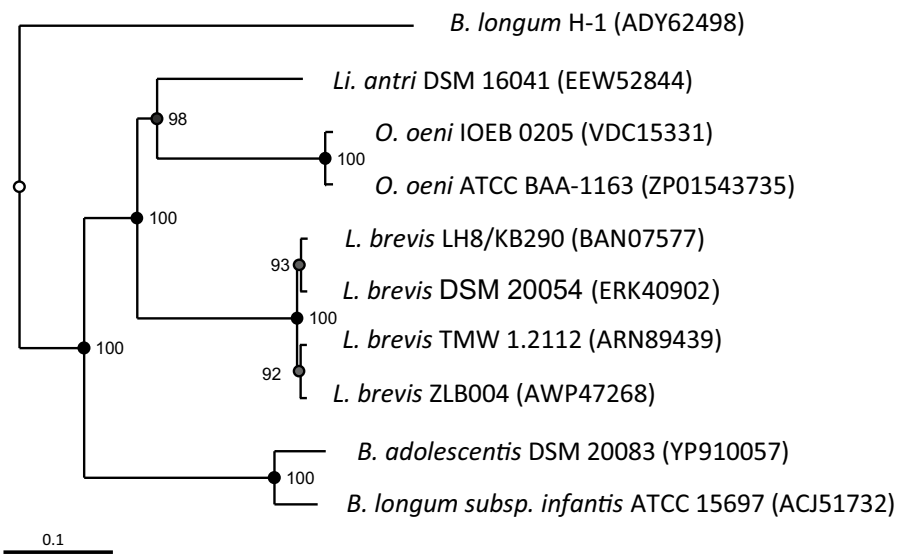

Fig. 1 Neighbour-joining tree of characterised GH 3 β-glucosidases of LAB. Amino acid sequences of *L. brevis* TMW 1.2112 (ARN89439), *L. brevis* LH8 (KB290) Bgy1 (BAN07577.1), *L. brevis* ZLB004 (AWP47268), *L. brevis* DSM 20,054 (ATCC 14,869) (ERK40902), *Li. antri* DSM 16,041 (EEW52844), *O. oeni* IOEB 0205 (VDC15331), *O. oeni* ATCC BAA-1163 (ZP_01543735), *B. adolescentis* DSM 20,083 (ATCC 15,703) (YP_910057), and *B. longum subsp. infantis* ATCC 15,697 (ACJ51732) were used for alignment and phylogenetic analysis with Bionumerics V7.6.2. Bootstrap values above 50% are shown on each node and were calculated from 1000 replications. *B. longum* H-1 (ADY62498) is used as an outgroup. The bar indicates 1% sequence divergence

spoiled wheat beer (TMW 1.2112) and kimchi (LH 8). Though only *L. brevis* TMW 1.2112 carry the *gtf2* gene for β-glucan formation (Fraunhofer et al. 2018a; Michlmayr et al. 2015; Quan et al. 2008). The Bifidobacteria and *Li.* antri DSM 16,041 were isolated from gastrointestinal tract of humans and *gtf2* negative (Mattarelli et al. 2008; Reuter 1963; Roos et al. 2005). The phylogenetic analysis revealed that GH 3 β-glucosidases appear in LAB of different origins not specifically related to EPS production ability of the strains. In past studies possible degradation of EPSs by glycoside hydrolases of LABs was observed as decreased EPS yields over fermentation and lowered viscosity e.g., by *Lacticaseibacillus rhamnosus* R (formerly *Lactobacillus rhamnosus* R (Zheng et al. 2020)) and *Streptococcus thermophilus* LY03 (Cerning et al. 1992; Degeest et al. 2002; Pham et al. 2000; Vuyst et al. 1998; Zannini et al. 2016). However, the lack of hydrolytic enzymes from EPS forming LABs associated with its degradation was also described (Badel et al. 2011; Patel et al. 2012).

Expression and purification of recombinant β-glucosidase

Within the sequence of *bgl*B no signal peptide sequence was predicted and only the stop codon was removed regarding Ni–NTA affinity purification via the poly-histidine tag coded within the expression vector. The sequence of *bgl*B was amplified by PCR and integrated into the expression vector pBAD/*Myc*-His and expressed in *E. coli* BL21. To maximize the protein yield, different inducing agent concentrations and inducing temperatures were tested and resulted an optimum concentration of 0.25% L-arabinose (v/v) at 15 °C overnight (García-Fraga et al. 2015; Sørensen et al. 2005). The intracellular formed enzyme was purified with Ni–NTA from the crude cell extract. The molecular mass of the enzyme was calculated via the amino acid sequence and resulted 83.5 kDa which corresponded with the bands of the elution fractions in SDS-PAGE gel stained with Coomassie (Fig. 2).

Substrate spectrum

Seven different pNP substrates were used analysing the specific enzyme activity at 37 °C within 2 h with a microtiter plate reader (Table 1). The results for BglB indicated specificities for β-D-linked glycosides.

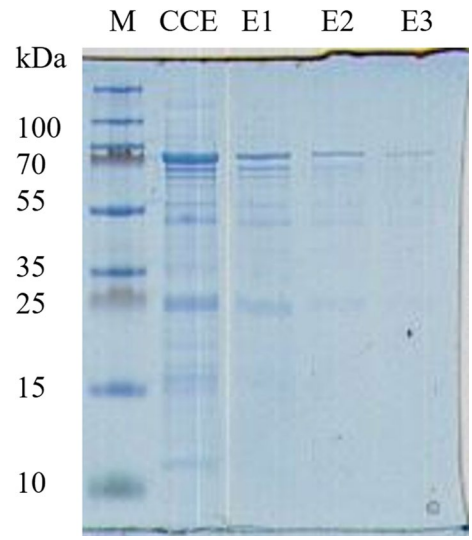

Fig. 2 Coomassie brilliant blue-stained SDS-PAGE from crude cell-free extract (CCE) and purified protein fractions eluted from the Ni–NTA resins after three (E1–E3) rounds of purification; M, molecular mass marker (kDa), as indicated on the left

Furthermore, the cell lysate of untransformed *E. coli* Bl21 was tested for enzymatic activity using the pNP substrates, which was negative i.a. for pNPβGlc. A significantly higher specificity of BglB was observed with pNPβGlc compared to the other substrates tested. This was confirmed by API® ZYM analyses resulting a strong colour change (≥ 40 nM substrate hydrolysed) and subsequently a positive enzyme reaction. However, a difference was observed for the β-galactosidase activity which was negative with the API® test and positive using pNPβGal. This might be associated with the different substrates type used in both analyse. The specificity of β-glucosidases for pNPβGlc is well described in several studies of different bacterial hosts (Chen et al. 2017; Fusco et al. 2018; Méndez-Líter et al. 2017; Michlmayr et al. 2010a, 2010b; Zhong et al. 2016a). Due to the high affinity of the enzyme to pNPβGlc this substrate was used in the following analysis.

Effects of temperature and pH on the enzyme activity and stability

The pH stability (Fig. 3A) of the recombinant β-glucosidase was analysed at a range of pH 4–11 and resulted in a hight stability at pH values between

Table 1 Substrate specificity of the GH 3 β-glucosidase from *L. brevis* TMW 1.2112. Values are means of biological duplicates including standard deviations

Enzymatic activity	Substrate	Hydrolytic activity	Relative activity [%]
β-glucosidase	pNPβGlc	+	100 ± 0.0
α-fucosidase	pNP α-L-fucopyranoside	−	0
β-fucosidase	pNP β-D-fucopyranoside	+	2.5 ± 0.2
α galactosidase	pNP α-D-galactopyranoside	−	0
β-galactosidase	pNP β-D-galactopyranoside	+[a]	1.4 ± 0.0
β-maltosidase	pNP β-D-maltoside	−	0
API® ZYM reaction			Activity 0–5
Phosphatase alkaline	2-naphthyl phosphate	+/−[a]	1
Esterase lipase (C 8)	2-naphthyl caprylate	−	0
Lipase (C 14)	2-naphthyl myristate	−	0
Leucine amino-peptidase	L-leucyl-2-naphthylamide	+/−[a]	1
Valine amino-peptidase	L-valyl-2-naphthylamide	+/−[a]	1
Cystine amino-peptidase	L-cystyl-2-naphthylamide	−	0
Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	−	0
Chymo-trypsin	N-glutamine-phenylalanine-2-naphthylamide	−	0
Phosphatase acid	2-naphthyl phosphate	+/−[a]	4
Phospho-amidase		+/−[a]	2
α-galactosidase	6-Br-2-naphthyl-α-D-galactopyranoside	−	0
β-galactosidase	2-naphthyl-β-D-galactopyranoside	−	0
β-glucuronidase	Naphthol-AS-Bl-β-D-glucuronide	+[a]	2
β-glucosidase	6-Br-2-naphthyl-β-D-glucopyranoside	+	5
β-glucosaminidase	1-naphthyl-N-acetyl-β-D-glucosaminide	−	0
α-mannosidase	6-Br-2-naphthyl-α-D-mannopyranoside	−	0
α-fucosidase	2-naphthyl-α-L-fucopyranoside	−	0

[a]Was additionally positive for *E. coli* BL21

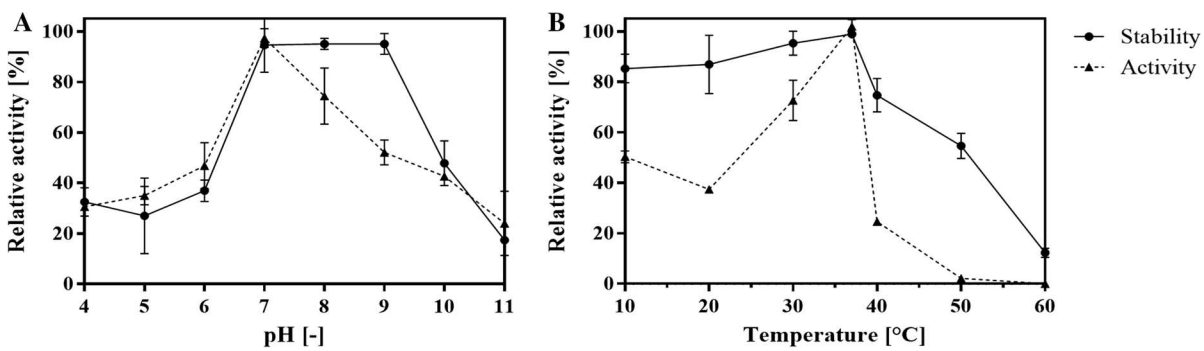

Fig. 3 **A** Effects of pH changes, **B** effects of temperature changes on enzyme stability and activity of the recombinant β-glucosidase. Values are means of triplicates including standard deviations

7 and 9 with ≥ 95% relative activities. Under acidic conditions (pH 4–6) the enzyme stability decreased and was < 40%, the stability values at pH 10 and 11 were similar. The optimum pH for enzyme activity was observed at pH 7. Next to the pH conditions, the enzyme stability at different temperatures (10–60 °C) was determined and displayed the maximum at 37 °C (Fig. 3B). Between 10 and 37 °C the relative activity was ≥ 80% decreasing to 12% at 60 °C. The temperature optimum for enzymatic activity was measured at 37 °C, temperatures above or below resulted only ≤ 40% relative activity. The described β-glucosidases of *L. brevis* SK3 and *L. brevis* LH8 showed optimal activities at pH 5.5 and 45 °C and pH 6–7 and 30 °C, respectively. Furthermore, characteristics of described GH 3 β-glucosidases including *O. oeni* species, Bifidobacteria and other LAB were compared (Table 2). Revealing that the temperature optima of β-glucosidases from *L. brevis* strains were in average lower compared to thermostable β-glucosidases of Bifidobacteria or *O. oeni* strains. In general, the pH optima ranged between 4.5 and 7 and temperature optima between 30 and 55 °C (Michlmayr et al. 2010b, 2015; Zhong et al. 2016b).

Kinetic parameters

The kinetic parameters of BglB were calculated by Lineweaver–Burk plot using pNPβGlc as substrate at various concentrations. The enzyme had a high affinity for the substrate revealed by a low K_m which was 0.22 mM. The maximal rate (V_{max}) was 77 µM · min^{-1}, k_{cat} was 59.58 s^{-1} and the catalytic efficiency (k_{cat}/K_m) was 8.3 · 10^3 s^{-1} mM^{-1}. The K_m value of the β-glucosidase from *L. brevis* SK3 measured with pNPβGlc was 0.22 mM (Michlmayr et al. 2010a). Further K_m values of GH 3 β-glucosidase (Table 2) from LABs ranged between 0.17 mM and 16 mM using pNPβGlc as substrate (Coulon et al. 1998; Sestelo et al. 2004).

Enzymatic hydrolysis of β-1,3-linked glucan by recombinant β-glucosidase

The motivation of this study was to characterise the carbohydrate active enzyme BglB of the β-1,3-linked glucan producing LAB *L. brevis* TMW 1.2112. The involvement of BglB in the degradation of cell-own EPS was additionally ivestigated. Three β-glucan isolates including cell-own β-glucan of *L. brevis* TMW 1.2112 were incubated with the purified recombinant enzyme and released D-glucose was quantified (Fig. 4). Curdlan a linear β-1,3-linked glucan resulted a negligible amount of free D-glucose after incubation with the enzyme which was rather a result of dissolving than enzymatic activity. Furthermore, since curdlan is insoluble in water, this could affect the availability of the polymer for enzymatic degradation (Koumoto et al. 2004; Zhang et al. 2014). Released D-glucose from β-glucan produced by *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 were significantly higher, however the D-glucose concentration were still low with a maximum of ~ 8 µg D-glucose · mL^{-1} (*L. brevis* TMW 1.2112 β-glucan). The solubility of the isolated bacterial β-glucans was likewise low which could be caused by the extraction conditions, structure, and degree of polymerization (Bohn et al. 1995; Havrlentova et al. 2011; Virkki et al. 2005). Furthermore, the purification process in some cases affects the structure integrity due to harsh chemicals and physical methods as used in this study e.g. ethanol precipitation, benchtop homogenizer, and freeze drying with subsequent resuspending (Goh et al. 2005). In addition, *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 synthesize likewise high-molecular weight β-glucans similar to that of *P. parvulus* 2.6R and *O. oeni* IOEB 0205, with molecular mass of 3.4 · 10^4 to 9.6 · 10^6 Da and 8.0 · 10^4 to ≥ 1 · 10^6 Da, respectively. (Ciezack et al. 2010; Dols-Lafargue et al. 2008; Werning et al. 2014). High-molecular β-1,3-linked glucan are described as insoluble in water (Bohn et al., 1995). Moreover, the degradation of β-glucan is more likely performed by more than one hydrolytic enzyme, especially as the characterized β-glucosidase (AZI09_02170) is an intracellularly expressed enzyme of *L. brevis* TMW 1.2112. Furthermore, in our previous study, we showed that the decrease in viscosity of *L. brevis* TMW 1.2112 culture broth could not be explained by the degradation of late expressed enzymes including BglB. However, the viscosity decrease indicated the degradation of high-molecular β-glucan which may have been caused by so far unknown enzymes of this strain (Bockwoldt et al. 2022).

According to the finding of this study and by the comparison of the GH 3 β-glucosidases from other LAB, BglB seemed to be an exo-active enzyme able to hydrolyse terminal, non-reducing β-D-glycosyl

Table 2 Properties of GH 3 β-glucosidases from lactic acid bacteria

Organism	K_m [mM][a]	V_{max} [µM min^{-1}]	k_{cat} [s^{-1}]	pH optimum [−]	Temp. optimum [°C]	Substrate spectra	Reference
L. brevis TMW 1.2112 *	0.22	77	60	7	37	pNPβGlc, pNPβFuc, pNPβGal, (further substrates are listed in Table 1)	This study
L. brevis SK3	0.22	n.d	n.d	5.5	45	pNPβGlc, pNP-β-D-xylopyranoside (pNPβXyl), pNP-α-L-arabinopyranoside (pNPαAra)	(Michlmayr et al. 2010a)
L. brevis ATCC 14,869 = DSM 20,054*	0.63	47	66	4.5	45	Cellobiose, Salicin, pNPβGlc, pNPβXyl, pNPαAra, n-Octyl-β-D-glucopyranoside, Deoxynivalenol-3-O-β-D-glucopyranoside, Nivalenol-3-O-β-D-glucopyranoside, HT-2-toxin-3-O-β-D-glucopyranoside	(Michlmayr et al. 2015)
L. brevis LH 8 Bgy1*	n.d	n.d	n.d	6	30	oNPβGlc, pNPβGlc	(Zhong et al. 2016a)
L. brevis LH 8 Bgy2	n.d	n.d	n.d	7	30	oNPβGlc, pNPβGlc	(Zhong et al. 2016b)
Limosilactobacillus antri DSM 16,041	n.d	n.d	n.d	6	45	pNPβGlc	(Kim et al. 2017)
Lacticaseibacillus casei ATCC 393	16	n.d	n.d	6.3	35	pNPβGlc, pNPαGlc, oNPβGlc, pNPβGal, Methyl-β-D-glucoside, Salicin, Prunassin, Cellobiose,	(Coulon et al. 1998)
O. oeni IOEB 0205 (UBOCC-A-315001)*	n.d	n.d	n.d	n.d	n.d	pNPβGlc, pNPαGlc, pNPβXyl, pNPαAra, p-nitrophenyl-α-L-rhamnopyranoside	(Gagné et al. 2011)
O. oeni ATCC BAA-1163*	0.17	n.d	n.d	5.5	45–50	pNPβGlc, pNPβXyl	(Michlmayr et al. 2010b)
O. oeni ST81	0.38	0.00521	n.d	5.0	40	pNPβGlc	(Mesas et al. 2012)
O. oeni 31MBR	1.05	0.00096	n.d	4.5–5	45	pNPβGlc	(Dong et al. 2014)
Bifidobacterium adolescentis DSM 20,083 (ATCC 15,703)*	0.32	0.00037	88	6.5	45	pNPβGlc, pNPβXyl	(Florindo et al. 2018)

Table 2 (continued)

Organism	K_m [mM][a]	V_{max} [µM min^{-1}]	k_{cat} [s^{-1}]	pH optimum [−]	Temp. optimum [°C]	Substrate spectra	Reference
Bifidobacterium adolescentis DSM 20,083 (ATCC 15,703)*	1.1	68	94	5.5	55	pNPβGlc, pNPβXyl, pNPαAra, pNPβGal, Cellobiose, Salicin, Quercetin-3-O-β-D-glucopyranoside n-Octyl-β-D-glucopyranoside Deoxynivalenol-3-O-β-D-glucopyranoside Nivalenol-3-O-β-D-glucopyranoside HT-2-toxin-3-O-β-D-glucopyranoside	(Michlmayr et al. 2015)
Bifidobacterium longum subsp. infantis ATCC 15,697*	0.27	n.d	24	6	30	pNPβGlc, pNPβXyl, pNPαAra	(Matsumoto et al. 2015)
Bifidobacterium longum H-1	0.83	57	n.d	5.5	35–37	pNPβGlc, Ginsenoside Rb1, Loganin, Arctin, Arbutin	(Jung et al. 2012)

Temp. = temperature, *n.d.* not determined

*Sources included in Neighbour-joining tree of characterized GH 3 β-glucosidases

[a]K_m was analysed using pNPβGlc, as substrate

residues of substrates. This restricted hydrolytic activity could be an explanation of the low released D-glucose amounts from β-glucan. Moreover, the β-glucosidase is most likely active on smaller carbohydrates and not high-molecular weight β-glucan. However, it might be involved to a later stage in polymer degradation e.g., after digestion with an endo-glucanase or if (partial) cell lysis occurs (Degeest et al. 2002; Pham et al. 2000). In preliminary experiments endo- and exo-glucanases of different origin (*Trichoderma* sp., and *Aspergillus oryzae*) further including a β-glucosidase from *Aspergillus niger* were used for the hydrolysis of the isolated bacterial β-glucan. Among others the GEM-assay (Danielson et al. 2010) was performed and resulted similar low D-glucose amounts after enzymatic digestion (data not shown) which again could be associated to the hurdles of β-glucan purification and resuspension.

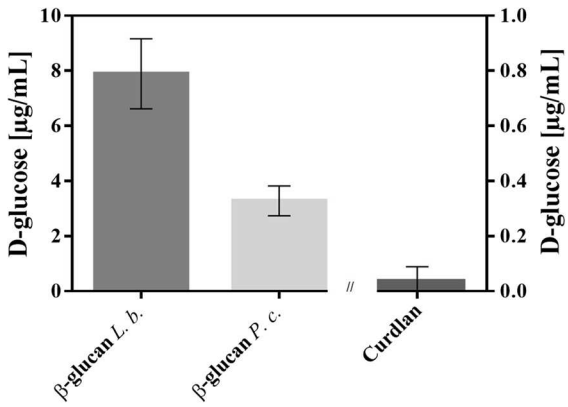

Fig. 4 β-glucans of 3 different sources (*L. brevis* (L. b.), *P. claussenii* (P. c.) and curdlan) were incubated with the recombinant β-glucosidase for 4 h at 37 °C, released D-glucose concentrations based on enzymatic determination. Values are means of triplicates including standard deviations

In conclusion, we have identified and characterised the β-glucosidase BglB of the beer spoiling and β-glucan forming *L. brevis* TMW 1.2112 with a molecular mass of 83.5 kDa which strongly favoured substrates with β-glycosidic bonds and is apparently an exo-active enzyme. Even though the start of β-glucan degradation was observed and might be in greater extent after a longer incubation period, the in vivo identification of involved enzymes in bacterial

β-glucan degradation e.g., by proteomic analysis is more favourable. Thus, the weak solubility of isolated β-glucan and feasible structural changes are eliminated and analysis of the enzymes activity under native conditions is enabled. However, it also looks like, given the phylogenetic analysis and characterization of GH 3 β-glucosidases from LABs, that this very enzyme family is not explicitly relevant to the EPS degradation.

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Declarations

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4.4 Proteomic analysis reveals enzymes for β-D-glucan formation and degradation in *Levilactobacillus brevis* TMW 1.2112

In the past, next to its formation, the degradation of β-glucan was characterized by a decrease in polymer concentration or viscosity. However, neither enzymes nor mechanisms responsible for its degradation have yet been identified. In general, the EPS biosynthesis by microorganisms is associated with, among others, biofilm formation, quorum sensing, protection from environmental effects, and energy storage compounds. The advantages of β-glucan biosynthesis are rather unknown, but the connections to the isolation sources of the producing LAB strains being alcoholic beverages already led to the suggestion that it has a protective function.

L. brevis. TMW 1.2112, previously reported as a stable β-glucan producer, was selected for analysis identifying degradative enzymes. This study focused on carbohydrate-active enzymes involved in both the formation and potential degradation of *O2*-substituted (1,3)-β-D-glucan including enzyme of the glycosyltransferase (GT) and glycoside hydrolase (GH) families. *L. brevis* TMW 1.2112 was cultured for 10 days and i.a. the β-glucan concentration, viscosity, and D-glucose content were analyzed, showing a decrease in viscosity but not in the β-glucan concentration. The growth parameters were also used for correlations with generated secretome and proteome data to identify enzymes or even whole pathways possibly involved in β-glucan biosynthesis and degradation. While several enzymes correlated with the formation of β-glucan, like maltose phosphorylase, the β-1,3-glucan synthases (GT2) were constantly expressed. Also, three maltose phosphorylase operons were identified and also expressed in the presence of maltose. In contrast, no enzymes could be clearly associated with the viscosity decrease; however, as a large part of proteins from the secretome were unknown, it cannot be ruled out that there are EPS-degrading enzymes among them. In addition, physical mechanisms such as pH changes or cell lysis could be associated with the viscosity decrease. Moreover, the analysis of the proteomic data revealed new possible research approaches regarding i) the capsule formation and binding to the cell surface by members of the LytR-Cps2A-Psr (LCP) protein family and ii) putative attachment or adhesion processes involving moonlighting genes which were discovered to be arranged in one operon. Moonlighting proteins of other lactobacilli were described i.a. to act in adhesion processes. In summary, it can be assumed that β-glucan is not used by the cells themselves as an energy store and probably has more of a protective function and/or is involved in attachment processes. The supplementary file (Figure S 3) of the publication is attached to the appendix in Chapter IV. Additional appendices are not listed here due to their extensive data size and can be found at the following address: https://www.mdpi.com/article/10.3390/ijms23063393/s1.

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Proteomic Analysis Reveals Enzymes for β-D-Glucan Formation and Degradation in *Levilactobacillus brevis* TMW 1.2112

Julia A. Bockwoldt [1], Chen Meng [2], Christina Ludwig [2], Michael Kupetz [3] and Matthias A. Ehrmann [1,*]

[1] Lehrstuhl für Mikrobiologie, Technische Universität München, 85354 Freising, Germany; julia.bockwoldt@tum.de
[2] Bayerisches Zentrum für Biomolekulare Massenspektrometrie (BayBioMS), Technische Universität München, 85354 Freising, Germany; chen.meng@tum.de (C.M.); tina.ludwig@tum.de (C.L.)
[3] Lehrstuhl für Brau- und Getränketechnologie, Technische Universität München, 85354 Freising, Germany; michael.kupetz@tum.de
* Correspondence: matthias.ehrmann@tum.de

Abstract: Bacterial exopolysaccharide (EPS) formation is crucial for biofilm formation, for protection against environmental factors, or as storage compounds. EPSs produced by lactic acid bacteria (LAB) are appropriate for applications in food fermentation or the pharmaceutical industry, yet the dynamics of formation and degradation thereof are poorly described. This study focuses on carbohydrate active enzymes, including glycosyl transferases (GT) and glycoside hydrolases (GH), and their roles in the formation and potential degradation of *O2*-substituted (1,3)-β-D-glucan of *Levilactobacillus* (*L.*) *brevis* TMW 1.2112. The fermentation broth of *L. brevis* TMW 1.2112 was analyzed for changes in viscosity, β-glucan, and D-glucose concentrations during the exponential, stationary, and early death phases. While the viscosity reached its maximum during the stationary phase and subsequently decreased, the β-glucan concentration only increased to a plateau. Results were correlated with secretome and proteome data to identify involved enzymes and pathways. The suggested pathway for β-glucan biosynthesis involved a β-1,3 glucan synthase (GT2) and enzymes from maltose phosphorylase (MP) operons. The decreased viscosity appeared to be associated with cell lysis as the β-glucan concentration did not decrease, most likely due to missing extracellular carbohydrate active enzymes. In addition, an operon was discovered containing known moonlighting genes, all of which were detected in both proteome and secretome samples.

Keywords: *Levilactobacillus brevis* TMW 1.2112; β-glucan; exopolysaccharide; glycosyltransferase; glycosyl hydrolase; moonlighting proteins; secretome; proteome

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1. Introduction

Exopolysaccharide (EPS) formation of lactic acid bacteria (LAB) has been massively studied for structural and sensory effects in the food industry and as drug delivery agents, bio-absorbents, and probiotics in the pharma industry [1–12]. The advantage of EPSs from LABs is that they are generally recognized as safe (GRAS) and could be used under in vitro or in vivo conditions [4]. EPSs are high-molecular-weight polymers, either secreted into the surrounding environment or acting as capsular polysaccharides attached to the cell surfaces (CPS) [13]. The EPSs produced by LAB are classified into homopolysaccharides (HoPSs) such as glucans and fructans, formed by repeating units of the same monosaccharide or heteropolysaccharides (HePS), which are mainly composed of D-glucose, D-galactose, and L-rhamnose. Most HoPSs (e.g., dextran, mutan, inulin, or levan) are polymerized extracellularly by glucansucrases or fructansucrases, whereas HePSs and β-glucans (consisting exclusively of D-glucose monomers) are formed intracellularly by transmembrane glycosyltransferases from nucleotide-activated sugars and released to the extracellular environment during polymerization [2,14–17]. The purposes of EPS and CPS formation are often described as protectors against biotic and abiotic stress (e.g., temperature, pH,

osmotic stress, or antimicrobial compounds) and as important agents in biofilm formation and cell–cell interaction [18–22].

On the contrary, although a decrease in EPS was observed in many studies by physical processes of the culture or enzymatic activity, the functions of EPS degradation by LAB remain unclear [23–29]. In general, the enzymatic hydrolysis of polysaccharides is performed by enzymes of the glycoside hydrolases (GH) families. GHs belong to the numerical classification EC 3.2.1.- and possess hydrolytic activity to glycosidic bonds of carbohydrates and non-carbohydrate fractions [30,31]. However, some EPS-producing LABs do not even possess hydrolytic enzymes enabling EPS degradation [4,32].

Levilactobacillus (*L.*) *brevis* TMW 1.2112 is a heterofermentative LAB isolated from spoiled beer due to capsular *O2*-substituted (1,3)-β-D-glucan formation [33,34]. *L. brevis* TMW 1.2112 was applied to in-situ-enriched EPS sourdough, to investigate the structural and sensory characteristics of the dough and the subsequent baked goods [35,36]. Furthermore, the physiological effects of isolated β-glucan from *L. brevis* TMW 1.2112 as well as plant-based and yeast β-glucans were analyzed in a comparative study [37]. Previous studies have described the polymerization of β-glucan by transmembrane glycosyltransferase-2 (Gtf-2) family members called β-1,3 glucan synthases (GT2), e.g., in *Pediococcus* (*Pe.*) *parvulus*, *Pediococcus* (*Pe.*) *claussenii*, *Paucilactobacillus* (*Pa.*) *suebicus*, and *L. brevis* sp. [35,38–43]. The Gtf-2 enzymes belong to the numerical classification EC 2.4.-, which comprises several glycosyl transferases (GTs). Enzymes of this class catalyze the transfer of sugar moieties from activated donor molecules to acceptor molecules, resulting in the formation of glycosidic bonds [44,45]. Moreover, Fraunhofer et al. (2018) postulated a putative pathway for β-glucan biosynthesis by *L. brevis* TMW 1.2112 through the genome sequence [46].

Homologies to the β-glucan and Gtf-2 of LAB were observed for the β-1,3 β-1,2 glucan capsule and glycosyltransferase Tts branched from *Streptococcus* (*S.*) *pneumoniae* 37 [47]. An immunoagglutination test using *S. pneumoniae* serotype 37 antibodies enables the identification of β-glucan capsules of LAB and was positive for *L. brevis* TMW 1.2112 [34], *Pe. claussenii* TMW 2.340 [35], *Oenococcus oeni* [42], and *Pediococcus damnosus* [48]. However, proteins involved in the attachment of the β-1,3-glucan capsule to the cell surface of LAB are so far undescribed. It has been discussed that the LytR-Cps2A-Psr (LCP) protein family and the Wzy pathways could be involved in polysaccharide attachment to the peptidoglycan of Gram-positive bacteria [49–53]. Still, the knowledge about LCPs in Lactobacilli is limited. Furthermore, moonlighting proteins are known to overtake multiple functions based on their cellular position, e.g., moonlighting proteins from commensal lactobacilli were described as acting in adhesion processes [54–57]. LCP and moonlighting proteins might interact with the CPS of *L. brevis* TMW 1.2112 regarding attachment and adhesion processes, but such phenomena have not yet been described.

Although a decrease in the β-glucan amount and viscosity effects in sourdough and culture broth fermented by *L. brevis* TMW 1.2112 was observed, the responsible factors are unknown [34,36]. To avoid weak solubility and possible structural changes due to the multistage isolation processes of isolated β-glucan, the in vivo expression of GTs and GHs during fermentation of *L. brevis* TMW 1.2112 can be studied by proteomic analysis [58,59]. Consequently, we aimed to identify the enzymes involved in β-glucan formation and the subsequent presumed degradation by the regulation of differentially expressed proteins during the exponential, stationary, and early death phases. We hypothesize that the observed changes in viscosity and β-glucan concentration are reflected in the proteome and correlate with the presence of relevant enzymes (GTs, GHs, and β-glucan biosynthesis). This study revealed a pathway for the biosynthesis of (1,3)-β-D-glucan of *L. brevis* TMW 1.2112 and demonstrated a lack of enzymatic activity for the polymer utilization as energy sources.

2. Results

2.1. Growth Characteristics of L. brevis TMW 1.2112 and β-Glucan Content in Culture Broth

L. brevis TMW 1.2112 was cultivated in a chemically defined medium (CDM) for 10 days. During the fermentation process, growth parameters, e.g., cell count, pH value,

viscosity, and the amount of β-glucan and D-glucose, were analyzed. After inoculation, the cell growth entered the exponential phase immediately and reached the stationary phase within 24 h (Figure 1A). During fermentation, the cell count increased with its maximum after 3 days with 5.5×10^8 cfu (colony forming units)/mL. After 5 days, the cell count decreased to 4.8×10^8 cfu/mL. The pH value decreased from the initial pH 6.2 to 5.1 after 24 h and to 3.7 after 7 days. Values of the optical density (OD) increased within 2 days in fermentation to 1.5 and was 1.7 after 10 days.

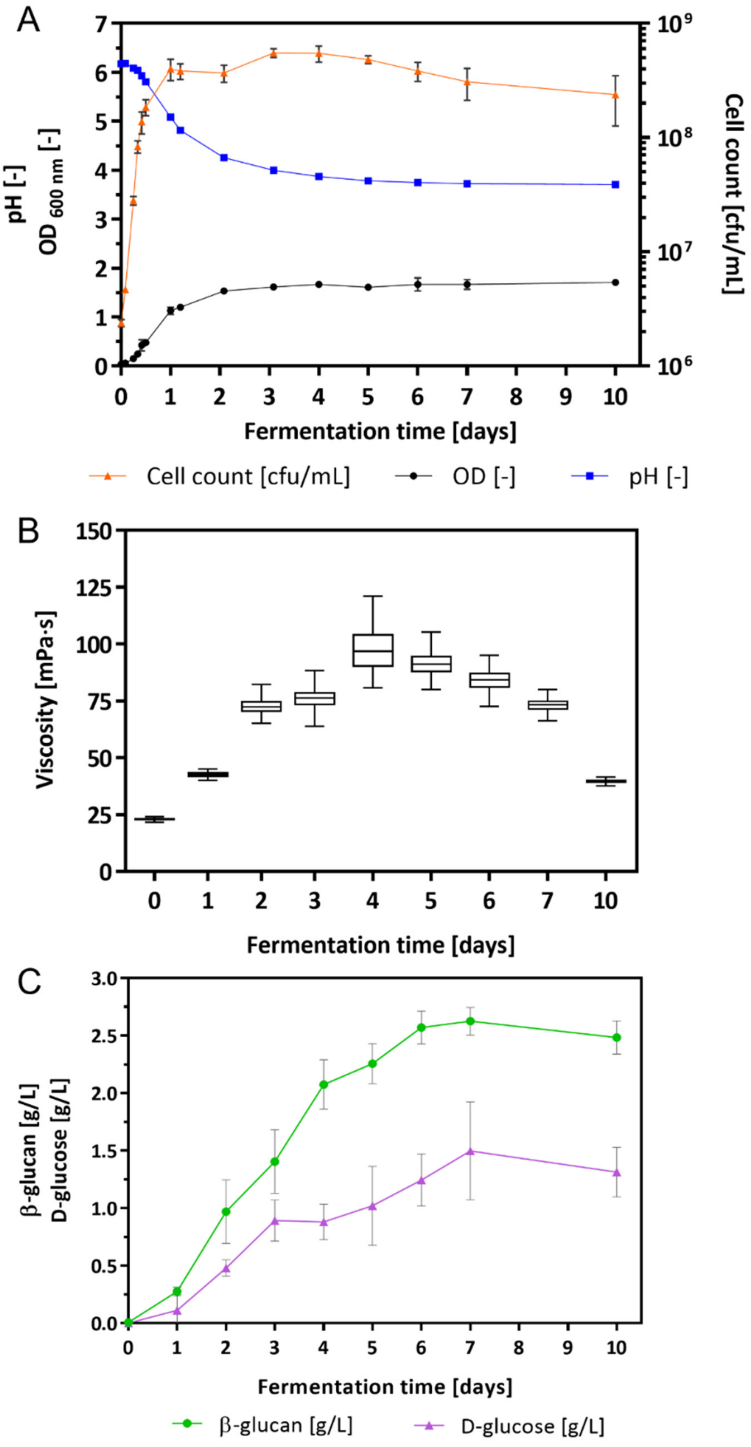

Figure 1. Growth characteristics of the fermentation broth: (**A**) cell count in cfu/mL, OD, and pH, (**B**) changes in the viscosity, and (**C**) β-glucan and D-glucose concentration in culture supernatants. Values are mean values of four-fold biological replicates including standard deviations.

The changes in viscosity were determined by a rotational viscometer once per day (Figure 1B). After inoculation of the CDM with *L. brevis* TMW 1.2112, the viscosity of the culture broth was 22.8 ± 0.9 mPa·s increasing continuously to 97.2 ± 26.4 mPa·s within 4 days. An increase in viscosity resulted in an increase in variance because of the heterogeneous viscoelastic characteristic of the culture broth. With progress in fermentation the viscosity values and the corresponding variances decreased again to finally 39.5 ± 2.5 mPa·s. Within 10 days the viscosity of the culture broth increased significantly (until 4 days) and subsequently approached towards the initial value.

In addition, the amount of β-glucan and D-glucose (monomeric units of β-glucan) was measured by an immunological and an enzymatical method (Figure 1C). The initial concentrations of both compounds were 0.00 g/L in the supernatant. Within 4 days, the β-glucan concentration was 2.08 ± 0.19 g/L and increased further to 2.63 ± 0.10 g/L within 7 days. A similar trend was observed for the D-glucose with an increase in the concentration after 4 and 7 days, resulting in 0.88 ± 0.13 g/L and 1.50 ± 0.37 g/L, respectively. After 4 days, the increase in the β-glucan became slower, and the accumulation of D-glucose slowed down after 3 days. At the end of fermentation (10 days), the concentrations of both compounds declined marginally to 2.48 ± 0.13 g/L (β-glucan) and 1.31 ± 0.19 g/L (D-glucose). The chemically defined medium with only maltose as a carbon source was used as a blank for both assays.

2.2. Glycosyl Transferases (GT) and Glycoside Hydrolases (GH) in L. brevis TMW 1.2112

The genome of *L. brevis* TMW 1.2112 c (GenBank accession No.: CP016797) [34,60] was additionally annotated by RAST and eggNOG-Mapper for clusters of orthologous groups (COG) and functionality, resulting in 2184 annotated proteins [61–63]. The genome sequence comprised 49 glycosyltransferases (GT; EC 2.4.-) and glycoside hydrolases (GH; EC 3.2.1.-), which were identified and characterized by several databases (NCBI BLASTx, UniProt, CAZy (Carbohydrate-Active enZYmes) and eggNOG-Mapper) for their protein classification and molecular functions (Table 1) [30,61–65]. SignalP-5.0 predicted six of the 49 annotated GHs and GTs as secreted enzymes and mostly were associated with cell wall biosynthesis [66]. Of note, the endo-β-1,3-glucanase (AZI09_02135), β-1,3-glucosidase BglB (AZI09_02170), and Gtf-2 proteins (AZI09_03685, AZI09_12985, AZI09_07565, AZI09_06585, AZI09_04045, AZI09_12875, and AZI09_10605) are enzymes potentially involved in the formation and degradation of β-glucan [15,41]. The transmembrane Gtf-2 (AZI09_12770), which is encoded on the plasmid pl12112-4 (GenBank accession No.: CP016801) of *L. brevis* TMW 1.2112, was considered particularly relevant [38,40].

2.3. Proteomic Analysis

2.3.1. Secretome: Protein Secretion from Exponential to Early Death Phases

Considering that β-glucan is an extracellular EPS and that its degradation is most likely initiated in the extracellular environment by GHs, we measured the secretome of *L. brevis* TMW 1.2112 in the exponential phase (8 h) and at the end of fermentation (7 days) to study the functions and roles of proteins associated with EPS degradation. We identified more proteins by their gene locus IDs at the end of fermentation (307 detected proteins) compared to the exponential phase (50 proteins detected). The proteins reproducibly detected in at least three out of four replicates were retained in the downstream analysis. The in-silico analysis using SignalP-5.0 predicted 199 secreted proteins, including endo-β-1,3-glucanase (AZI09_02135). Figure 2 shows the cluster of orthologous groups (COG) and functional characterization of the in-silico secretome and both time points (8 h and 7 days). Energy production and conversion, carbohydrate metabolism, and coenzyme metabolism generated larger shares within the analyzed samples compared to the in silico secretome, and the share of proteins with unknown function was reduced at the same time. Proteins of the carbohydrate metabolism group made up 13% during the exponential phase and only 7% during the death phase. Approximately 25% of the detected proteins in both samples were previously assigned as hypothetically secreted proteins.

Table 1. GTs (EC 2.4.-) and GHs (EC 3.2.1.-) enzymes within the genome sequence of *L. brevis* TMW 1.2112 (CP016797).

#	Description	CAZy	EC No.	Gene Locus	Protein Accession Number
1	polysaccharide biosynthesis protein	GT	2.4.1.-.	AZI09_03705	A0A1W6N8G4
2	glycosyltransferase family 2	GT2	2.4.1.-.	AZI09_03685	A0A0C1PWD9
3	glycosyltransferase family 2	GT2	2.4.1.-.	AZI09_12985 [a]	-
4	glycosyltransferase family 2	GT2	2.4.1.-.	AZI09_07565	A0A1W6NA30
5	glycosyltransferase family 2	GT2	2.4.1.-.	AZI09_06585	A0A1W6N9Q8
6	glycosyltransferase family 2	GT2	2.4.1.-.	AZI09_04045	A0A1W6N8N8
7	glycosyltransferase family 2	GT2	2.4.1.-.	AZI09_12770	A0A1W6NCZ8
8	glycosyltransferase family 2	GT2	2.4.1.-.	AZI09_12875	Q6I7K0
9	glycosyltransferase family 2 (*ykoT*)	GT2	2.4.1.-.	AZI09_10605	A0A1W6NC01
10	exosortase G system-associated	GT2	2.4.1.-.	AZI09_06670	A0A1W6N9Q3
11	glycosyltransferase family 8	GT8	2.4.1.-.	AZI09_12575	A0A1W6NCV8
12	nucleotide-diphospho-sugar transferases superfamily	GT8	2.4.1.-.	AZI09_12410	A0A1W6NCU7
13	glycosyltransferase family 1	GT1	2.4.1.52	AZI09_12995	A0A1W6NDG0
14	poly(glycerol-phosphate) α-glucosyltransferase	GT1	2.4.1.52	AZI09_04905	A0A1W6N8Q2
15	poly(glycerol-phosphate) α-glucosyltransferase	GT1	2.4.1.52	AZI09_04910	A0A1W6N980
16	poly(glycerol-phosphate) α-glucosyltransferase	GT1	2.4.1.52	AZI09_04920	A0A1W6NCH6
17	poly(glycerol-phosphate) α-glucosyltransferase	GT1	2.4.1.52	AZI09_04940	A0A1W6N8Q6
18	poly(glycerol-phosphate) α-glucosyltransferase	GT1	2.4.1.52	AZI09_04945	A0A1W6N8W8
19	UDP glucose-poly(glycerol-phosphate) α-glucosyltransferase (*tagE_6*)	GT1	2.4.1.52	AZI09_04950	A0A1W6N952
20	maltose phosphorylase	GH65	2.4.1.8	AZI09_04670	A0A1W6N8P0
21	maltose phosphorylase	GH65	2.4.1.8	AZI09_01010	A0A1W6N6Y3
22	maltose phosphorylase	GH65	2.4.1.8	AZI09_10320	-
23	endo-β-1,3-glucanase	GH8	3.2.1.-	AZI09_02135 [a]	-
24	glycosyl hydrolase family 18	GH18	3.2.1.14	AZI09_03025 [a]	A0A1W6NI26
25	glycosyl hydrolase family 88	GH88	3.2.1.172	AZI09_11545	A0A1W6NC28
26	glycosyl hydrolase family 31	GH31	3.2.1.177	AZI09_02865	A0A1W6N888
27	α-xylosidase	GH31	3.2.1.177	AZI09_09820	A0A1W6NBL2
28	glucohydrolase	GH13	3.2.1.10	AZI09_00950	A0A1W6N750
29	glucohydrolase (*malL_2*)	GH13	3.2.1.10	AZI09_10575	A0A1W6NBX6
30	α-glucosidase	GH31	3.2.1.20	AZI09_08630	A0A1W6NAK3
31	α-glucosidase	GH31	3.2.1.20	AZI09_11465	A0A1W6NBZ8
32	α-glucosidase	GH31	3.2.1.20	AZI09_12510	A0A1W6NCT8
33	β-1,3-glucosidase (*bglB*)	GH3	3.2.1.21	AZI09_02170	A0A1W6N7S3
34	xylan 1,4-β-xylosidase	GH39	3.2.1.37	AZI09_11985	A0A1W6NC86
35	β-xylosidase (*xynB*)	GH43	3.2.1.37	AZI09_11935	A0A1W6NCN2
36	glucosylceramidase	GH30	3.2.1.45	AZI09_00755	A0A1W6N733
37	α-L-arabinofuranosidase 1	GH51	3.2.1.55	AZI09_03165	A0A1W6N7 × 2
38	α-N-arabinofuranosidase (*abfA_1*)	GH51	3.2.1.55	AZI09_00785	A0A1W6N6T5
39	6-phospho-β-glucosidase	GH1	3.2.1.86	AZI09_09805	A0A1W6NB21
	GHs and GTs Associated with Cell Wall Biosynthesis				
40	DD-transpeptidase	GT51	3.4.16.4	AZI09_04800	A0A1W6N956
41	penicillin-binding protein	GT51	2.4.1.129	AZI09_06425	A0A1W6N9H6
42	N-acetylglucosaminyldiphosphoundecaprenol N-acetyl-beta-D-mannosaminyltransferase	GT26	2.4.1.187	AZI09_09370	A0A1W6NAY4
43	UDP-N-acetyl-D-mannosamine transferase	GT26	2.4.1.187	AZI09_03665	A0A1X0XQ74
44	poly(glycerol-phosphate) α-glucosyltransferase	GT1	2.4.1.208	AZI09_04170	A0A1W6N8F4
45	1,2-diacylglycerol 3-glycosyltransferase (*pimA*)	GT1	2.4.1.337	AZI09_04165	A0A1W6N8G5
46	N-acetylglucosaminyltransferase (*murG*)	GT28	2.4.1.227	AZI09_05030	A0A1W6N9A0
47	N-acetylmuramide glycanhydrolase	GH25	3.2.1.-	AZI09_10600 [a]	A0A1W6NBH8
48	N-acetylmuramoyl-L-alanine amidase	GH73	3.2.1.96	AZI09_04775 [a]	A0A1W6N923
49	N-acetylmuramoyl-L-alanine amidase (*atl_1*)	GH73	3.2.1.96	AZI09_02505 [a]	A0A1W6N829

[a] Putative signal peptide proteins.

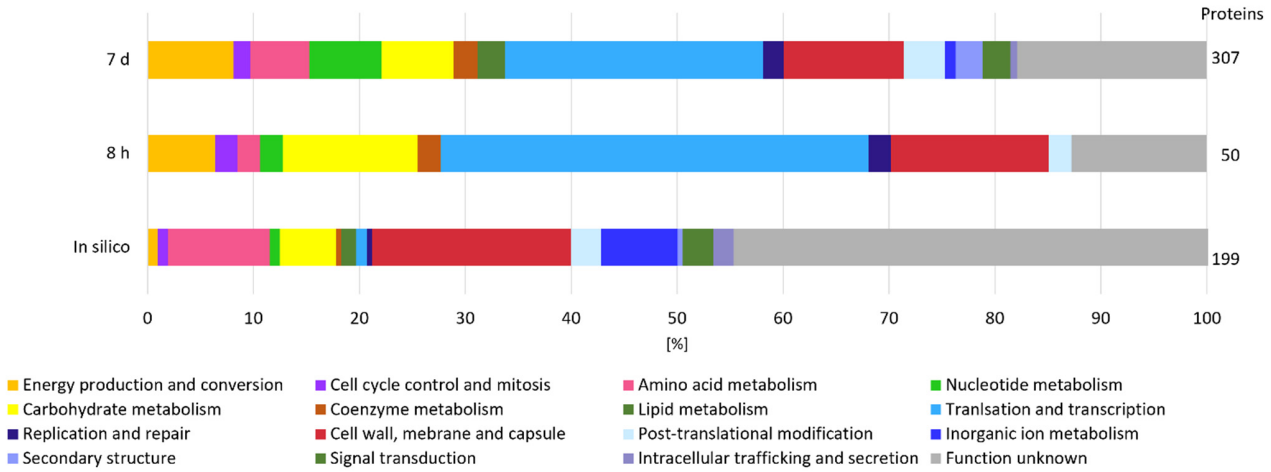

Figure 2. Secretome analysis by COG classification. The in silico analyzed secretome was compared with samples from the beginning (8 h) and end (7 days) of fermentation of at least three out of four replicates. On the right, the total numbers of identified gene locus IDs are stated.

A Student *t*-test was used to identify proteins that were differentially secreted between 8 h and 7 days (Figure 3). In total, 8 GHs and GTs were detected in our experiment, including two GH65 family proteins (AZI09_10320 and AZI09_04670) maltose phosphorylases (hereinafter referred as MP), two GH73 family proteins N-acetylmuramoyl-L-alanine amidase (AZI09_02505 and AZI09_04775), the GT51 family protein penicillin-binding protein (AZI09_06425), two GT8 family proteins (AZI09_12410 and AZI09_12575), and GH31 family protein α-glucosidase (AZI09_12510). Enzymes of the GT8 family have been described as acting as nucleotide-diphospho-sugar glycosyltransferases [67]. The endo-β-1,3-glucanase (AZI09_02135), which was assumed to be involved in β-glucan degradation, was not detected. All GHs and GTs were detected after 7 days, except GH73 N-acetylmuramoyl-L-alanine amidase (AZI09_04775), which was also present after 8 h.

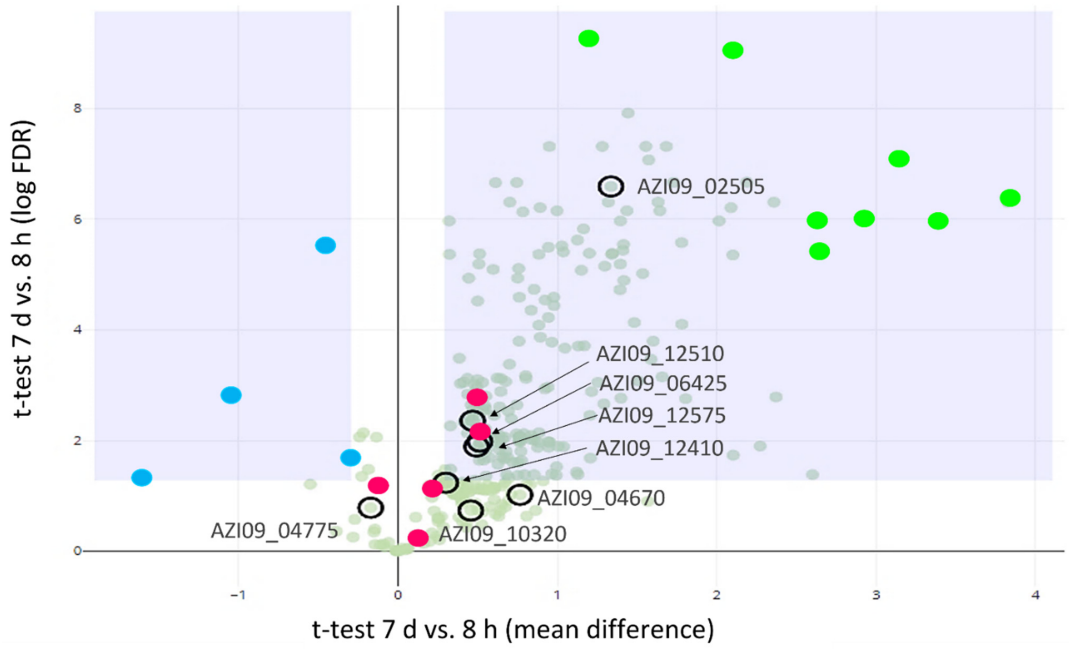

Figure 3. Proteomics analysis of secreted proteins. Volcano plot for the differential abundance analysis of 8 h vs. 7 d secretomes: GHs and GTs (**encircled**), proteins with higher abundance at 8 h (**blue dots**), proteins with higher abundance at 7 days (**light green dots**), and potential moonlighting proteins (**red dots**).

The secretion of four proteins were higher after 8 h compared to 7 days. Those four proteins were annotated as phosphoketolase (AZI09_09710), NlpC P60 family (AZI09_02850 and AZI09_01220), and LSU ribosomal protein L3p (L3e) (AZI09_03445) (Figure 3, blue dots). After 7 days, the number of secreted proteins increased significantly, including the ABC transporters (AZI09_01955 and AZI09_00135), ribosomal proteins (AZI09_03485 and AZI09_05315), and uncharacterized proteins (AZI09_10715 and AZI09_11665) (Figure 3, green dots).

Moreover, several proteins, which were previously described as moonlighting proteins in bacteria were detected, such as enolase (AZI09_08765), triose-phosphat isomerase (TPI) (AZI09_08770), phosphoglycerate kinase (PGK) (AZI09_08775), glyceraldehyde-3-phosphate dehydrogenase (GADPH) (AZI09_08780), and elongation factor (EF) Tu (AZI09_05335). Those proteins were detected within both samples, except TPI, which was only detected after 7 days (Figure 3, red dots) [68].

Additionally, the secretome was analyzed for proteins involved in β-glucan biosynthesis and proteins associated with polysaccharide capsules and stress response. This resulted in the detection of β-phosphoglucomutase (β-PGM) (AZI09_04665), MFS maltose transporter, MalT (AZI09_10325), UTP–glucose-1-phosphate uridylyltransferase (UGP) (AZI09_08865), and glucokinase (AZI09_07205) after 7 days. The previous named enzymes are members of the postulated pathway for β-glucan formation by *L. brevis* TMW 1.2112 [46]. According to capsular polysaccharide attachment, two proteins of LytR-CpsA-Psr (AZI09_03640 and AZI09_03715) were detected after 7 days [49–52]. An arginine deiminase (ADI) (AZI09_01860) was present after 8 h and 7 days in the secretome. Several proteins identified by the secretome analysis are common intracellular proteins. After 7 days, the number increased, which could be due to cell lysis according to the growth phase.

The number of uncharacterized proteins accounted for 47% of the identified secretome, by only two proteins of the secretome after 8 h (Figure S1). The top ten proteins of the 8 h secretome-comprised cell wall and the cell-surface-related proteins were expressed. Further, only AZI09_01220, which codes for N1pC/P60 proteins, a family of cell-wall peptidases, was present within the 8 h sample, but not after 7 days. The highest expression (iBAQ intensity [69]) within both secretome samples was an uncharacterized protein (AZI09_12405) with a molecular weight (MW) of 49.9 kDa. Bioinformatic characterization (SWISSMODEL and PredictProtein) showed that this protein might be a cell wall or a surface layer protein [70,71]. Moreover, after 7 days in fermentation, the protein with the second highest expression was an ABC transporter binding protein (AZI09_01995) with an MW of 25.1 kDa and a putative signal peptide.

2.3.2. Cell Lysate: Changes in Protein Expression over Fermentation Time

To examine how protein expression changes during fermentation, we measured the proteome of *L. brevis* TMW 1.2112 at five time points during fermentation, in the exponential (8 h), stationary (24 h to 4 d), and early death phases (7 d). *L. brevis* TMW 1.2112 genome contains 2537 predicted protein coding genes, and 1641 proteins were identified by proteomic analysis. This corresponds to a proteome coverage of approximately 65%, which is in the range of other label-free quantitative proteomic analyses of LABs [72,73]. Principal component analysis (PCA) revealed a high similarity between biological replicates (Figure 4A). The samples of 4 days and 7 days in fermentation clustered together, while the sample of 8 h was the most distinguishable from other time points. To continue the global comparison of the five time points, a heat map was generated, and the samples were hierarchically clustered using Pearson correlation distance (Figure 4B). Most differences were obtained for the 8 h time point. The clusters of 7 days and 4 days were highly similar.

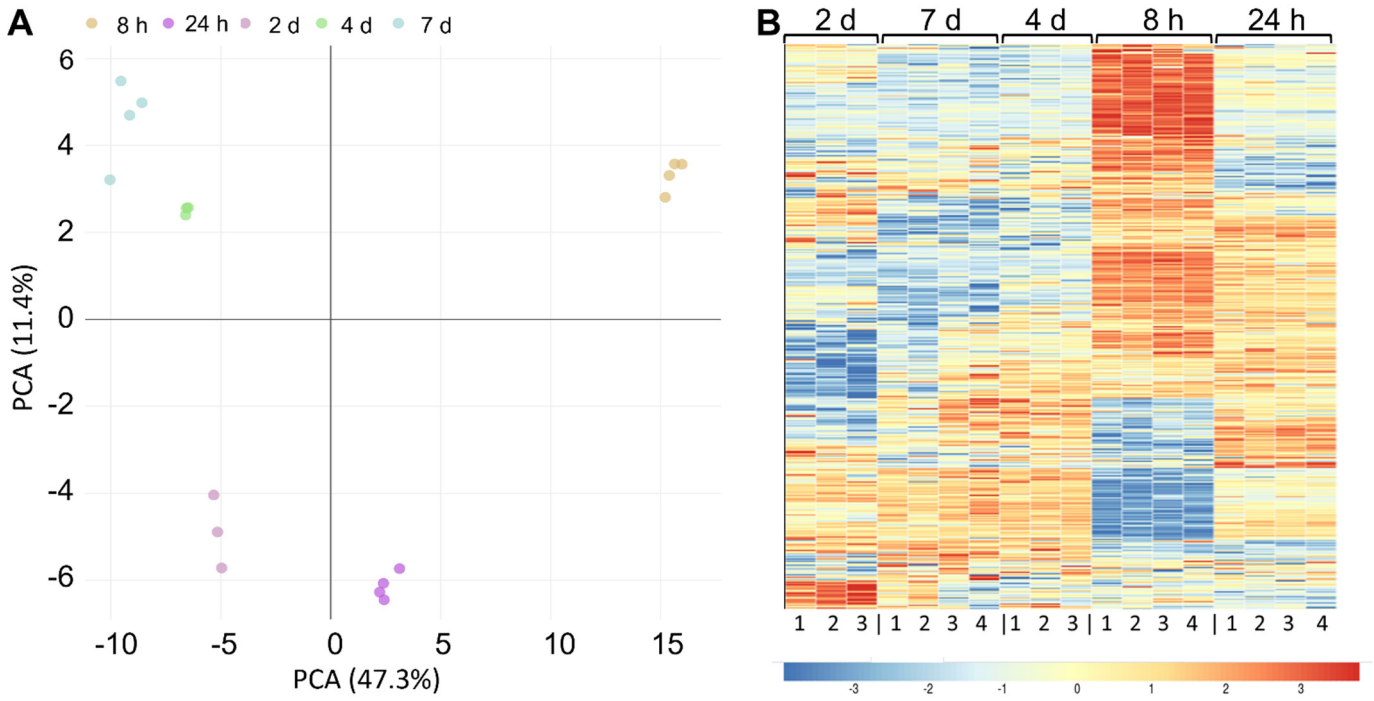

Figure 4. Proteomics analysis of cellular proteins over fermentation. (**A**) Principal component analysis (PCA) of the five fermentation time points. (**B**) Heat map using Pearson correlation displaying the protein abundance at five different time points (8 h, 24 h, 2 days, 3 days, and 7 days) after the hierarchical clustering of 1641 proteins. Three- to four-fold biological replicates were used for the analysis. The changes in enzyme expression are depicted by color intensity, as indicated below the figure.

The proteome analyzed in silico counted 2184 annotated proteins, which were used for COG and functional characterization (Figure 5). COG prediction of the in silico proteome showed a pattern similar to that of the expressed proteins at the five time points. A total of 30% of the expressed proteins could not be categorized (unknown function). Between the five time points (8 h, 24 h, 2 days, 3 days, and 7 days), no significant difference was observed according to the distribution of the groups. The COG analyses of the expressed proteins accounted for translation and transcription ~20% each: ~7% were related to carbohydrate metabolism and approximately 5% were clustered for energy production and conversion. Proteins of the last two groups are considered to be related to polysaccharide formation and degradation, among others.

Fraunhofer, Jakob, and Vogel (2018) postulated a putative pathway for the β-glucan biosynthesis based on analyses of the *L. brevis* TMW 1.2112 genome sequence. The β-glucan biosynthesis from maltose as a substrate included a transporter for maltose, MP, glucokinase, phosphoglucomutase, UGP, UDP kinase or nucleoside-diphosphate kinase (NDPK), and Gtf-2 [46]. Several pathway members have been measured in our proteomics data, e.g., MalT (transporter) (AZI09_10325), MPs (AZI09_04670 and AZI09_10320), glucokinase (AZI09_07205), β-PGM (AZI09_04665 and AZI09_02330), UGP (AZI09_08865), NDPK (AZI09_05450), and Gtf-2 family proteins (AZI09_04045, AZI09_12875, and AZI09_12770). According to β-glucan degradation, β-1,3-glucosidase BglB (AZI09_02170) was detected during the whole fermentation. The endo-β-1,3-glucanase (AZI09_02135) was only expressed at 4 days (late stationary phase). Genes associated with polysaccharide encapsulation, stress response, and moonlighting proteins as previously described with respect to the secretome were also present within the cell lysate samples.

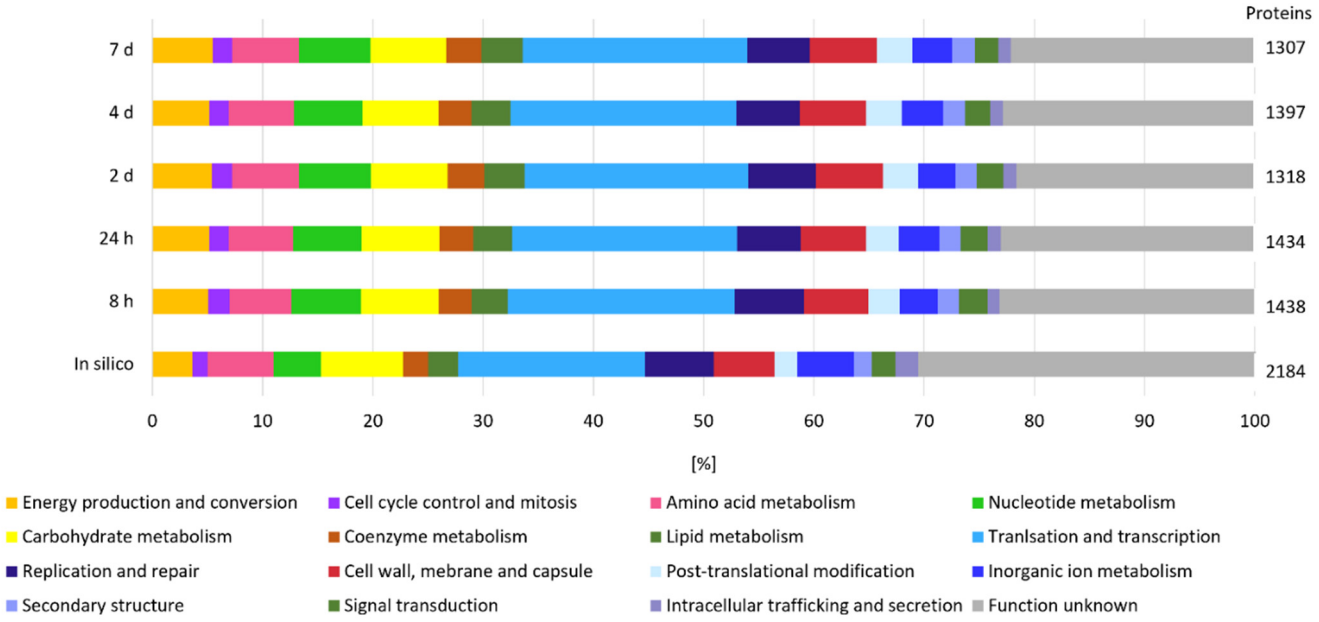

Figure 5. Cellular proteome analysis by COG classification. The in silico proteome was compared at five time points (8 h, 24 h, 2 days, 3 days, and 7 days) in at least three out of four or two out of three replicates.

2.3.3. Cell Lysate: Expression of GHs and GTs and Their Correlation with Viscosity, β-Glucan, and D-Glucose Concentrations

In total, 32 of the previously described 49 GHs and GTs were detected in our proteomic data. We manually assigned these proteins into three clusters according to their expression over time (Figure 6A).

The first cluster (orange cluster, Figure 6A) included enzymes that were increased during the early growth phase and decreased in the further course of fermentation. The second cluster (purple cluster, Figure 6A) contained enzymes with higher expressions during the early growth phase and/or the beginning of the stationary phase. The third cluster (red cluster, Figure 6A) represented enzymes that increased towards the end of fermentation. Additionally, the correlation between protein expression and viscosity, β-glucan, and D-glucose concentration were examined (Figure 6B). During exponential and the early stationary phase, the GT1 family protein 1,2-diacylglycerol 3-glycosyltransferase, *pimA* (AZI09_04165) was more abundant compared to the late stationary and death phases, which also resulted in an inverse correlation with changes in viscosity (Figure 6B), β-glucan, and D-glucose. Members of this enzyme family catalyze the transfer of sugar moieties from nucleotide-sugar donors to membrane-associated acceptor substrates and are involved in plasma membrane synthesis [74]. Gtf-2 family proteins AZI09_12875 and AZI09_04045 were assigned to the second cluster, and Gtf-2 AZI09_12770 was grouped into the third cluster. The expression of the two MPs (GH65) AZI09_04670 and AZI09_10320 increased with progress in fermentation (8 h to 7 days). However, only the MP AZI09_04670 correlated strongly ($r \geq 0.8$) with changes in viscosity and D-glucose concentration, while MP AZI09_10320 showed no correlations [75]. Furthermore, the β-1,3-glucosidase (AZI09_02170) as a member of the GH3 family was expressed on a constant level after 24 h, with medium to low correlations with the growth characteristics. The GH8 family protein endo-β-1,3-glucanase (AZI09_02135) was only expressed after 4 days when the viscosity of the fermentation broth decreased continuously (Figure 1).

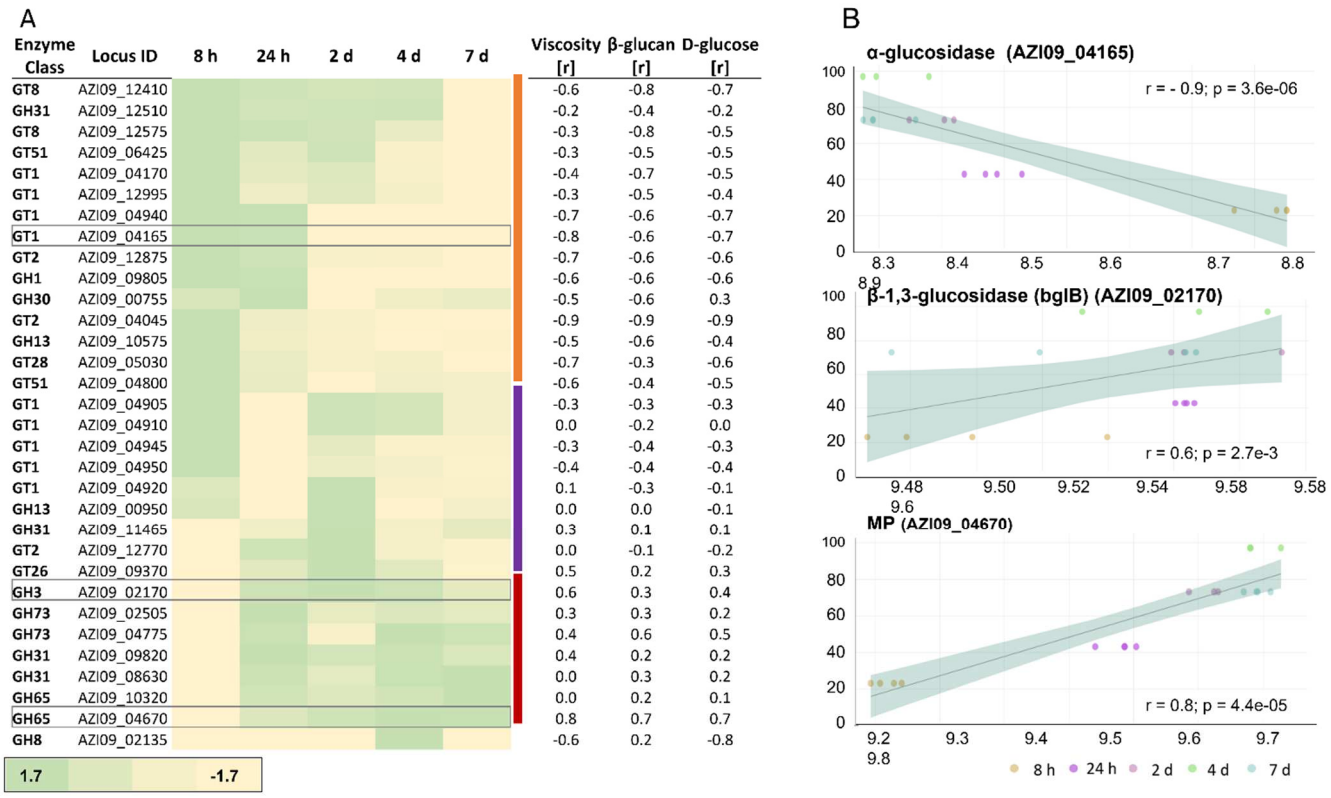

Figure 6. Expression of GHs and GTs in the cell lysate. (**A**) Heat map of the expressed enzymes over time. The three clusters of proteins are indicated by the color bar on the right (orange, purple, and red). Relative fold change of protein expression compared to the mean depicted by color intensity, as stated below the figure (a range between −1.7-fold and 1.7-fold). Correlation coefficient values with viscosity, β-glucan, and D-glucose are listed next to the specific GHs and GTs. (**B**) The correlation of the expression of α-glucosidase AZI09_04165, β-1,3-glucosidase (bglB) AZI09_02170, and the MP AZI09_04670 with viscosity, including correlation coefficient values (r) and *p*-values (*p*).

2.3.4. Correlation between Protein Expression and Growth Characteristics

In addition to the correlations of GHs and GTs described in the previous section, the expression patterns of all other proteins were correlated with viscosity, β-glucan, and D-glucose to reveal potential novel proteins with a function related to β-glucan formation or degradation. Proteins of the pathway for β-glucan biosynthesis resulted in moderate to strong correlations ($0.6 \leq r \leq 0.8$), e.g., β-PGM (AZI09_04665) and NDPK (AZI09_05450), with all growth characteristics. The sequence of MP (AZI09_04670) is encoded downstream to β-PGM and showed similar correlation coefficients. Moonlighting-associated proteins, e.g., enolase (AZI09_08765), TPI (AZI09_08770), PGK (AZI09_08775), and GADPH (AZI09_08780), showed low to strong correlations ($0.4 \leq r \leq 0.8$) with all three characteristics. Strong inverse correlations ($r \leq -0.7$) were observed for LytR-CpsA-Psr (AZI09_03640) and ADI (AZI09_01860) with D-glucose, and only moderate inverse correlations with β-glucan were observed. The results of the correlation are listed in a Supplementary Excel file (Table S1).

To obtain a comprehensive understanding of the gene expression during β-glucan biosynthesis or degradation, we performed an overrepresentation analysis using proteins significantly correlated with these parameters ($r \geq 0.7$ and $r \leq -0.7$). In total 118, 433, and 404 proteins correlated with D-glucose, β-glucan, and viscosity, respectively. The resulting gene ontology (GO) terms were categorized in three groups: molecular function (MF), cellular component (CC), and biological process (BP) (Figure 7). The GO term oxidoreductase activity (MF) correlated with all three growth characteristics. Among others, nucleic acid binding (BP), cell division (BP), and other terms associated with cell growth

correlated inversely with viscosity and β-glucan. Carbohydrate-metabolism-associated terms, e.g., glycolytic process (BP), carbohydrate process (BP), and carbohydrate binding (MF), correlated positively with the analyzed characteristics, while the macromolecular catabolic process (BP) correlated inversely with D-glucose concentration. However, only three GHs and GTs were identified within the terms: the MP (GH65) AZI09_04670, part of the gene set of the term carbohydrate binding, the GH8 family protein endo-β-1,3-glucanase (AZI09_02135) of the macromolecular catabolic process, and the GT8 family protein (AZI09_12410) of coils coils. Moreover, enolase (AZI09_08765), a putative moonlighting protein and an enzyme of glycolysis, was identified in BP terms correlating with the β-glucan concentration, e.g., the organic acid metabolic process, the cellular amino acid catabolic process, and the glycolytic process. The results of the overrepresentation analysis are listed in a Supplementary Excel file (Table S2).

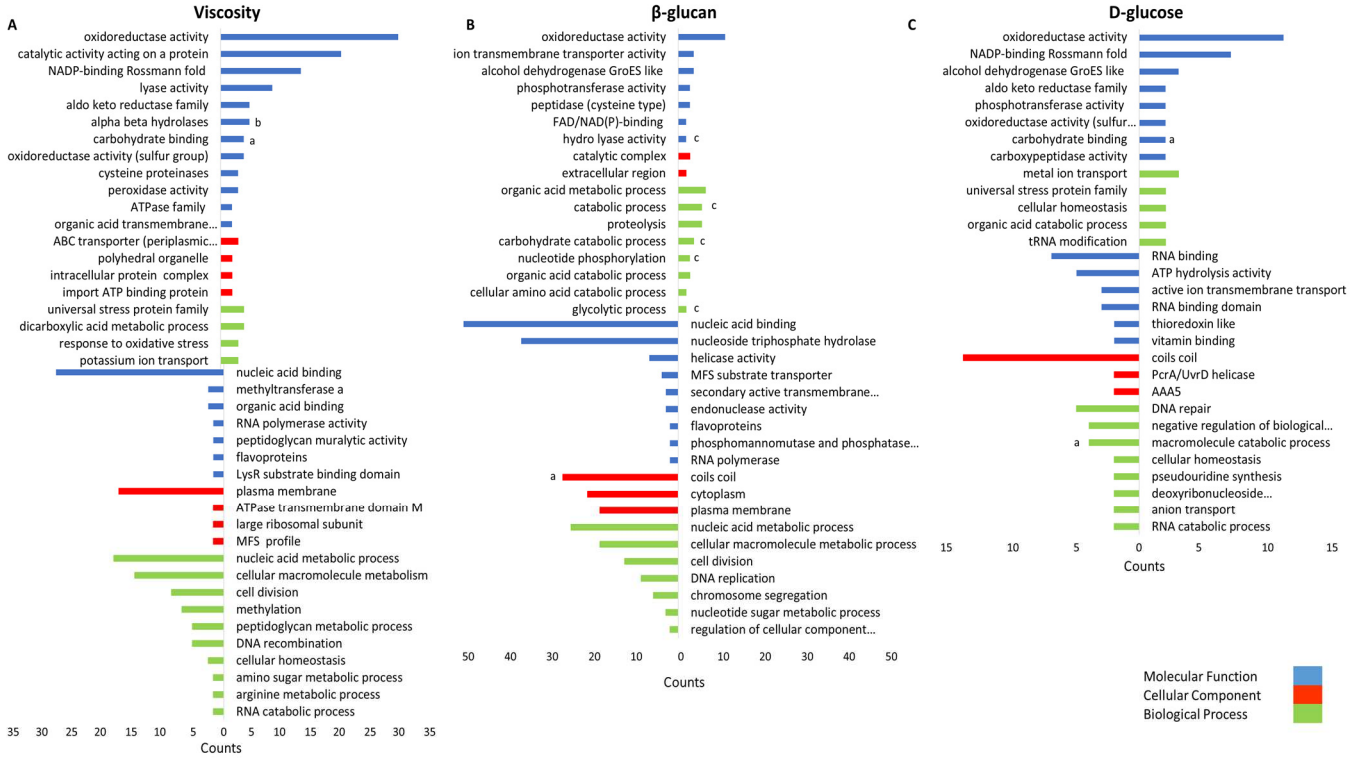

Figure 7. Gene set overrepresentation analysis of proteins correlating with (**A**) viscosity, (**B**) β-glucan content, and (**C**) D-glucose content. Top bar charts indicated the positively and bottom bar chart the negatively correlating proteins with corresponding GO terms (*y*-axis). The *x*-axis represents the gene counts. GO terms comprising proteins of interest are indicated by (a) GHs and GTs; (b) β-glucan-biosynthesis-associated proteins (other than GHs and GTs), and (c) moonlighting-associated protein enolase (AZI09_08765).

3. Discussion

In this study, proteomics based on state-of-the-art LC-MS/MS was used to analyze the biosynthesis and potential degradation of *O2*-substituted (1,3)-β-D-glucan by *L. brevis* TMW 1.2112 on a molecular level. The focus of the study was to identify correlations between the expression of carbohydrate-active enzymes and changes in the EPS state (viscosity, β-glucan, and D-glucose concentration) by biosynthesis and degradation. Since β-glucan forms a polysaccharide capsule (CPS) around the cells, proteomic data of both the cell lysate and secretome were analyzed to actively identify the involved GHs and GTs [35,38,76].

Pathways for the biosynthesis of β-glucan by *L. brevis* TMW 1.2112 from several mono- and disaccharides have been postulated [46]. With maltose as a sole energy source, the proteomic data were analyzed for enzymes related to this putative pathway. In sum-

mary, all proteins described in the putative pathway were identified within the cell lysate, and some were identified within the secretome (Figure 8A). Transporters for maltose uptake in LAB, e.g., the maltose ABC transporter or the MFS maltose transporter, have been described in *Lactiplantibacillus* (*La.*) *plantarum* or *Fructilactobacillus* (*F.*) *sanfranciscensis*, respectively [77,78]. Our analyses resulted in the MFS maltose transporter, MalT (AZI09_10325), expressed within the cell lysate and secretome (7 days). The ORF of MalT was next to the ORF of the MP (AZI09_10320) and the ORF of the transcriptional regulator MalR (AZI09_10330), resulting in one operon. These proteins were detected at all time points of the cell lysate and after 7 days within the secretome. In addition, the ORFs of the MP AZI09_04670 and β-PGM (AZI09_04665) could be associated with a second operon, and a second β-PGM (AZI09_02330) was also identified (Figure 8B). Two maltose operons were described for *F. sanfranciscensis*: one contains an MP (*mapA*) and β-PGM and is induced by the presence of maltose acting as a major catabolic enzyme, and the second includes, in addition to an MP (*mapB*) and β-PGM, a permease and epimerase. The mapB system is under the control of a constitutive promotor and a microorganism, and only the mapA system usually has a prolonged lag phase [79]. The cell growth of *L. brevis* TMW 1.2112 entered the exponential phase immediately without a lag phase, which could also be due to the faster utilization of the free amino acids of the CDM.

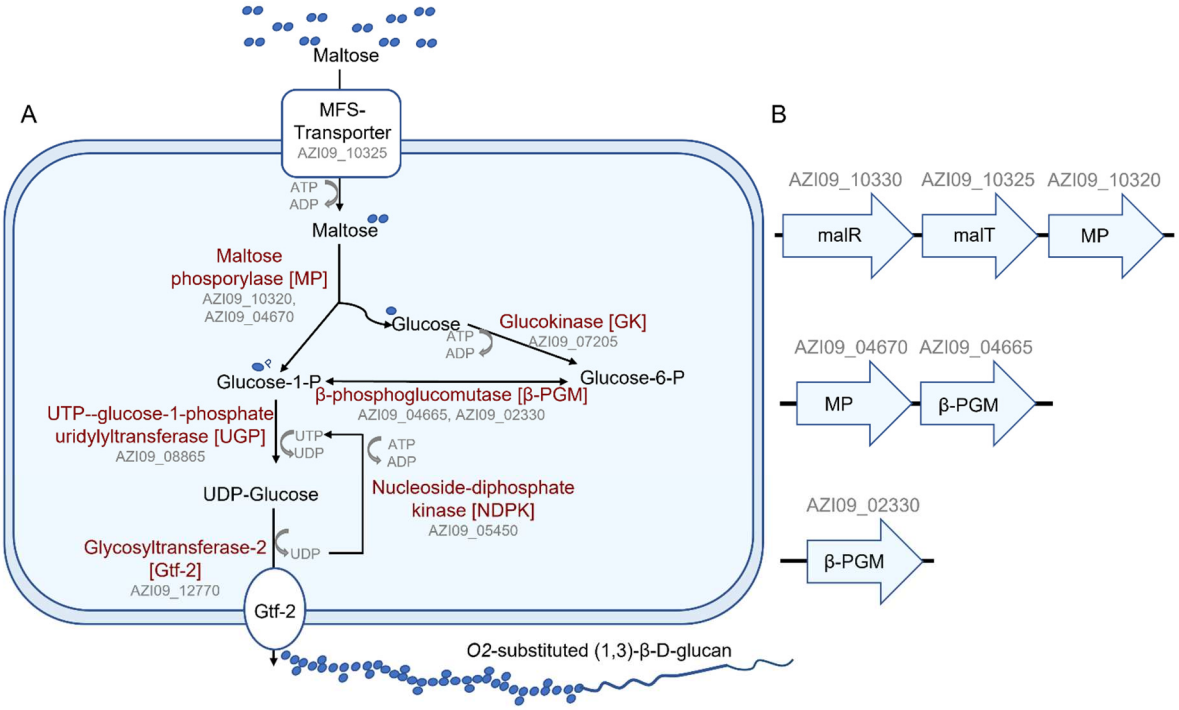

Figure 8. β-glucan biosynthesis of *L. brevis* TMW 1.2112. (**A**) Metabolic pathway of the β-glucan biosynthesis, as suggested from genomic data and proteomic analysis. (**B**) Suggested maltose operons: malR = transcriptional regulator, MP = maltose phosphorylase, malT = MFS maltose transporter and β-PGM = β-phosphoglucomutase.

The β-glucan biosynthesis began simultaneously with the cell growth. While the cell count slowed down after one day, the β-glucan concentration continued to increase significantly, leading to the assumption that β-glucan formation was independent from the cell count and accumulated over time. This behavior has been observed in other β-glucan-forming LABs [43,80,81]. Proteome analysis revealed three expressed GT2 family proteins: two chromosomal encoded GT2 proteins (AZI09_04045 and AZI09_12875), both significantly more abundant during the exponential phase, inversely correlated with viscosity and D-glucose concentration, and a plasmid-encoded GT2 protein (AZI09_12770), which was most likely constantly expressed. The transmembrane β-1,3 glucan synthase (GT2

family) is described as the key enzyme in the bacterial β-glucan biosynthesis [15,41,46,48]. The GT2 family comprises thousands of sequences with at least 12 distinct GT functions, making a functional prediction by homology difficult [82]. However, Llamas-Arriba et al. (2018) compared the nucleotide sequences of 13 β-1,3 glucan synthases of *Pediococcus* spp., *Oenococcus oeni*, and *Lactobacillus* spp., including *L. brevis* TMW 1.2112 (AZI09_12770). The study revealed that β-1,3 glucan synthase sequences are highly conserved and mostly plasmid-encoded [41,83]. Sequence alignments of the two chromosomally encoded GT2s) from *L. brevis* TMW 1.2112 resulted in low identities (data not shown). Consequently, only AZI09_12770 is a β-1,3 glucan synthase and responsible for β-glucan biosynthesis.

The start of β-glucan production during the exponential phase has been observed in *Pe. parvulus* spp. and *Pe. damnosus* IOEB8801 [81,84]. In addition, *Pa. suebicus* CUPV221 started β-glucan production during the stationary phase [43]. Nevertheless, the strains have one thing in common with *L. brevis* TMW 1.2112: the continuous increase in β-glucan concentration even up to 14 days and the lack of its decrease [43,81,84]. What differed, however, was the viscosity of *L. brevis* TMW 1.2112 cultures, as it decreased significantly after 4 days, unlike the viscosities of *Pediococcus* strains and *Pa. suebicus*, which increased steadily until 7 days or more and remained high [43,85]. The decrease in the viscosity and heterogeneous viscoelastic characteristics might be associated with the enzymatical or physical effects degrading high-molecular EPS ($\geq 9.6 \times 10^6$ Da) into smaller fractions (at least 6.6×10^6 Da), which could still be detected by ELISA [86]. A broad detection range of the immunological assay could also result in an overestimation of the polymer concentration. The endo-β-1,3-glucanase (AZI09_02135) was, despite the identification of a signal peptide, only detected within the cell lysate during the stationary phase (4 days). Sequence analysis revealed two stop-codon mutations within the last 30 amino acids of the 367 amino acid sequence of the endo-β-1,3-glucanase (AZI09_02135), which might affect enzyme activity or secretion. Furthermore, the overrepresentation analysis resulted in an inverse correlation for D-glucose and the term macromolecular catabolic process. This in turn means that changes in the D-glucose concentration were not a result of endo-β-1,3-glucanase activity. Most of the GHs were identified as intracellular enzymes, such as β-1,3-glucosidase (BglB) (AZI09_02170), which was also not present in the secretome, though it was expressed during the fermentation period. Moreover, the arginine deiminase (ADI), known for its role in the defense against acidic stress, was also expressed during fermentation [87,88]. This could lead to the assumption that a low pH value might have interfered with the viscosity, but physical effects due to self-produced organic acids appeared to be unlikely, as this was so far not observed for other β-glucan-producing LABs such as pediococci [43,84,85]. Further, capsular EPSs are described as protectors against environmental stress such as acidity [34,89,90]. Nevertheless, the fermentation broth underwent structural changes during fermentation, which could not be explained by the enzymes listed in Table 1. The secretome contained so far uncharacterized enzymes that may affected the viscosity, since many proteins could not be clearly categorized. Though changes in the heterogeneous character of the viscosity might be attributed to the formation of a β-glucan-cell-network, lysed cells reduced the network integrity during the late stationary and early death phases, and this in turn might have led to the reduced viscosity [34,35]. Autolysis, which is strain-dependent and preferentially triggered under stress conditions or in late growth phases, is a well-known phenomenon in lactic acid bacteria [91,92]. Moreover, the increased number of proteins in the secretome after 7 days compared to 8 h could also be explained by the autolysis of *L. brevis* TMW 1.2112 cells.

Potential β-1,3-glucan degradation could be represented by an increase in the D-glucose concentration as the monomeric unit [15,38,76,80]. An increase in the D-glucose concentration was detected but with a similar curve progression as the β-glucan concentration, which means that the release of D-glucose started already during the exponential phase and simultaneously with β-glucan biosynthesis. This in turn means that it is rather a product of maltose utilization than of β-glucan degradation. Glucose secretion after the phosphorolytic cleavage of maltose is described in other lactobacilli [93]. The utilization

of maltose strains and the subsequent glucose release by beer-spoiling *L. brevis* has been described [94]. In conclusion, β-glucan is not used as a storage compound by *L. brevis* TMW 1.2112. The CPS therefore might have mostly a protective function according to the strain's origin from beer with present ethanol, low pH, or antimicrobial hop compounds [34,95].

The proteins responsible for the attachment of the β-1,3-glucan capsule to the cell surface of LAB have not been described. Members of the LytR-Cps2A-Psr (LCP) protein family are discussed for their role in the attachment of polysaccharides to the peptidoglycan of Gram-positive bacteria [49–52]. According to sequence analyses, *L. brevis* TMW 1.2112 possess three LCP family proteins, two of which were constantly expressed according to the cell lysate data and after 7 days within the secretome. Both proteins (AZI09_03640 and AZI09_03715) were identified as BrpA (Biofilm regulatory protein A) proteins, with AZI09_03715 containing a putative signal peptide. The knowledge about *Lactobacilli* LCPs is currently limited. Nevertheless, it was assumed that the Wzy pathways could be involved in the coupling of CPS to the cell surface peptidoglycan [52,53,96]. Genes of the Wzy pathway were not detected within the proteomic data or the genome sequence, but CDSs for a chain-length determining protein (AZI09_03645) and an EPS biosynthesis protein (AZI09_03650) are encoded next to BrpA (AZI09_03640). However, neither protein was detected by MS. Further experiments are necessary to identify specific enzymes involved in the attachment of the capsules to cell surfaces.

Several proteins that have been described as acting as moonlighting proteins in other bacteria emerge regularly within the proteomic data of the cell lysate and secretome, including an enolase (AZI09_08765), triose-phosphate isomerase (TPI) (AZI09_08770), phosphoglycerate kinase (PGK) (AZI09_08775), glyceraldehyde-3-phosphate dehydrogenase (GADPH) (AZI09_08780), and elongation factor Tu (EF-Tu) (AZI09_05335). Most moonlighting proteins are primarily enzymes of the glycolytic and metabolic pathways or molecular chaperones [54,68] and overtake multiple functions based on their cellular position, for example, when released into the extracellular milieu [54,68]. For example, the enolase is grouped in terms associated with metabolic processes, which is not surprising, as enolases are involved in the glycolysis. Interestingly, all proteins except TPI (AZI09_08770) were detected after 8 h within the secretome, even though these proteins are usually intracellular proteins. The moonlighting proteins GADPHs, EF-Tu, and the enolases of commensal lactobacilli have been described as acting in adhesion processes and might contribute to probiotic traits [54–57]. Further, four of these so-called moonlighting genes were located within an operon including a transcriptional regulator (AZI09_08785), which was also detected within the cell lysate samples (Figure 9). An operon of moonlighting proteins was previously found in *Staphyloccocus aureus* [97], and while moonlighting proteins of pathogens and probiotics are generally described to be highly conserved [54], it could be assumed that these genes, which are also grouped with an operon, might have moonlighting functions in *L. brevis* TMW 1.2112. However, the effect of these genes, e.g., on the β-glucan-cell-network, on the adhesion to surfaces, or even on probiotic actions, requires further studies to identify their specific roles in the extracellular milieu.

Figure 9. Operon of putative moonlighting proteins. GADPH (glyceraldehyde-3-phosphate dehydrogenase), PGK (phophoglycerate kinase), TPI (triose-phosphat isomerase), and enolase.

In the present study, we have shown that, over a period of fermentation lasting 10 days, the glucose concentration and the amount of β-glucan in the supernatant increased, but the viscosity decreased. The biosynthesis of β-glucan is closely linked to the

maltose metabolism via an MP. Genes encoding enzymes involved in maltose utilization are organized in three operons. The continuous increase in glucose is probably due to the phosporolytic cleavage of maltose rather than the degradation of the β-glucan to the monomer. Thus, we assume that β-glucan is not used as a storage substance or degraded by the cells for further utilization. In addition, a limitation of ELISA differentiating between high- and low-molecular-weight β-glucan might cause misinterpretations of the concentrations and cause its actual decrease to evade detection. There are probably several reasons for the decrease in viscosity. The only carbohydrate active enzyme that strongly correlated with viscosity, β-glucan, and D-glucose concentrations was an MP, and none of the enzymes with a β-glycosidic bond preference as the endo-β-1,3-glucanase or β-1,3-glucosidase (BglB). Aside from an enzymatic cleavage by GHs secreted or released by partial autolysis, destruction of the β-glucan cell network due to a detachment of capsular β-glucan bound to cell surfaces also seems plausible. Moreover, two new study approaches focusing the extracellular polysaccharide encapsulation and cell–cell adhesion were identified with respect to the LCP protein family and moonlighting proteins, respectively. Furthermore, the secretome contained proteins with potential degradable functions on β-glucan with respect to the viscosity that have hitherto been uncharacterized. This study provides important insight into growth characteristics associated with the β-glucan formation of *L. brevis* TMW 1.2112 and creates a basis for future investigations of EPS-forming LAB in which the role of growth characteristics and EPS biosynthesis is studied.

4. Materials and Methods

4.1. Strain, Medium, and Growth Conditions

L. brevis TMW 1.2112 is a LAB isolated from wheat beer. The strain was cultivated in a modified chemically defined medium (CDM). The CDM (pH 6.2) was mixed from a 970 mL base medium with a 10 mL vitamin solution, a 10 mL metal solution, and a 10 mL nucleic acid base solution, as previously described by Otto et al. (1983) and Sánchez et al. (2008) with further modifications. The base medium contained (quantities per liter of distilled water) 20 g of maltose, 2.5 g of $K_2HPO_4 \cdot 3\ H_2O$ (VWR International, Radnor, PA, USA), 3 g of KH_2PO_4 (VWR International, Radnor, PA, USA), 0.6 g of di-ammonium hydrogen citrate (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 1 g of sodium acetate (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 0.25 g of cysteine-HCl (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 5 g of casamino acids (MP Biomedicals GmbH, Germany), and 1 g of Tween 80® (Merck KGaA, Darmstadt, Germany). The vitamin solution (pH 7.0) contained (quantities per liter of distilled water) 100 mg of nicotinic acid, 100 mg of thiamine-HCl, 100 mg of riboflavin, 100 mg of pantothenic acid, 1 g of aminobenzoic acid, 1 g of D-biotin, 100 mg of folic acid, 100 mg of vitamin B_{12}, 500 mg of orotic acid, 500 mg of thymidine, 500 mg of inosine, 250 mg of lipoic acid, 500 mg of pyridoxine (all chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA, except for folic acid (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), and 100 mg of vitamin B_{12} (AppliChem GmbH, Darmstadt, Germany). The metal solution contained (quantities per liter of distilled water) 20 g of $MgCl_2 \cdot 6\ H_2O$ (Sigma-Aldrich, St. Louis, MO, USA), 5 g of $CaCl_2 \cdot 2\ H_2O$ (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 0.5 g of $FeCI_2 \cdot 4\ H_2O$ (Fluka Chemie GmbH, Buchs, Switzerland), 0.5 g of $ZnCl_2$ (Merck KGaA, Darmstadt, Germany), and 0.25 g of $CoCl_2 \cdot 6\ H_2O$ (Sigma-Aldrich, St. Louis, MO, USA). The nucleic acid base solution contained (quantities per 10 mL 0.1 M NaOH) 10 mg of adenine sulfate (SERVA Electrophoresis GmbH, Germany), 10 mg of uracil (Sigma-Aldrich, St. Louis, MO, USA), 10 mg of xanthine (Sigma-Aldrich, St. Louis, MO, USA), and 10 mg of guanine (Fluka Chemie GmbH, Buchs, Switzerland) [98,99].

4.2. Fermentation and Monitoring of Cell Growth

To investigate the formation and degradation of β-glucan by *L. brevis* TMW 1.2112 intra- and extracellularly, two sets, each with four biological replicates, were inoculated with an initial $OD_{600\ nm}$ of 0.05, aliquoted (50 mL) and incubated at 30 °C as static cultures

in 50 mL reaction tubes (Sarstedt AG & Co., Darmstadt, Germany). Aliquots of the first set were used for proteomic analyses, while the second set was used for viscosity analyses. Cell growth was monitored for 10 days based on OD measurements, cell count analysis, and changes in the pH values. The colony forming units (cfu) were analyzed with inoculated mMRS agar plates incubated at 30 °C for 48 h before counting as previously described [35]. All experiments were carried out using four biological replicates.

4.3. Proteomic Analysis

4.3.1. Proteomic Sample Preparation

The *L. brevis* TMW 1.2112 fermentation broths (50 mL) were centrifuged at 17,000× *g* for 30 min at 4 °C. For cellular proteome measurements, the cell pellets were processed, while the supernatants were collected for secretome analyses.

Cellular proteomes: Bacterial cell pellets were washed once with a 50 mL saline solution (Ringer tablets, Merck KGaA, Darmstadt, Germany). After a second centrifugation step, the cell pellets were shock-frozen in liquid nitrogen and stored at −80 °C. For cell lysis, between 300 and 1600 µL of lysis buffer (8 M urea (Gerbu Biotechnik GmbH, Heidelberg, Germany), 100 mM NH_4HCO_3 (Sigma-Aldrich, St. Louis, MO, USA), 1 mM dithiothreitol (DTT, Gerbu Biotechnik GmbH, Heidelberg, Germany) in water, pH 8.0), and acid-washed glass beads (Ø 2.85–3.45 mm, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) were added, depending on the previous OD of the culture broth. The cell pellet of the 50 mL culture broth with an OD_{600} of 1 was resuspended in 1 mL of lysis buffer. The cells were disrupted with a benchtop homogenizer (FastPrep®-24 MP, MP Biomedical Inc., Eschwege, Germany) in five cycles of 45 s each at 5 $m \cdot s^{-1}$. Between the cycles, the samples were stored on ice to cool (1 min). Cell lysates were collected after centrifugation (17,000× *g*, 10 min, 4 °C), and the total protein concentrations were determined using the Coomassie (Bradford) protein assay kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. For each sample, 15 µg of protein extract was reduced with 10 mM DTT (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and carbamidomethylated with 55 mM chloroacetamide (CAA, Merck KGaA, Darmstadt, Germany). Subsequently, proteins were digested twice with 0.15 µg of trypsin (Roche Deutschland Holding GmbH, Penzberg, Germany), first for 2 h and then overnight at 37 °C. Digested peptide samples were desalted and resuspended in 2% acetonitrile (VWR International, Radnor, PA, USA), 98% H_2O, and 0.1% formic acid (CARLO ERBA Reagents GmbH, Emmendingen, Germany) for a final concentration of 0.1 µg/µL.

Secretomes: For secretome analyses, 500 µL of the fermentation medium were mixed at 2:1 with NuPAGE™ LDS Sample Buffer (4×) (ThermoFisher Scientific, Waltham, MA, USA) and heated for 10 min at 70 °C. In-gel trypsin digestion was performed according to standard procedures [100]. Briefly, the samples were run on a NuPAGE™ 4–12% Bis-Tris Protein Gel (Thermofisher Scientific, Waltham, MA, USA) for 3 min. Subsequently, the still not size-separated single protein band per sample was cut out of the gel, reduced (50 mM DTT), alkylated (55 mm CAA), and digested overnight with trypsin (trypsin-gold, Promega, Madison, WI, USA). The sample was freshly re-suspended before MS measurement in 25 µL of 2% acetonitrile and 0.1 formic acid, and 3 µL were injected into the mass spectrometer per measurement.

4.3.2. LC-MS/MS Measurements

LC-MS/MS measurements were performed on an Ultimate 3000 RSLCnano system coupled to a Q-Exactive HF-X mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). For proteome analyses, ca. 0.2 µg of peptides were delivered to a trap column (ReproSil-pur C18-AQ, 5 µm, 20 mm × 75 µm, self-packed, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) at a flow rate of 5 µL/min in HPLC grade water with 0.1% formic acid. After 10 min of loading, peptides were transferred to an analytical column (ReproSil Gold C18-AQ, 3 µm, 450 mm × 75 µm, self-packed, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) and separated using a 50 min gradient from 4% to 32% of Solvent

B (0.1% formic acid in acetonitrile and 5% (*v*/*v*) DMSO (Sigma-Aldrich, St. Louis, MO, USA)) at a 300 nL/min flow rate. Both nanoLC solvents (solvent A = 0.1% formic acid in HPLC grade water and 5% (*v*/*v*) DMSO) contained 5% DMSO to boost MS intensity. The Q-Exactive HF-X mass spectrometer was operated in data-dependent acquisition (DDA) and positive ionization mode. MS1 spectra (360–1300 m/z) were recorded at a resolution of 60,000 using an automatic gain control (AGC) target value of 3e6 and a maximum injection time (maxIT) of 45 ms. Up to 18 peptide precursors were selected for fragmentation for full proteome analyses. Only precursors with a charge state from 2 to 6 were selected, and a dynamic exclusion of 25 s was enabled. Peptide fragmentation was performed using higher energy collision induced dissociation (HCD) and a normalized collision energy (NCE) of 26%. The precursor isolation window width was set to 1.3 m/z. The MS2 resolution was 15,000 with an automatic gain control (AGC) target value of 1e5 and a maximum injection time (maxIT) of 25 ms.

4.3.3. LC-MS/MS Data Analysis

Peptide identification and quantification were performed using the software MaxQuant (version 1.6.3.4) with its built-in search engine Andromeda [101,102]. MS2 spectra were searched against the proteome database of *L. brevis* TMW 1.2112 (GenBank accession No.: CP016797), including 2537 coding sequences supplemented with common contaminants (built-in option in MaxQuant). Trypsin/P was specified as a proteolytic enzyme. Precursor tolerance was set to 4.5 ppm, and the fragment ion tolerance was 20 ppm. Results were adjusted to a 1% false discovery rate (FDR) on a peptide spectrum match (PSM) level and a protein level employing a target-decoy approach using reversed protein sequences. The minimal peptide length was defined as 7 amino acids, and the "match-between-run" function was enabled (matching time window: 0.7 min; alignment window: 20 min). Carbamidomethylated cysteine was set as a fixed modification and oxidation of methionine and N-terminal protein acetylation as variable modifications. To compare relative protein abundances in the cell lysate time course experiment, label-free quantification (LFQ) was used. The LFQ assumes that the overall protein abundance across samples is comparable. This assumption is clearly violated in the secretome experiment; therefore, we used intensity-based absolute quantification (iBAQ) in this analysis [103]. Each sample type was analyzed in biological triplicates (at minimum).

4.3.4. Statistical Analysis of Proteomics Data

In the downstream analysis, the proteins identified as "only identified by site", "reversed", and "potential contaminants" were removed first. The iBAQ intensites (for the secretome experiment) were centered across samples based on the proteins shared between 8 h and 7 days. The LFQ intensity was already well normalized and therefore not further changed. The intensities were logarithm-transformed on base 10. Proteins identified in less than 3 replicates out of 4 were excluded from the statistical analysis, and the remaining missing values were imputed using the lower detection limit method [104]. The missing values of a protein expression were replaced by the constant, which was half of lowest detected values. If the imputed value was higher than the 15% quantile of all the detected values, the missing value was replaced by the 15% quantile. The rationale for this is based on the fact that the missing values are more likely to result from low abundant proteins.

A Student *t*-test was used to identify proteins that were significantly differentially expressed between 8 h and 7 days in the secretome experiment. For the cell lysate time course experiment, Pearson correlation analysis was used to identify proteins whose intensity well correlates (positively and negatively) with viscosity, β-glucan, and D-glucose abundance. Fisher's exact test was used in the enrichment analysis. PANNZER2 [105] and InterProScan [106,107] were used to predict the functions associated with proteins. All the statistical analyses were performed in an R statistical environment (version 3.6.3) [108,109].

The proteome database of *L. brevis* TMW 1.2112 was additionally analyzed by the RAST (rapid annotations using subsystems technology, version 2.0) software [61], eggNOG-

Mapper (version 2), a functional annotation tool using the default settings for clusters of orthologous groups (COG), pairwise orthology predictions, and functional annotation [62,63]. Putative signal peptides, which provide an indication of secretory proteins, were identified using SignalP(version 5.0) [66].

4.4. Viscosity Analysis

Changes in the viscosity were analyzed daily over the 10-day fermentation period using the rotational viscometer 'Super 4 Rapid-Visco-Analyser' (RVA, Perten Instruments, PerkinElmer Company, Waltham, MA, USA) [110,111]. For each measurement, 40 ± 0.01 g of the fermentation broth were weighed in a RVA sample can and analyzed at 20 °C. The RVA configuration was set to 160 rpm with an initial 2 min equilibration phase for a temperature adjustment of the fermentation broth and a subsequent 5 min analysis period under stable conditions. The recorded viscometric data were exported and further evaluated with V. 6.01 GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). All experiments were carried out using four biological replicates.

4.5. Quantification of the β-Glucan and D-Glucose Concentrations

The β-glucan concentration of the supernatant was analyzed by a competitive enzyme-linked immuno-sorbent assay (ELISA) based on *Streptococcus* (*S.*) *pneumoniae* serotype 37 antibodies for the quantification of the bacterial β-glucan [86]. The assay was performed as previously described [36]. The D-glucose concentrations of the supernatant samples were determined using a glucose oxidase/peroxidase assay (GOPOD, Megazyme Ltd., Bray, Ireland) according to the manufacturer's protocol. The assay was adapted to a microtiter plate volume with a 50 µL sample volume and 150 µL of the GOPOD reagent. A standard curve was used for D-glucose (Megazyme Ltd., Bray, Ireland) calculations. All experiments were carried out using four biological replicates.

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5 Complementary Results

Within the scope of this work, other results and findings were collected that were not included in the previously listed publications. To make a global discussion as comprehensive as possible, some complementary results are presented within this chapter. These additional results thereby support and connect the individual studies.

5.1 Screening of LAB for β-glucan formation and sourdough fermentation

Next to *L. brevis* TMW 1.2112, *P. claussenii* TMW 2.340, and the two *Δgtf-2* mutants, *L. brevis* TMW 1.2320 and *P. claussenii* TMW 2.2123, additional LAB were also analyzed for β-glucan formation. This included further beer isolates, like *Furfurilactobacillus* (*F.*) *rossiae* TMW 1.2512 and isolates from other food sources, such as sourdough and meat samples (Table 18). All isolates were phenotyped and genotyped for β-glucan formation. For this purpose, liquid cultures and colony-forming units were tested for their viscosity and ropy properties, respectively. It was shown that all isolates with beer origins, such as wheat beer, exhibited the characteristic viscosity increase of culture broths and ropy colonies. In contrast, the isolates from sourdough and meat samples did not exhibit any of these features.

Table 18. Strains screened for β-glucan formation.

Species	Strain	Source	β-glucan formation	Accession No.	Reference
Levilactobacillus (*L.*) *brevis*	1.240	Beer	Yes	-	(Preissler et al., 2010)
L. brevis	1.2111	Wheat beer	Yes	CP019743	(Fraunhofer et al., 2017)
L. brevis	1.2113	Brewery surface	Yes	CP019750	(Fraunhofer et al., 2017)
L. brevis	1.2114	Wheat beer	Yes	-	(Fraunhofer, 2018)
L. brevis	1.2115	Wheat beer	Yes	-	
L. brevis	1.2147	Wheat beer	Yes		
Furfurilactobacillus (*F.*) *rossiae*	1.2152	Wheat beer	Yes	-	
L. brevis	1.2153	Wheat beer	Yes	-	
L. brevis	1.2154	Wheat beer	Yes	-	
L. brevis	1.2155	Beer	Yes	-	
F. rossiae	1.1307	Sourdough	No	-	-
F. rossiae	1.590	Sourdough	No	-	-
F. rossiae	1.2227	Meat	No	-	-
L. brevis	1.839	Sourdough	No	-	-
L. brevis	1.1205	Sourdough	No	-	(Kern et al., 2014)
L. brevis	1.592	Sourdough	No	-	-
L. brevis	1.1786	Sourdough	No	-	-
L. brevis	1.2235	Sourdough	No	-	-
L. brevis	1.100	Sourdough	No	-	(Kern et al., 2014)

In addition, the genomic DNA of all strains was isolated and analyzed for the presence of the glycosyltransferase 2 family (*gtf-2*) sequence using PCR and specific primers (Gtf-2 fwd and Gtf-2 rev). This analysis resulted in the same results as previously observed by screening for phenotypes and growth behavior. Of the remaining β-glucan-positive strains, some strains were selected for the persistence experiments in sourdough fermentations of wheat and rye flours.

The β-glucan-positive strains, *L. brevis* TMW 1.2113, *L. brevis* TMW 1.2114, *L. brevis* TMW 1.2115, and *F. rossiae* TMW 1.2152, were selected and analyzed as previously described in Chapter 4.1. With initial cell counts being between 10^6 and 10^7 CFU/g dough after inoculation of the wheat and rye doughs. Table 19 summarizes the results after 72 hours of fermentation including two back-slopping steps of the wheat and rye sourdoughs. The analyses revealed that several β-glucan-forming *L. brevis* strains possess the ability to manufacture sourdoughs including the required acidification and persistence. In contrast, *F. rossiae* TMW 1.2152 was not able to persist against the endogenous sourdough microbiota as indicated by the species identity. Since *F. rossiae* could not establish itself in the fermentation of the wheat and rye doughs, capsule detection was difficult, and the same applied to the determination of ropy colonies. Lactic acid and acetic acid formation were quantified by HPLC for FQ calculations. The FQ of wheat and rye sourdoughs fermented by *L. brevis* TMW 1.2112 and *L. brevis* TMW 1.2320 (Δ*gtf-2*) was 9.44 ± 0.30 and 4.62 ± 0.04 (wt) and 4.58 (Δ*gtf-2*), respectively. The FQ of homofermentative *P. claussenii* was not calculated due to very low acetic acid amounts (approx. 0.4 mM) compared to formed lactic acid amounts (approx. 32.5 mM) in wheat and rye sourdough. *L. brevis* TMW 1.2114 and *L. brevis* TMW 1.2115 resulted lower FQ compared to the other *L. brevis* strains. The FQ of wheat fermented with *F. rossiae* TMW 1.2152 was significantly higher compared to all other samples. Detected traces of acetic acid in *P. claussenii* sourdough samples were probably formed by concomitant heterofermentative LAB. In rye sourdough the FQ were at least half of the wheat sourdough FQ with 4.25 (*L. brevis* TMW 1.2113), 2.65 (*L. brevis* TMW 1.2114), 2.83 (*L. brevis* TMW 1.2115), and 4.31 (*F. rossiae* TMW 1.2152).

Table 19. Analyses of the persistence of additional β-glucan forming LAB in sourdoughs.
Results of wheat and rye sourdough fermentation after 72 h including two back-slopping steps.

	***L. brevis* TMW 1.2113**		***L. brevis* TMW 1.2114**		***L. brevis* TMW 1.2115**		***F. rossiae* TMW 1.2152**	
	Wheat	Rye	Wheat	Rye	Wheat	Rye	Wheat	Rye
Species identity [%]	100	100	100	86	100	73	9	0
pH	3.7	3.8	3.9	3.9	3.9	3.9	3.9	4
Cell count [CFU/g]	$1.5 \cdot 10^9$	$3.4 \cdot 10^9$	$1.5 \cdot 10^9$	$2.9 \cdot 10^9$	$4.9 \cdot 10^8$	$1.7 \cdot 10^9$	$8.2 \cdot 10^8$	$2.3 \cdot 10^9$
FQ	10.8	4.25	5.80	2.65	7.23	2.83	28.83	4.31
Capsule	+		+		+		/	
Ropy CFU [%]	100%		≤ 90%		≤ 80%		≤ 9%	

The formation of EPS capsules in sourdough fermentation was identified by capsule-staining (Figure 3). Here, next to the identified capsules of the *L. brevis* strains, the different cell morphologies were

remarkable. In particular, the strain *L. brevis* TMW 1.2115 (Figure 3 C) stood out due to significantly longer rods compared to the other analyzed samples. Microscopic analyses of *L. brevis* TMW 1.2114 cells from culture broths revealed that the majority of the cells showed similar cell morphologies as *L. brevis* TMW 1.2115. Wheat sourdough samples initially inoculated with *F. rossiae* TMW 1.2152, were analyzed for the presence of capsules, which was negative (Figure 3 D).

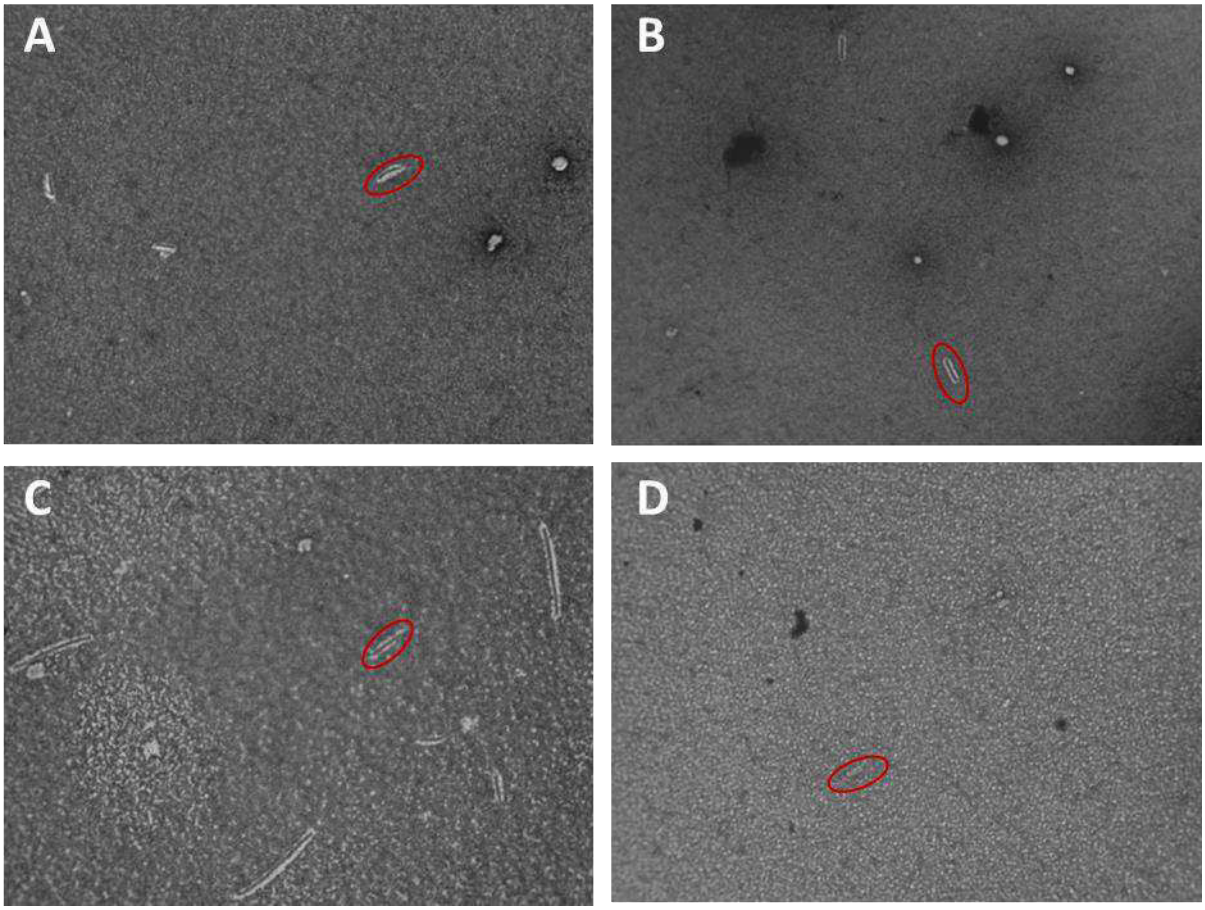

Figure 3. Capsule staining of β-glucan-forming LAB isolated from wheat sourdough.
A) *L. brevis* TMW 1.2113, B) *L. brevis* TMW 1.2114, C) *L. brevis* TMW 1.2115, and D) *F. rossiae* TMW 1.2152

The rheological effects were analyzed for selected strains including β-glucan-forming *L. brevis* TMW 1.2113 and EPS-negative sourdough isolate *L. brevis* TMW 1.1205 (Figure 4). The rheological analyses demonstrated that *L. brevis* TMW 1.2113 significantly increased the viscosity of wheat sourdough, similar as previously reported for *L. brevis* TMW 1.2112. In contrast, *L. brevis* TMW 1.1205 did not improve the rheological properties. Similar results were observed for wheat doughs fermented by the *Δgtf-2* mutants *L. brevis* TMW 1.2320 and *P. claussenii* TMW 2.2123.

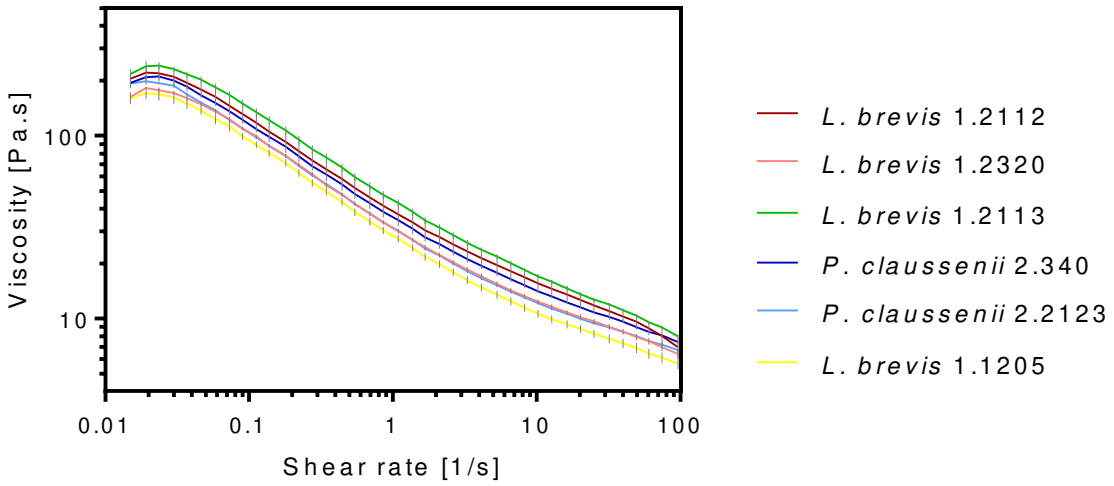

Figure 4. Flow curves obtained for wheat sourdoughs.
(■) *L. brevis* TMW 1.2112, (■) *L. brevis* TMW 1.2320 (Δgtf-2), (■) *L. brevis* TMW 1.2113, (■) *P. claussenii* TMW 2.340, (■) *P. claussenii* TMW 2.2123 (Δgtf-2), and (■) *L. brevis* TMW 1.1205. Values are means of triplicates with standard deviations except for the artificial acidified sourdough which is represented by duplicates.

The flow behavior indices (n) of *L. brevis* TMW 1.2113 and *L. brevis* TMW 1.1205 were determined by the Herschel-Bukley model of the power law to be 0.632 and 0.679, respectively. Statistical analysis, using one-way ANOVA, demonstrated a significantly higher viscosity in wheat sourdoughs fermented by *L. brevis* TMW 1.2112 and TMW 1.2113 compared to other samples. The viscosities of wheat sourdoughs with *P. claussenii* TMW 2.340, both *Δgtf-2* mutants*, and L. brevis* TMW 1.1205 resulted no significant differences in their viscosities.

Table 20. Power law constants of wheat sourdough samples. Listed data were recorded at 20 °C and obtained with a conical concentric cylinder at 5.917 mm gap width.

Strain	K (Pa s^n)	n	r^2
L. brevis TMW 1.2112	35.398	0.650	0.999
L. brevis TMW 1.2320 (Δgtf-2)	26.143	0.693	0.999
L. brevis TMW 1.2113	35.428	0.632	0.997
P. claussenii TMW 2.340	25.728	0.681	0.998
P. claussenii TMW 2.2123 (Δgtf-2)	21.125	0.700	0.998
L. brevis TMW 1.1205	19.715	0.679	0.998

In addition, wheat and rye sourdoughs were analyzed for spontaneously occurring yeast species. In both dough types, yeasts of the species *Candida kefyr*, *Candida krusei,* and *Candida glabrata* were identified by MALDI-TOF MS.

5.2 Tolerance and stress response of beer-spoiling lactic acid bacteria: growth and β-glucan formation

An analysis was conducted on the tolerance of *L. brevis* TMW 1.2112, *P. claussenii* TMW 2.340, and the two *Δgtf-2* mutants against stress factors associated with common compounds of alcoholic beverages, such as iso-α-acids from beer and the polyphenol (+) catechin from wine. In addition, the

effect of ethanol concentration of 5 and 10% (v/v) regarding the survival of the strains was analyzed. Hereby, putative protective effects of the β-glucan capsule were identified and have further clarified the background on the benefits of this EPS. The strains were cultivated in an mMRS medium (adapted to the experiment) with initial $OD_{600\ nm}$ values of 0.1. First, the strains were tested for their resistance to different pH values (Figure 5).

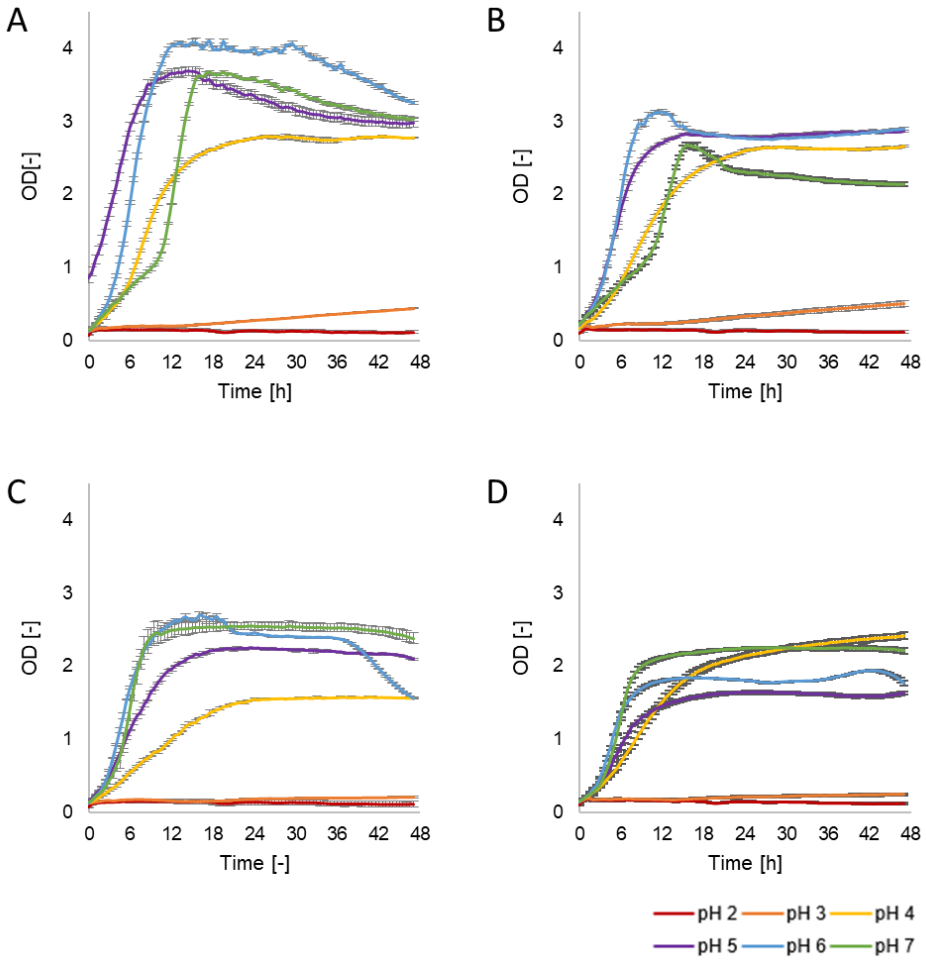

Figure 5. Bacterial growth in mMRS medium with different pH values.
A) L. brevis TMW 1.2112, B) *L. brevis* TMW 1.2320 (Δgtf-2), C) *P. claussenii* TMW 2.340, and D) *P. claussenii* TMW 2.2123 (Δgtf-2). Values are means of biological triplicates.

Here, *L. brevis* TMW 1.2112 resulted in the maximum $OD_{600\ nm}$ values with up to 4.0 at a pH value of 6, while the β-glucan deficient mutant *L. brevis* TMW 1.2320 showed a maximum value of 2.8 at a pH value of 5 and 6 after 24 h. Lower growth rates were observed for both *L. brevis* strains in mMRS media with pH 3 and 4 with an OD value between 0.4-0.5 after 48 h at pH 3. In general, lower OD values were detected for the β-glucan-deficient mutant *L. brevis* TMW 1.2320 at all tested pH values compared to *L. brevis* TMW 1.2112, except at pH 4 with both strains growing to an OD of ~2.7. The β-glucan-producing strain, *P. claussenii* TMW 2.340, reached maximum OD values between 2.2 and 2.5 in mMRS media with pH values from 5 to 7. In contrast, the β-glucan-deficient mutant, *P. claussenii* TMW 2.2123 reached maximum values between 1.6 and 2.3. For mMRS medium pH 4, the β-glucan-deficient

P. claussenii TMW 2.2123 exhibited higher OD values than the wild type. Similar to both *L. brevis* strains low growth was observed at pH 2 and 3 with OD values between 0.1 and 0.3.

Moreover, the effects of iso-α-acids (HHV, Hallertauer Hopfenveredelungsgesellschaft mbH, Mainburg, Germany) on cell growth were analyzed at different concentrations (Figure 6) of this hop bitter compound in an mMRS medium (pH 6.2).

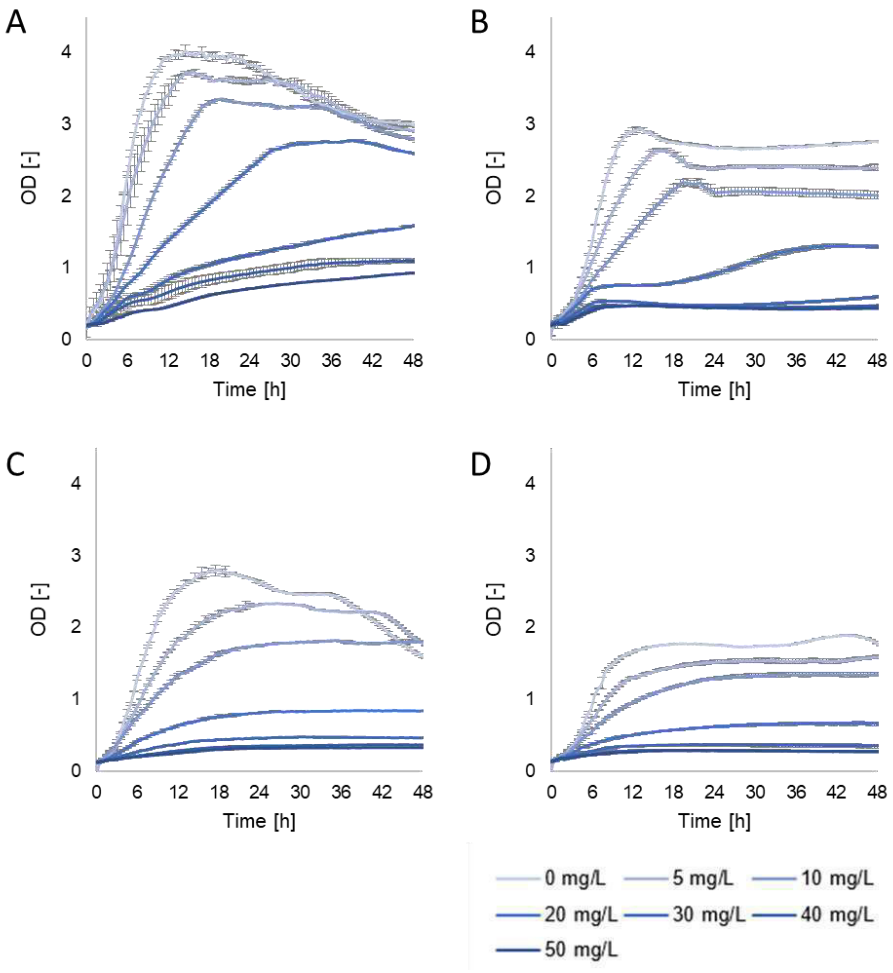

Figure 6. Bacterial growth in mMRS medium (pH 6.2) at different concentrations of iso-α-acids.
A) *L. brevis* TMW 1.2112, B) *L. brevis* TMW 1.2320 (Δgtf-2), C) *P. claussenii* TMW 2.340, and D) *P. claussenii* TMW 2.2123 (Δgtf-2). Values are means of biological triplicates.

L. brevis TMW 1.2112 resulted in maximum OD values of 3.6 (5 mg/L) and 3.3 (10mg/L) after 24 h. Compared to the medium without iso-α-acids, the maximum was 3.8 after 24 h. In comparison, the β-glucan-deficient mutant, *L. brevis* TMW 1.2320, showed OD values between 2.4 (5 mg/L) and 2.1 (10mg/L). Especially at 20 mg/L of iso-α-acids, the differences in growth rates and maximum OD values were remarkable, with more than twice the OD for the wild type (2.4) compared to the mutant (0.9) within 24 h. Both strains showed the lowest growth at a range of 30 mg/L to 50 mg/L. *P. claussenii* TMW 2.340 reached a maximum OD value of 2.3 within 24 h when 5 mg/L iso-α-acids were added and OD 1.7 using 10 mg/L iso-α-acids. In comparison, the OD was 2.3 without the addition of the hop bitter compound. The mutant *P. claussenii* TMW 2.2123 showed values of 1.8, 1.5, and 1.3 at 0 mg/L, 5 mg/L, and 10 mg/L, respectively. At iso-α-acids concentrations of 20 mg/L and higher, both strains showed

significantly lower growth and therefore OD. In comparison, it could be observed that *L. brevis* strains were able to tolerate higher concentrations of iso-α-acids than the *P. claussenii* strains.

Another known compound from wine is (+) catechin (Sigma-Aldrich, St. Louis, MO, USA) and this one was also used to identify the tolerances of these strains against a polyphenol (Figure 7). The maximum OD values of *L. brevis* TMW 1.2112 were OD 4.0 with mMRS (pH 6.2) without (+) catechin and with 100 mg/L (+) catechin, respectively. In comparison, the OD values of *L. brevis* TMW 1.2320 were in general lower, hovering around 2.7 (100 mg/L). With the increasing concentration of 700 mg/L (+) catechin, both strains showed lower growth rates and OD values of 1.8 ± 0.1 after 48 h. Concentrations of 1000 mg/L (+) catechin almost completely inhibited the growth of both strains.

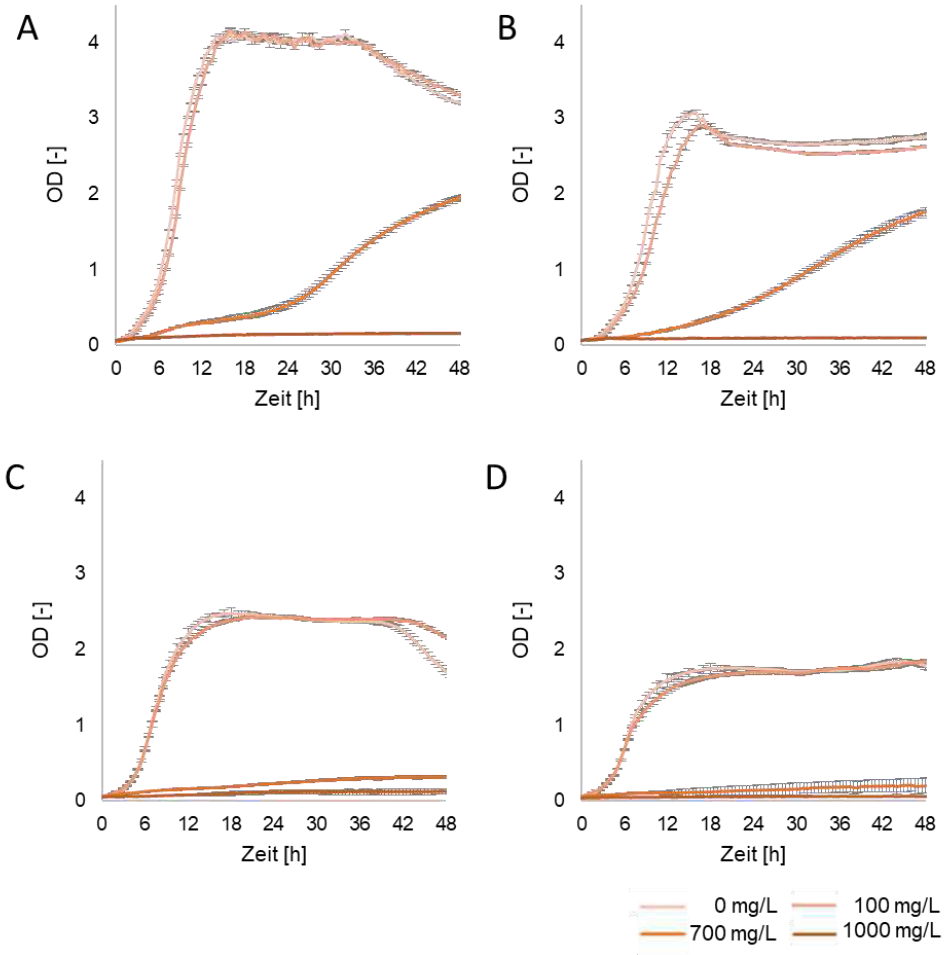

Figure 7. Bacterial growth in mMRS medium (pH 6.2) at different concentrations of the polyphenol (+) catechin.
A) *L. brevis* TMW 1.2112, B) *L. brevis* TMW 1.2320 (Δgtf-2), C) *P. claussenii* TMW 2.340, and D) *P. claussenii* TMW 2.2123 (Δgtf-2). Values are means of biological triplicates.

The OD values of *P. claussenii* TMW 2.340 were approx. 2.4 at concentrations ranging from 0 to 100 mg/L (+) catechin, compared to OD values of 1.7 for the β-glucan-deficient mutant *P. claussenii* TMW 2.2123. At a concentration of 700 mg/L (+) catechin, OD values significantly decreased with both strains and resulted in values of approx. 0.3, whereas with 1000 mg/L, little growth was recorded in *P. claussenii* TMW 2,340, with a maximum OD value of 0.12 after 48 h, a concentration which inhibited the growth of the mutant.

Analyses to determine the effect of mMRS medium spiked with ethanol at 5% and 10% (v/v) concentrations on bacterial growths resulted in reduced cell counts with the higher ethanol concentrations after a 48 h (Figure 8). *L. brevis* 1.2320 resulted in significantly lower cell counts compared to the β-glucan-producing strain, *L. brevis* TMW 1.2112, with both 5% and 10% ethanol concentrations. However, differences between β-glucan-forming and non-forming *P. claussenii* strains were not observed.

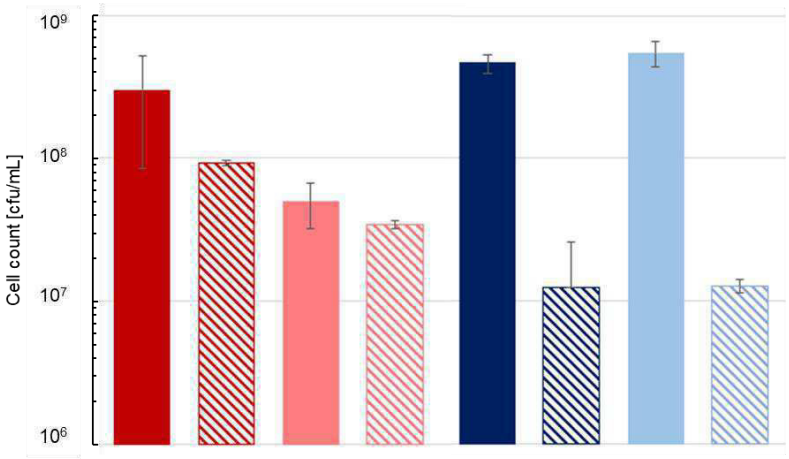

Figure 8. Effect of ethanol on bacterial cell growth.
Cell count of *L. brevis* TMW 1.2112 (▮), *L. brevis* TMW 1.2320 (▮), *P. claussenii* TMW 2.340 (▮), and *P. claussenii* TMW 2.2123 (▮) after 72 h incubation in mMRS medium (pH 6.2) containing 5% (filled) and 10% (striped) ethanol (v/v). Values are means of biological triplicates.

Finally, the percentage of ropy colonies of *P. claussenii* TMW 2.340 when incubated with different stressors was analyzed. In previous experiments, fluctuations in ropy colonies and β-glucan concentrations were observed for this strain but not for *L. brevis* TMW 1.2112. This experiment could help to identify a selection pressure for the maintenance of the β-glucan capsule of *P. claussenii* (Table 21).

Table 21. Effect of different growth media on the share of ropy colonies of *P. claussenii* TMW 2.340.
The sample size was 100 colonies per time point using biological triplicates.

	Non-ropy cfu [%]		
Stressor	**0 h**	**24 h**	**48 h**
mMRS, pH 6.2	3.3 ± 1.6	3.7 ± 3.0	4.0 ± 1.0
mMRS, pH 4.7	4.7 ± 1.5	6.7 ± 2.6	19.0 ± 2.3
Iso-α-acids (20 mg/L)	0.0	1.0 ± 1.5	2.0 ± 0.3
(+) Catechin (400 mg/L)	1.3 ± 0.3	2.0 ± 1.0	2.0 ± 2.0
Rye sourdough (DY 200)	2.6 ± 1.8	5.3 ± 3.1	11.4 ± 2.7
Wheat sourdough (DY 200)	3.4 ± 1.4	5.7 ± 0.3	11.8 ± 3.3

P. claussenii TMW 2.340 was cultivated for 48 h in an mMRS medium, with variations that included additives (pH 6.2) and others that did not, and sourdough samples (back-slopped every 24 h). The share of ropy colonies was then determined from inoculated agar plates. The mMRS medium with a pH of 4.7 and in addition the wheat and rye sourdough samples resulted in an increase of non-ropy colonies with increasing fermentation time. The pH values of the wheat dough were approx. 5.7-6.0 after refreshing

and 3.8 after 24 h. Rye doughs resulted 5.9-5.6 after refreshing and 5.7 after 24 h of fermentation. Within 48 h, the share of non-ropy cfu increased from 4.7 ± 1.5% to 19.0 ± 2.3% when *P. claussenii* TMW 2.340 was cultivated in an mMRS medium with a pH of 4.7. In contrast, mMRS media (pH 6.2) containing iso-α-acids (20 mg/L) and (+) catechin (400 mg/L) resulted in the lowest number of non-ropy colonies in the analyses.

5.3 Enzymatic β-D-glucan degradation using endo- and exoglycanases

The enzymatic hydrolysis of bacterial β-glucan by a commercially available endo-β-1,3-D-glucanase (*Trichoderma* sp.), exo-β-1,3-D-glucanases (*Aspergillus oryzae*; *Trichoderma* sp.), lyticase (*Arthrobacter luteus*), and a β-glucosidase (*Aspergillus niger*) was tested in different experiments (Table 22). Therefore, the β-glucan of *L. brevis* TMW 1.2112 was isolated. The ethanol-precipitated β-glucan was dialyzed by using a 3.5 kDa dialysis tube and subsequently freeze-dried. The D-glucose released from enzymatic digestion was also quantified enzymatically.

In the first experiment, an endo-1,3-D-glucanase (*Trichoderma* sp.) or an exo-β-1,3-D-glucanase (*Aspergillus oryzae*) was used for β-glucan digestion. The β-glucan samples were either thermally or physically pretreated before the actual enzymatic treatment to bring the lyophilized samples back into the solution. This resulted in β-glucan contents ranging from 0.00% to a maximum of 2.04%. Here, it was found that pretreatment with ultrasound and 40 °C of the freeze-dried samples resulted in the highest of the determined values.

Table 22. Enzymatical β-glucan quantification.

	Room temperature		**40°C**		**40°C ultrasonic**		**GEM-Assay**	
	β-glucan content [%]							
Enzyme	pH 4.3	pH 6.2	pH 4.3	pH 6.2	pH 4.3	pH 6.2	pH 4.3	pH 6.2
endo-β-1,3-D-glucanase	0.00 - 0.22	0.44 - 0.72	0.26 - 0.88	0.42 - 0.81	0.11 - 0.29	0.81 - 2.04		
exo-β-1,3-D-glucanase	0.06 - 0,20	0.14 - 0.35	0.00 - 0.06	0.00 - 0.19	0.00 - 0.69	0.30 - 1.05		
Lyticase, exo-1,3-β-D-glucanase and β-glucosidase							3.73	3.76 - 3.90

The same isolated β-glucan samples were used in the β-glucan enzyme assay (GEM assay). Again, pretreatment of the samples was performed in this case by gelatinization; the soaking of the β-glucan could improve the enzyme availability for hydrolytic processes. Compared to the previous method, an increase of 3.7 to 3.9% was measured in β-glucan content. Further, this method demonstrated that pretreatment of isolated β-glucan to improve its solubility had positive effects.

6 Discussion

In recent years, we can increasingly observe the turnaround from processed food and matrices to natural food products without artificial additives. As a result, food fermentations gained renewed attention not only due to sensory and digestibility benefits but also for advantages for the shelf life of the fermented food. This turnaround was also intensified at the latest with the onset of the corona pandemic when people began to engage in the production of their own food. In particular, the trend of sourdough fermentation and people baking their own breads was observed, just by the shortage of flour and baker's yeast in supermarkets. Many of the new self-proclaimed bakers were able to experience how different the qualities of sourdough breads can be, even though they actually do it like everyone else. A reason for this is the sourdough microbiota which plays a central role by influencing the sensory and technological quality of the bread. In particular, EPS-forming LAB can be used for sourdough fermentation to improve the shelf life and delay staling.

The scope of this work was to investigate LAB that can enrich sourdoughs with β-glucan without any other additives. Enriched sourdoughs and breads were analyzed according to structural, technological, sensory, and physiological alterations. For this purpose, different LAB isolates from beer and brewery environments, among others, were analyzed for their applicability as sourdough starters. In addition, exogenous factors influencing the β-glucan production and advantages in growth were studied. Moreover, the intrinsic mechanisms of its formation and especially the feasibility of β-glucan degradation were investigated. Clarifications of these mechanisms were considered necessary to optimize fermentation processes employing β-glucan producing LAB. The previously defined working hypotheses (Chapter 2) were used as the basic framework of this work and will be discussed in this chapter.

6.1 Beta-glucan producing LAB as sourdough starters

As the first step in this work, LAB isolates from beer (Fraunhofer, 2018), sourdoughs (Kern et al., 2014), and meat were re-evaluated for β-glucan-forming properties. The viscosity effects in liquid and solid media were analyzed by increased viscosities of the culture broths and occurrence of ropy colonies, respectively. The presence of *gtf-2* was tested by a PCR method. Llamas-Arriba et al. (2018) demonstrated that *gtf-2*, which codes for a β-1,3 glucan synthases, is highly conserved among β-glucan-forming LAB. Based on these analyses, it was shown that only the beer isolates produced β-glucan. In contrast to the beer isolates, the meat and sourdough isolates analyzed in this study did not exhibit any of these properties, meaning none of them formed any other EPS. The formation of EPS is associated with the protection of bacteria against environmental factors (Caggianiello et al., 2016, Dertli et al., 2016). Therefore, this result is not very surprising since, compared to meat or sourdough, the microorganisms in beer are exposed to harsher conditions, including the ethanol content and hop bittering substances such as iso-α-acids (Shimokawa et al., 2016, Suzuki, 2011). A more extensive discussion of the formation and putative benefits of EPS, especially β-glucan to microorganisms, is provided later in this discussion. Based on the initial analyses, a selection of strains was used for persistence studies in sourdough fermentations. In addition, *L. brevis* TMW 1.2320 and *P. claussenii*

TMW 2.2123 both *Δgtf-2* mutants, were added to the set as negative controls. Using these strains, the effects of β-glucan in sourdoughs could be identified. *L. brevis* TMW 1.2320 resulted from a serial dilution with fructose as the sole carbon source in mMRS medium. The β-glucan deficiency of this strain resulted from an insertion of an IS30-related transposon within the *gtf-2* coding region located on plasmid pl12112-4 (accession: NZ_CP016801). In contrast, *P. claussenii* TMW 2.2123 deficiency was due to the loss of the plasmid pPECL- 7 (accession: CP003144), which carried the *gtf-2* gene (Jakob et al., 2021, Bockwoldt et al., 2020).

6.1.1 The persistence of beer-spoiling LAB in sourdough fermentations

The analyses of the designated strains' persistence in wheat and rye dough fermentations showed that *F. rossiae* TMW 1.2152 was not the dominant strain even after high initial cell counts were used for dough inoculation. The strain was displaced by the endogenous flour microbiota, e.g. *Weissella confusa* and *Leuconostoc citreum* although *F. rossiae* spp. are well-known members of sourdough microbiota (Corsetti et al., 2005). The microbiota of spontaneous sourdoughs often consists of LAB like *Fr. sanfranciscensis*, *La. plantarum*, *L. brevis*, *Leuconostoc citreum*, *Pediococcus* (*P.*) *pentosaceus* and *Weissella confusa*, and yeasts like *Saccharomyces cerevisiae*, or *Candida humillis* (Bartkiene et al., 2020, Leroy and De Vuyst, 2004). Unfortunately, the growth rate of *F. rossiae* TMW 1.2152 was generally low regardless of whether it was inoculated in a cultivation medium, or dough compared to *L. brevis* and *P. claussenii*. The low growth rates therefore were disadvantageous for *F. rossiae* to prevail, and subsequently the spontaneous dough microbiota displaced it. Also *P. claussenii* TMW 2.340 and TMW 2.2123 showed a tendency to be displaced with increasing back-slopping trials (Bockwoldt et al., 2020). The progressive displacement of *P. pentosaceus* by well-adapted sourdough species, e.g., *La. plantarum*, *Limosilactobacillus fermentum*, *Fr. sanfranciscensis*, and *L. brevis* in propagated sourdoughs was already observed in the past (Van der Meulen et al., 2007, Oshiro et al., 2020). However, *P. claussenii* spp. are so far not described as a typical sourdough species. In contrast, the selected *L. brevis* strains proved to be persistent after several back-slopping trials (Bockwoldt et al., 2020). However, *L. brevis* TMW 1.2114 and *L. brevis* TMW 1.2115 were slowly displaced by the endogenous microbiota. Both exhibited altered cell morphologies, which might have caused the lower numbers of ropy colonies and reduced persistence potential compared to other *L. brevis* strains. The altered morphology showed that the cells were exposed to stressors in this environment, which they are not able to cope with.

The adaptability and persistence of *L. brevis* spp. and *P. claussenii* spp., in both wheat and rye sourdoughs, might be related to their isolation source. The tested strains were isolated from beer (Fraunhofer, 2018, Dobson et al., 2002). Comparing sourdough and beer, the basic commonality is the cereal origin of both products with comparable available nutrients. Carbohydrate sources such as maltose, sucrose, glucose, and fructose in wheat and rye flours are about 1.55% and 1.84% in comparison, the wort for brewing beer contains the same carbohydrates with additional maltotriose. However, maltose and maltotriose alone account for up to 70% of the total sugar content, with concentrations of up to 60 g/L of maltose in wort (Martínez-Anaya, 1996, McCance et al., 1945, Preedy, 2011, Brickwedde et al., 2017). The maltose concentration in beer, on the other hand, is much lower

since the yeasts utilize the sugars during the fermentation process. And this results in a residual maltose content in beer between 0.05 and 1.5 g/L, depending on the type of beer (Nogueira et al., 2005, Caballero et al., 2003, Ferreira et al., 2005). In addition to carbohydrate availability, sourdoughs and beer have further similarities, e.g., the pH decreases during the fermentation process and the bacteria living under anaerobic conditions (Hammes and Gänzle, 1998). Beer has even more unfavorable conditions for bacterial growth, since the bacteria must be tolerant to hop bitter compounds, high ethanol concentrations, and carbon dioxide (Sakamoto and Konings, 2003, Suzuki, 2011). Altogether, the investigated *L. brevis* and *P. claussenii* strains showed that they can adapt well to the living conditions in sourdoughs, which also had a positive effect on their persistence in the dough matrices. Their persistence might be reflected in the cell count results, which reached up to 10^9 cfu/g in rye and 10^8 cfu/g in wheat sourdoughs within 24 hours (Bockwoldt et al., 2020). These results are also consistent with those cell densities found in the literature (≥ 10^8 cfu/g dough) of traditional sourdoughs (De Vuyst and Neysens, 2005). Moreover, the high growth rates were reached without the use of any additives to the dough-water mixture, e.g., sugars as a substrate. Accordingly, this corresponds to a clean label product.

6.1.2 Acidification activity of β-glucan-producing LAB

The dominant LAB species in a sourdough that either possesses a hetero- or homofermentative metabolism influences the sensorial and technological properties of the doughs and later breads. As acetic acid contributes to the hardening of gluten, lactic acid, on the other hand, tends to have a positive influence and supports more elastic gluten (Corsetti and Settanni, 2007). Both, wheat and rye doughs inoculated with any of the β-glucan forming LAB resulted in dough acidifications within the optimal range (pH 3.5 and 4.3) (Esteve Collar et al., 1994). The formation of lactate and acetate by LAB was observed by HPLC measurements calculating the FQ (molar ratio lactate/acetate), resulting in heterofermentative *L. brevis* strain ranges between 5.80 and 10.80; and 2.65 and 4.25 in wheat and rye sourdough, respectively. Though the strains *L. brevis* TMW 1.2114 and TMW 1.2115 resulted in the lowest FQ (= higher acetate concentrations) among analyzed *L. brevis* strains in wheat and rye sourdoughs. This might also be associated with their limited cell growth. Wheat sourdoughs fermented with *F. rossiae* TMW 1.2152 had significantly higher FQ than *L. brevis* spp., which in turn means low acetate concentrations, even though *F. rossiae* spp. are heterofermentative LAB (Corsetti et al., 2005). Since *F. rossiae* was not persistent and *L. brevis* TMW 1.2114 and TMW 1.2115 showed the tendency to be displaced during sourdough fermentation and exhibited altered cell morphologies presumably affecting EPS formation, these strains were excluded from further analysis. Sourdough samples inoculated with homofermentative *P. claussenii* contained meager acetic acid amounts compared to lactic acid. These trace amounts of acetic acid were most likely produced by spontaneously growing LAB in the sourdough since *P. claussenii* was slightly displaced by heterofermentative LAB, like *Weissella confusa* and *Leuconostoc citreum* (Bockwoldt et al., 2020).

The FQ is an indicative value for bread flavor and quality. FQ ranges between 1.0 to 9.57 for wheat sourdoughs and 1.5 and 4.0 in rye sourdoughs are usually recommended. However, the studies of Barber et al. 1991 resulted in an FQ of approx. 4.0 for best bread quality, and Hammes and Gänzle (1998) even recommend an FQ between 2.0 and 2.7. (Barber et al., 1991, Spicher et al., 1986, Oura et

al., 1982, Spicher and Stephan, 1987, Hammes and Gänzle, 1998). Though, the FQ can rather be considered as a loose guideline since too many factors influence the mole ratio of lactate and acetate, i.a. the used flour, sourdough starters (homo- and/or heterofermentative LAB), fermentation temperature, DY, external electron acceptors availability, pH value, and fermentation time (Brandt et al., 2004, Gobbetti, 1998, Gerez et al., 2009). Moreover, wheat sourdoughs fermented by *L. brevis* TMW 1.2112 resulted in a lactic acid concentration 6.5 times higher than acetic acid (Bockwoldt et al., 2020). In contrast, levan-producing *Fr. sanfranciscensis* TMW 1.392 resulted in a lactate concentration only 2.5 times higher than acetate in wheat sourdough (DY 200). The levansucrase deletion mutant of *Fr. sanfranciscensis* TMW 1.392, however, resulted in a 1.6-fold decrease in the acetate concentration. Heterofermentative LAB use fructose as electron acceptor to gain additional ATP, forming acetate instead of ethanol. The deletion mutant produced lower fructose concentrations due to the missing hydrolytic activity of the levansucrase which subsequently resulted in lower acetate concentration. Levan production in general requires the availability of sucrose therefore, this sugar needed to be added for the *in-situ* enrichment of the sourdough. These findings imply that sucrose feeding during sourdough fermentation to enable levan production results simultaneously excessive acetic acid formation (Kaditzky et al., 2007). As previously mentioned, acetic acid also contributes to the hardening of gluten which was described to have adverse effects on the gluten network and later bread volume. The residual sweetness of unused sucrose could also have a negative effect on the later product. Consequently, the application of β-glucan-forming LAB avoids any problem of residual sweetness and excessive acetic acid affecting bread taste and quality (Bockwoldt et al., 2020). Especially, *P. claussenii* could exhibit unique technological and sensorial advantages due to its homofermentative metabolism. In summary, the strains *L. brevis* TMW 1.2113, and *L. brevis* TMW 1.2112 with the isogenic mutant *L. brevis* TMW 1.2320, as well as *P. claussenii* TMW 2.340 with the isogenic mutant *P. claussenii* TMW 2.2123, proved to be persistent and do not need substrates such as sucrose for growth.

6.1.3 *In situ* β-glucan formation during sourdough fermentation

In addition to the demonstrable persistence and acidification of the wheat and rye doughs by β-glucan-forming LAB, the *in situ* β-glucan formation and potential rheological effects triggered thereby were of particular importance (Bockwoldt et al., 2020). Since beer-spoiling LAB were previously described to form capsules from the EPS and further form a network-like cell structure, this fact was used for the analyses (Fraunhofer et al., 2018a). Here, the strains with isogenic β-glucan-deficient strains (*L. brevis* TMW 1.2320 and *P. claussenii* TMW 2.2123) were included since the latter were suitable as negative controls for lacking EPS formation. The formation of capsules by the LAB, and the fact that these are β-glucan capsules, were proven by capsule staining and the agglutination test, respectively (Bockwoldt et al., 2020). Bacterial capsules could be visualized by using a negative staining method in combination with counterstaining of cells subsequently observed by electron or light microscopy (Ferreira et al., 2002, Bayer, 1990, James and Swanson, 1977). Using counterstaining, the capsules appear as halos around the cells. The capsule staining of *L. brevis* TMW 1.2112 and TMW 1.2113 demonstrated the presence of such capsules, either isolated from solid cultivation medium or wheat sourdough samples. However, this was not possible for *P. claussenii* TMW 2.340 and the negative controls (*gtf-2*-deficient mutant). For

P. claussenii TMW 2.340, the cell and capsule sizes were most likely responsible that capsules were not observed (Bockwoldt et al., 2020). The agglutination test confirmed that *P. claussenii* TMW 2.340 formed capsules, which was also accounted for *L. brevis* TMW 1.2112 and TMW 1.2113. This assay is based on the similarity between capsular polysaccharide (β-1,3-glucan) of *S. pneumoniae* serotype 37 and the capsular *O2*-substituted (1,3)-β-glucan of LAB (Llull et al., 2001, Werning et al., 2014). None of the negative control strains agglutinated in the presence of the antibodies, confirming that they indeed did not produce β-glucan capsules. Moreover, *L. brevis* TMW 1.2112 resulted in significantly more agglutinated cells compared to *P. claussenii* TMW 2.340, which is presumably related to the decreasing number of ropy colonies of this strain (≤ 40 % of ropy colonies after 24 h) due to the loss of the *gtf-2*-encoding plasmid. In contrast, in sourdoughs with *L. brevis* TMW 1.2112 and TMW 1.2113, the steadiness of β-glucan formation from the ropy colonies was striking, as those were always 100% (Bockwoldt et al., 2020). Dobson et al. (2002), who described the strain *P. claussenii* ATCC BAA-344^T = DSM 14800^T (= TMW 2.340), first already observed the loss of the ability to form ropy colonies derived from the formation of β-D-glucan. They observed the changes of the phenotype during serial cultivations in culture broths. Pittet et al. (2013) analyzed these ropy and non-ropy *P. claussenii* ATCC BAA-344^T (= TMW 2.2123) isolates and observed the loss of the *gtf-2*-encoding plasmid (pPECL-7) and two additional plasmids (pPECL-4 and pPECL-6) within non-ropy isolates.

The ability of the strains to produce β-glucan during sourdough fermentation could be demonstrated (Bockwoldt et al., 2021, Bockwoldt et al., 2020), however the potential of these strains acting as functional sourdough starters, needed analyses focusing on the effects of *in situ* β-glucan production, like dough rheology. Predominately, wheat and rye flours are used for sourdough fermentations and the manufacturing of baked goods, but other types such as amaranth, buckwheat or quinoa are also described (Hammes and Gänzle, 1998, Siepmann et al., 2018). In terms of the β-glucan content, wheat and rye are among low β-glucan cereals with about 0.5-3% β-glucan. On the other hand, barley and oat are rich in β-glucan with more than 10% however, the application of these cereals for dough preparations usually has negative technological effects, e.g., on the bread volume. It was also demonstrated that mixtures of wheat flour with oat flour or barley flour lead to qualitative losses, which were associated with the interference of the fibers on the formation of the gluten network and gas-holding capacity (Lazaridou and Biliaderis, 2007, Gill et al., 2002, Rieder et al., 2012). The β-glucans of barley and oat could be purified by various processes like extrudation (Sharma and Gujral, 2012), hot water extraction (Ahmad et al., 2009), enzymatic starch and protein removal with subsequent ammonium sulfate saturation (Irakli et al., 2004), methanol, ethanol and isopropanol precipitation, combined with coagulation and filtration (Redmond and Fielder, 2006), alkaline extraction (Dawkins and Nnanna, 1993), and others. However, since a lot of energy and/or solvents are necessary to purify the plant-based β-glucans, alternative enrichment processes via *in situ* β-glucan formation during food processing are highly desirable also regarding clean label products (Zhu et al., 2016, Asioli et al., 2017). Therefore, an enrichment of wheat and rye doughs by bacterial β-glucan, like *L. brevis* and *P. claussenii*, was considered as a possibility to save energy, costs, and excessive use of additives.

The impact of the capsular β-glucan on the viscosity of wheat sourdoughs was analyzed by rheological experiments. Significantly increased viscosity effects in wheat sourdoughs were determined for *L. brevis*

TMW 1.2112 and TMW 1.2113 with flow behavior indices (n) of 0.650 and 0.632, respectively (Bockwoldt et al., 2020). A flow behavior index of 1.0 describes Newtonian fluids (e.g., water or alcohol) while indices smaller than 1.0 comprise viscoplastic fluids (Herschel-Bukley model), such as polymer solutions exhibiting time-independent viscous shear-thinning behavior (Hernández-Ortiz, 2004, Sofou et al., 2008). Sourdough, toothpaste, or clay are typical viscoplastic materials since these materials are able to resist flowing until the applied shear stress exceeds the yield stress limit (Bingham model). Which means that these fluids behave like a firm material as long as the shear stress is less than the yield stress. Meaning, the consistency index increases if the polymer concentration increases and the flow behavior index gradually decreases (Song et al., 2006, Skelland, 1967). This also corresponds to the observation that wheat sourdough samples fermented by sourdough isolates *L. brevis* TMW 1.1205 (Kern et al., 2014), *P. claussenii* TMW 2.340, and the β-glucan-deficient mutants (*L. brevis* TMW 1.2320 and *P. claussenii* TMW 2.2123) resulted in higher flow behavior indices (0.679 - 0.700) with no significant rheological effects compared to *L. brevis* TMW 1.2112 and TMW 1.2113 (Bockwoldt et al., 2020). Emphasizing higher polymer concentration in wheat sourdough samples if using *L. brevis* TMW 1.2112 and TMW 1.2113 compared to the other strains. Coda et al. (2018) and Katina et al. (2009) reported the positive effect of other HoPS like dextran in sourdough samples but again sucrose addition was necessary for *in situ* production. In contrast, it was also reported that capsular HePS did not cause significant changes in viscosity of wheat sourdough samples, although such effects were previously observed in liquid media (Galle et al., 2011). According to Fraunhofer (2018), a sticky network of the capsular β-glucan and cells was responsible for increased viscosity effects of liquid cultures rather than just the release of the polysaccharide. This network could thus contribute to the rheological properties of sourdoughs. Moreover, the i*n-situ* enrichment of sourdough samples with EPS of LAB was described as more effective than an artificial enrichment of doughs by EPS (Brandt et al., 2003, Tieking et al., 2003a, Katina et al., 2009). The consequence of artificial enrichment with β-glucan would be the lack of the β-glucan-cell-network and thus potential losses in rheological properties. Therefore, the significant effect of *L. brevis* TMW 1.2112 and TMW 1.2113 on the viscosity of wheat sourdough could be attributed to the *in-situ* formation of such a cell network. This could be a similar effect as previously observed for EPS interacting with proteins of dairy products optimizing the casein network stability (Daba et al., 2021, Hassan et al., 2004, Mende et al., 2016). However, it must also be considered that different LAB species with different morphologies (cocci vs. rods) and metabolisms (heterofermentative and homofermentative) were studied. These properties are likely to have an additional impact on dough rheology next to the EPS formation itself.

The β-glucan-producing strains, as well as the corresponding *gtf-2*-deficient mutants of *L. brevis* and *P. claussenii*, resulted in high cell counts in wheat and rye sourdoughs, acidified the wheat and rye doughs within the characteristic ranges for sourdoughs. Moreover *L. brevis* TMW 1.2112 and TMW 1.2113 caused significant rheological effects by increasing the viscosity of wheat sourdough samples. Furthermore it was demonstrated that the β-glucan did not provide any selective advantage regarding survival or persistence of cells under the prevailing conditions in sourdough (Bockwoldt et al., 2020). This could also explain the increased ejection rate of the *gtf-2*-coding plasmid of *P. claussenii* TMW 2.340 and lower rheological effects. However, this could also simply confirm a low plasmid

stability, as previously described, which in turn is unfavorable for the β-glucan enrichment of sourdoughs (Dobson et al., 2002, Pittet et al., 2013). In order to increase the β-glucan yield during sourdough fermentation or to identify factors influencing it, analyses were carried out in advance to determine potential selection pressures.

6.2 Selective advantages of β-glucan formation

The β-glucan-producing LAB described so far have been isolated from alcoholic beverages, for example, *L. brevis* spp. and *P. claussenii* TMW 2.340 isolated from spoiled beer (Fraunhofer, 2018, Dobson et al., 2002). Beer-spoiling microorganisms are used to unfavorable conditions, including ethanol concentrations between 0.5 and 10% (v/v), hop bitter compounds (17 bis 55 ppm) such as iso-α-acids, carbon dioxide (approx. 0.5%), and low pH values (3.8 - 4.7) (Shimokawa et al., 2016, Suzuki, 2011, Suzuki et al., 2006, Preedy, 2011). It therefore stands to reason that the β-glucan capsules of beer-spoiling LAB could have a protective function as a passive resistance mechanism against antibacterial conditions. Fraunhofer (2018) reported that higher slime formation correlated with lower initial pH values compared to higher initial pH values, further assuming an increased activity of the transmembrane β-1,3 glucan synthase under acidic conditions.

L. brevis TMW 1.2112 and *P. claussenii* TMW 2.340 and the two *Δgtf-2* mutants (*L. brevis* TMW 1.2320 and *P. claussenii* TMW 2.2123) were analyzed according to their pH sensitivity. The assumed protective factor of the β-glucan capsules were determined based on comparisons between the wild type and the mutant. In general, the analyses resulted in lower OD values with both mutants compared to the β-glucan-forming strains. However, higher OD values were most likely related to increased polymer concentrations in samples of *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 (Fraunhofer et al., 2019). Decreases in growth rates were observed for all strains at pH 4 which also represents the lower pH range of beer (Shimokawa et al., 2016). Interestingly *P. claussenii* TMW 2.2123 (*Δgtf-2*) reached higher final OD-values compared to the wildtype at pH 4. In sum, no objective advantage could be determined for capsule-forming strains compared to mutant strains with respect to acidity. At a pH of 3, the growth was significantly decreased however, the strains were able to survive, and at pH 2 cell growth was inhibited. Garai-Ibabe et al. (2010a) reported that *P. parvulus CUPV22* and *Pa.* suebicus CUPV221 were able to survive at pH 3.1 and even 1.8 simulating gastric conditions, respectively. Great interest was given to the identification of a selective pressure to retain the *gtf-2* coding plasmid of *P. claussenii* TMW 2.340 since this strain showed fluctuations in ropy colonies due to the loss of the *gtf-2*-encoding plasmid (Bockwoldt et al., 2020). Cultivations of this strain in media at pH 4.7 and wheat and rye sourdoughs fermentations resulted in the highest proportions (up to 19%) of non-ropy colonies among the conditions tested. Which means that a low pH value did not influence the β-glucan formation or represent a selective pressure to maintain the plasmid.

Furthermore, hop bitter acids such as iso-α-acids as a beer ingredient are known having antibacterial effects. Hop bitter acids act as protonophores disrupting the transmembrane pH gradient of hop-sensitive LAB strains (Sakamoto and Konings, 2003, Behr and Vogel, 2009). The transmembrane pH gradient of LAB represents an important element of the proton motive force (PMF), by coupling energy

generation (ATP) and nutrient transport (Kashket, 1987). The nutrient transport and metabolic processes of the bacteria are influenced by the intracellular pH, which in turn can be lowered if hop bitter acids are present (Poolman et al., 1987). A resulting pH shift is discussed to lower nutrient transport efficiency which could cause the starvation of the bacteria. In addition, the activity of the hop bitter acids is pH-dependent, which means that their activity increases at low pH values (Sakamoto and Konings, 2003). The range of the hop content in beer ranges between 7-55 ppm, with wheat beer having a hop content between 8 and 15 ppm (Preedy, 2011). The tolerance of the β-glucan-forming strains compared to the mutants was analyzed towards iso-α-acids with concentrations between 5 and 50 ppm (= mg/L). *L. brevis* proved to be more tolerant against iso-α-acid than *P. claussenii*. In particular, *L. brevis* TMW 1.2112 reached OD of approx.1, even at concentrations of 50 mg/L iso-α-acid, while the growth of *P. claussenii* TMW 2.340 was more or less inhibited at 30 mg/L. Striking were the different growth rates of *L. brevis* TMW 1.2112 and *L. brevis* TMW 1.2320 at 20 mg/L, which demonstrated a growth-inhibiting effect for the latter. However, such observations were not made with *P. claussenii*. Cultivation experiments with *P. claussenii* TMW 2.340 in iso-α-acid spiked mMRS medium (20 mg/L) resulted in much lower shares of ropy colonies (up to 2%) compared to the pH test. This implied a putative effect on plasmid stability, nevertheless a growth advantage for the wildtype was not observed e.g., by tolerating higher iso-α-acids concentrations compared to *P. claussenii* TMW 2.2123, as it was observed with *L. brevis* TMW 1.2112. Hop-resistant LAB are further known to possess plasmid-encoded HorA (ATP dependent multidrug transporter) and/or HorC (PMF-dependent multidrug transporter). These transporters help to remove hop bitter compounds from the intracellular space and are widespread and almost exclusive among beer-spoiling lactobacilli or pediococci (Suzuki, 2011). *P. claussenii* ATCC BAA-344^T = DSM 14800^T (=TMW 2.340) possess *horA* (horA, plasmid: pPECL-8) but not *horC* (Haakensen et al., 2008). In contrast, *L. brevis* TMW 1.2112 possess *horA* (AZI09_12585, plasmid: pl12112-2) and *horC* (AZI09_12815, plasmid: pl12112-5). The presence of both genes in *L. brevis* and only one in *P. claussenii* might explain the enhanced tolerance of the first against higher iso-α-acid concentrations. Here, it should also be mentioned that the genome sequence of *P. claussenii* TMW 2.2123 proved the presence of *horA*, and *L. brevis* TMW 1.2320 was also harboring the plasmids encoding *horA* and *horB*. Therefore, the observed inhibitory effect with the *L. brevis* TMW 1.2320 can be attributed primarily to the missing capsules, rather than to the mutant missing those genes.

As previously mentioned, β-glucan-forming LAB are isolates from alcoholic beverages, including wine and cider-spoiling pediococci and oenococci (Dols-Lafargue et al., 2008, Walling et al., 2005b, Llauberes et al., 1990). Similar to beer, the growth conditions for bacteria in wine are difficult. The adverse characteristics include low pH, high alcohol content, high sulfur dioxide concentrations, low nutrient densities, and the presence of polyphenols (Bartowsky, 2009, Vaquero et al., 2007, Fischer and Noble, 1994). The flavonoid catechin belongs to the group of polyphenols and can be found in wine, fruits (apple and berries), and cocoa products (Donovan et al., 2006). In red wine, the catechin concentrations could range between 100 and 1500 mg/L (Fischer and Noble, 1994). Catechin and other phenolic compounds can influence bacterial growth and metabolism. Depending on their structure and concentration, these compounds have either an activating or an inhibiting effect on microbial growth (Arakawa et al., 2004, Vaquero et al., 2007, Alberto et al., 2002). The resistance of *L. brevis* and

P. claussenii against (+) catechin was analyzed to identify the role of the capsule against this polyphenol. Again, *L. brevis* proved to be more tolerant, against catechin than *P. claussenii*, which was more or less inhibited at concentrations of 700 mg/L. However, a protective effect or advantage of the β-glucan capsules was not observed. Still, the cultivation of *P. claussenii* TMW 2.340 using (+) catechin-spiked mMRS media (400 mg/L) resulted in much lower shares of ropy colonies (up to 2%) compared to the pH test (up to 19%), similar to the iso-α-acid (up to 2%). Considering previous studies which could not assign the stability to any mechanism, the different observed plasmid stabilities in this study could also be due to chance and not caused by applied stress (Dobson et al., 2002, Pittet et al., 2013).

The antibacterial action of alcohols like ethanol depends on the bacterial phenotype, including attributes such as the cell wall structure, EPS properties, and capsule formation. Ethanol is able to enter the cell by passive diffusion and further disrupt the cell membrane organization resulting in increased membrane permeability and leakage leading to the release of i.a. ions or macromolecules (Man et al., 2017, Russell and Gould, 2012). Since beer and wine contain ethanol concentration with up to 10% (v/v) and 16% (v/v), respectively, the protective function of β-glucan against ethanol was analyzed (Suzuki et al., 2006, Bartowsky, 2009). The cell count of *L. brevis* TMW 1.2320 was significantly lower compared to the wildtype *L. brevis* TMW 1.2112, indicating a protective effect which allowed the wildtype strain to grow to higher cell counts. Again, such effects were not observed for *P. claussenii*. These results correspond to the findings of Jakob et al. (2021) describing that *L. brevis* TMW 1.2112 increased its cell growth at ethanol concentrations of 10% (v/v) with simultaneous higher organic acid production. In contrast, the Δ*gtf-2* mutant *L. brevis* TMW 1.2320 did not show any of the effects but was able to grow at ethanol concentrations of 10% (v/v). In the same study *L. brevis* TMW 1.2112 was described as the dominant phenotype after co-cultivations with *L. brevis* TMW 1.2320 (Δ*gtf-2*), further assuming a selective advantage due to the β-glucan during colonization processes (Jakob et al., 2021).

The identification of a non-strain-specific selective advantage of LAB's β-glucan capsules was so far not accomplished. Although individual interactions with ethanol, iso-α-acids, and pH were observed. One reason for this could be that the evolutionary origin of these bacteria may not be in alcoholic beverages such as beer. At least isolates of *L. brevis* from beer, resulted in a considerable plasmidomal overlap with isolates from insects (Fraunhofer et al., 2019). Further Dimopoulou and Dols-Lafargue (2021) hypothesized that EPS-producing LAB need to survive in between alcoholic beverage production processes. Therefore, they might colonize the gastrointestinal tract of i.a. insects, which potentially represent their natural origin and the origin of spoilage.

6.3 Exogenous factors influencing *in-situ* β-glucan enrichment of sourdoughs

6.3.1 Selection of a suitable β-glucan quantification method

The persistence of several β-glucan producing and non-producing LAB during wheat and rye dough fermentation was demonstrated including beneficial technological effects, but without having actually quantified the amount of β-glucan in the doughs (Bockwoldt et al., 2020). Therefore, a chromatographic (HPLC) method and an immunological assay were evaluated to enable the quantification of β-glucan in sourdough samples. The latter proved to be suitable to analyze β-glucan amounts during sourdough

fermentations. Quantification by HPLC would have been based on the comparison between β-glucan producing and non-producing LAB. However, this was not possible anyway, as no significant differences between these groups were detected by this method (Bockwoldt et al., 2021). The immunological quantification of bacterial β-glucan in cereal-based matrices by ELISA was first described by Werning et al. (2014). This method takes advantage of the similarity of the branched β-1,3 β-1,2 glucan capsules produced by *S. pneumoniae* serotype 37 and the β-glucan of LAB, which leads to the fact that the antibodies of *S. pneumoniae* also react with *O2*-substituted (1,3)-β-glucan of LAB (Llull et al., 2001, Werning et al., 2014). Analyses including other EPS with those antibodies, e.g., curdlan (linear (1,3)-β-glucan), laminarin (*O6*-substituted (1,3)-β-glucan), and xanthan (HePS), demonstrated that laminarin and *O2*-substituted (1,3)-β-glucan were equal competitive (Werning et al., 2014). Since laminarin is a marine-based polysaccharide, this fact could be neglected for the β-glucan quantification in sourdough samples (Becker et al., 2020). Moreover, plain rye and wheat doughs were analyzed for cross-reactions with the *S. pneumoniae* antibody by Western blot. It was demonstrated that the antibodies interacted with components of the uninoculated wheat and rye dough samples. Figure A 1 (IV. Appendix) shows the Western blot which presented several bands in either wheat or rye doughs. Furthermore, sourdoughs of *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 and the two *Δgtf-2* mutants (*L. brevis* TMW 1.2320 and *P. claussenii* TMW 2.2123) were analyzed and revealed similar band patterns. Since all samples, whether fresh or fermented, with or without β-glucan producers, showed similar band patterns, a cross-reaction with components of the wheat and rye flours were assumed. In addition, an ELISA of uninoculated flour-water mixtures confirmed the result of the Western blot. Based on these results, negative controls of uninoculated doughs were used as blanks (Bockwoldt et al., 2021). In contrast, no cross-reactions were observed for barley, oat, and rice samples (Werning et al., 2014). Hereby, a cross-reaction with the plant β-glucan can be largely excluded.

6.3.2 Optimization of β-glucan yields in wheat and rye sourdoughs

By use of the immunological assay, endogenous parameters (inoculum size and cocultivation) and exogenous (temperature and time) were analyzed for their effects on β-glucan-formation by LAB during wheat and rye sourdough fermentation (Bockwoldt et al., 2021). Endogenous and exogenous factors are responsible for the modulation and interaction of the microbiota (LAB and yeasts) in dough fermentations and thus the product quality including flavor, texture, and shelf life (Catzeddu, 2011).

One of the tested parameters dealt with the effects of co-cultivating an α-amylase producing strain (*La. plantarum* TMW 1.2330) with a β-glucan producing strain. The background of this analysis was that due to the α-amylase activity the glucose and maltose concentration in the doughs might increases and thus at the same time the β-glucan formation increases too. Wheat and rye doughs were inoculated with *La. plantarum* TMW 1.2330 and *L. brevis* TMW 1.2112 or *P. claussenii* TMW 2.340, which resulted in significantly lower β-glucan concentrations compared to fermentations without the α-amylase producing strain (Bockwoldt et al., 2021). Further, the addition of 10% maltose to wheat and rye doughs fermented by *L. brevis* TMW 1.2112 demonstrated that β-glucan formation was not limited by substrate availability in both flour types (unpublished data). However, in the co-cultivation experiments employing the *Δgtf-2* mutants (*L. brevis* TMW 1.2320 and *P. claussenii* TMW 2.2123), the previously described phenomenon

of the dominant phenotype could be observed (Jakob et al., 2021). Since the β-glucan producers were represented in higher proportions compared to the mutants, like *P. claussenii* TMW 2.2123 which was three times lower detected as for *P. claussenii* TMW 2.340 when co-cultivated with *La. plantarum*. In conclusion, however, co-cultivation to increase substrate availability can be dismissed, based on the finding that there is no substrate shortage during sourdough fermentation resulting a reduced β-glucan formation.

Furthermore, the effect of the inoculum size was analyzed. It was demonstrated that this factor had no effect on the β-glucan concentrations in the fermented doughs. Only the time course of its formation was affected by means higher inoculated doughs reached earlier the maximum concentration. Moreover, the results demonstrated that the cell count and EPS formation were independent of each other, implying external factors triggering β-glucan biosynthesis. This was particularly noticeable for *P. claussenii* TMW 2.340 since a high plasmid instability still resulted in high β-glucan concentrations (Bockwoldt et al., 2021). Factors accounting for the high production efficiency might include the plasmid copy number, the transcriptional efficiency, and Gtf-2 activity (Degeest and De Vuyst, 2000, Escalante et al., 1998, Grobben et al., 1996, Franz et al., 2006). The repeatedly plasmid loss and high share of non-ropy *P. claussenii* TMW 2.340 colonies could be due to a trade-off evaluating a reduced fitness and keeping the plasmid or increase the fitness by segregationally loss of the plasmid (Dahlberg and Chao, 2003, Turner et al., 1998). At a low frequency of plasmid loss, the part of the population without the plasmid could reproduce faster due to fitness or cell division advantages (De Gelder et al., 2007). Comparing the genome sizes with *L. brevis* TMW 1.2112 having a genome of the size of 2.67 Mb including five plasmids (Fraunhofer, 2018) and *P. claussenii* TMW 2.340 having a genome of the size of 1.98 Mb including eight plasmids (Pittet et al., 2012), this trade-off could be imaginable.

The exogenous parameter which mainly influenced β-glucan formation and therefore the concentration was the fermentation temperature (Bockwoldt et al., 2021). The sourdough samples were either fermented at 25 °C and 28 °C both typical for type I sourdoughs (20 - 30 °C) or at 35 °C typical for type II sourdoughs (30 - 45 °C) (Gobbetti, 1998, De Vuyst and Neysens, 2005, Brandt, 2019). Hereby, species and flour-dependent differences were determined. *P. claussenii* TMW 2.340 resulted the highest β-glucan concentrations with approx. 625 mg/kg dough in rye and approx. 520 mg/kg dough in wheat sourdoughs fermented at 25 °C. These finding corresponded to observations with *P. pentosaceus* which preferred lower fermentation temperatures (≥ 17 °C) for the growth in sourdoughs with dough yields of 200 or even lower (Siepmann et al., 2018). The optimum fermentation temperature of *L. brevis* TMW 1.2112 was 35 °C in wheat (500 mg/kg dough) and 25 °C in rye sourdoughs (approx. 575 mg/kg dough) regarding β-glucan enrichment (Bockwoldt et al., 2021). The optimal fermentation temperature for *L. brevis* spp. (25 and 35 °C) was within the described optimal growth ranges of this species (Orla-Jensen, 1919). The measured β-glucan concentrations of this study are similar to those using *P. parvulus* 2.6 fermenting oat which resulted in approx. 660 mg/L β-glucan (Pérez-Ramos et al., 2017).

Overall, the fermentation temperature significantly influenced LAB's β-glucan formation in sourdoughs. Additionally *L. brevis* and *P. claussenii* produced sourdoughs characteristically of traditional sourdoughs according to the cell counts (≥10^8 CFU/g) and pH range (pH 3.5 - 4.3) even after process adaptations

(Esteve Collar et al., 1994, De Vuyst and Neysens, 2005). Eventually, β-glucan production possess the same restrictions as HePS due to an energy-consuming biosynthesis and its competition with the peptidoglycan biosynthesis. The yield of HePS are usually within the range of 25-600 mg/L, in comparison HoPS can reach 1 to 10 g/L and even 16 g dextran/kg wheat sourdough were reported (Galle et al., 2011, Gobbetti and Gänzle, 2013, Badel et al., 2011, Salazar et al., 2016, Ruas-Madiedo et al., 2010, Hu and Gänzle, 2018). Finally, yields in the range shown observed with HoPS are not to be expected for β-glucan. Nevertheless, a significant increase of the viscosity of wheat sourdoughs with *L. brevis* TMW 1.2112 and increased water binding capacity in rye sourdough breads with *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 were observed (Schlörmann et al., 2022, Bockwoldt et al., 2020). This suggests that even small amounts of β-glucan produce effects similar to those of other hydrocolloids (Tieking et al., 2003b, Arendt et al., 2007).

Previously, longer, and more acidified fermentation conditions, as for type II sourdoughs, were assumed to positively influence β-glucan formation (Bockwoldt et al., 2020). Here, even the opposite could be proven since a slower acidification of the doughs led to higher β-glucan formation. Moreover, it was shown that a prolonged fermentation period led to a significant decrease in the concentration of the *in situ* produced EPS and not an accumulation. Already within 16 hours of sourdough fermentation with *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 the start of β-glucan degradation was observed (Bockwoldt et al., 2021). These results could correspond to those of Mårtensson et al. (2002), who described that within 16 hours the viscosity decreased in an oat-based medium fermented by β-glucan-forming *L. brevis* G-77 and *P. damnosus* 2.6. Besides, *L. brevis* G-77 was described to form additionally an α-glucan next to β-glucan. However, the actual EPS concentrations were missing. Fraunhofer et al. (2018a) hypothesized the utilization of β-glucan as a carbohydrate source by *L. brevis* TMW 1.2112, since after prolonged fermentation periods, a decrease in culture broth's viscosity was observed. This led to the assumption that the EPS was degraded. The identification of several glycoside hydrolases including a β-endo-β-1,3-glucanase (AZI09_02135) and a β-1,3-glucosidase BglB (AZI09_02170) within the genome sequence of *L. brevis* TMW 1.2112 encouraged this theory (Fraunhofer et al., 2018a). However, analyses of the same strain revealed that this strain in fact did not utilize the polymer as an energy source. The β-glucan concentration within culture broth samples did not decrease during the entire fermentation period but rather increased and remained constantly high. However, a decrease in viscosity of the culture broths as described above were also observed (Bockwoldt et al., 2022). This on the other hand, would correspond to the study of Pérez-Ramos et al. (2017). They showed that within 64 hours, no decrease rather than an increase of the β-glucan concentration during oat, rice, and barley fermentation with *P. parvulus* 2.6 could be observed. However, these cereal matrices were heated to 95 °C before *P. parvulus* 2.6 was added to kill contaminations like yeasts and mesophilic bacteria (Pérez-Ramos et al., 2017, Russo et al., 2016). Presumably, endogenous enzymes could also have been inactivated too. The flour-water-mixture used in the study of Bockwoldt et al. (2021) was not pre-treated in anyway and if *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 were not responsible for the β-glucan degradation, the decreased concentrations must have been caused by other factors.

The most plausible contributors for the degradation are endogenous enzymes and/or spontaneously occurring yeasts able to degrade β-glucans (Rieder et al., 2015, Andersson et al., 2004). Rieder et al.

(2015) demonstrated that endogenous β-glucanases from wheat, rye, and barley flour caused a reduction of cereal high molecular weight β-D-glucans. Since cereal and LAB β-glucans are structurally different, this does not necessarily apply to the β-glucan produced by LAB. Cereal β-D-glucans are linear polymers build of β-(1,3) and β-(1,4) linked glucose monomers and the ones from LAB are *O2*-substituted (1,3)-β-glucans. Additionally, yeasts possess β-(1,3)-glucans with β-(1,6) side chains (McCleary and Codd, 1991, Llauberes et al., 1990, Kollár et al., 1997). Exo-β-glucanases hydrolyzing β-1,3- and β-1,6-glucans; endo-β-glucanases hydrolyzing β-1,3- and β-1,4-glucans, and β-glucosidases were described for different yeasts i.a. *Candida* species., *Hansenula anomala* and *Saccharomyces cerevisiae*, (Abd-el-Al and Phaff, 1968, Lopes et al., 2014, So and Rhee, 2010, Strauss et al., 2001). In this study the detected yeast species *Candida kefyr*, *Candida krusei,* and *Candida glabrata* of the wheat and rye doughs might be involved in the degradation of the LAB's β-glucan. The hydrolytic activity of yeasts on e.g. barley β-D-glucan, however, is contradictory. Moriartey et al. (2010) does not attribute any degradative activity to yeasts, while Andersson et al. (2004), Trogh et al. (2004), and Cleary et al. (2007) assumed this on the basis of the decrease of high molecular weight barley β-D-glucan. Further, Abd-el-Al and Phaff (1968) analyzed several yeasts for their hydrolytic activity towards oat β-glucan and β-(1,2)-glucans which turned out negative. Yet, the hydrolytic activity of yeasts on LAB's β-glucan is so far unknown.

In conclusion, the degradation of LAB's β-glucan during sourdough fermentation cannot be clearly assigned to either endogenous flour enzymes or yeasts. However, it was shown that within 24 hours the bacterial β-glucan concentration significantly decreased in sourdough samples, while even after a week the polymer concentration in culture broths was stable, which finally excluded the LAB themselves for utilizing actions on the EPS (Bockwoldt et al., 2021, Bockwoldt et al., 2022).

6.4 Sensorially and nutritional-physiological characteristics of β-glucan enriched sourdoughs

6.4.1 Sensorial analysis of β-glucan enriched baked goods

To determine consumers acceptance of β-glucan-enriched wheat and rye sourdough breads, those were sensorially analyzed by an untrained panel. Sourdough fermented by *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 as well as the isogenic β-glucan-negative mutants were used to prepare wheat and rye sourdough breads (Bockwoldt et al., 2021). Analysis of the β-glucan quantities in sourdough bread showed, that approx. 100 µg/g in wheat (*L. brevis* TMW 1.2112) and even up to 670 µg/g in rye bread (*P. claussenii* TMW 2.340) were detected (Schlörmann et al., 2022). Although no significant differences between wheat and rye sourdough breads with and without bacterial β-glucan were detected, the comparison between breads processed with *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340, and control bread samples (prepared without sourdough), were to some extent significant. The overall acceptance and airiness of wheat sourdough bread fermented with *L. brevis* were significantly higher than the ones with *P. claussenii* or the control (Bockwoldt et al., 2021). The increase of the dough viscosity due to *in situ* β-glucan formation was already demonstrated for *L. brevis* TMW 1.2112 and TMW 1.2113 (Bockwoldt et al., 2020). The positive influence of bacterial EPS on bread

volume, which has been described several times, might explain the described crumb airiness from the sensory analysis (Arendt et al., 2007, Brandt, 2007, Poutanen et al., 2009, Gänzle et al., 2007). On the other hand, rye sourdough breads with *L. brevis* resulted a sourer taste than those with *P. claussenii* (Bockwoldt et al., 2021). This is most likely associated with the homofermentative metabolism of *P. claussenii* compared to the heterofermentative metabolism of *L. brevis* which produces acetic acid next to lactic acid (Simpson and Taguchi, 1995, Damiani et al., 1996). These metabolisms associated differences (homo- and hetero-fermented) of rye sourdough breads were previously assessed by a trained panel. It was found that the overall flavor intensity of heterofermented rye sourdough bread samples including *L. brevis* spp. was more appreciated than homofermented bread samples (Banu et al., 2011, Lotong et al., 2000). Basically, both lactic and acetic acid contribute to bread quality, either technologically or sensory. Further it was described that a balanced ratio of lactate and acetate contributes to the costumer's taste experience (Leroy and De Vuyst, 2004, Korakli et al., 2003, Corsetti and Settanni, 2007). In conclusion, the sensory analysis proved that sourdough breads produced with both β-glucan producing LAB, *L. brevis* and *P. claussenii*, were accepted by the panel and even contributed significantly to the sensorial quality of the product.

6.4.2 Nutritional-physiological analysis of bacterial β-glucans

The health-beneficial effects of β-glucans from LAB have already been extensively described including prebiotic properties (Russo et al., 2012, Pérez-Ramos et al., 2017), cholesterol-binding capacity (CBC) (Mårtensson et al., 2005, Mårtensson et al., 2002), immunoregulatory effects (Notararigo et al., 2014, Šandula et al., 1999), and anti-Inflammatory effect (Notararigo et al., 2022). The study of Schlörmann et al. (2021) analyzed the health-beneficial effects from β-glucans produced by *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 i.a. CBC, chemopreventive potential associated with the production of short-chain fatty acids (SCFA) and ammonia concentrations, as well as their influence on the composition of the bacterial community during *in vitro* fermentations. This study also included i.a. β-glucans from oats and barley as well as *Δgtf-2* mutants cell preparations (*L. brevis* TMW 1.2320 and *P. claussenii* TMW 2.2123) (Schlörmann et al., 2021).

Beta-glucans from oat and barley resulted the highest CBC effects (Schlörmann et al., 2021) which were analyzed by means of the solubility of cholesterol in artificial micelles (Nagaoka et al., 2001, Lin et al., 2018). Health claims have been defined for β-D-glucans from oats and barley, stating that they have a positive effect on the regulation of cholesterol levels in the blood, reduce the risk of coronary heart diseases, and reduce the increase in blood glucose levels after a meal, but these effects are dependent on the consumed quantities (3 g/day to 4 g/meal) (EFSA Panel on Dietetic Products and Allergies, 2011, EFSA Panel on Dietetic Products and Allergies, 2009, FDA, 2022). The cholesterol-lowering properties of β-glucans from oats and barley result from viscosity increase of the digestive pulp, which offers an increased binding and excretion effect of bile acids. New cholesterol is synthesized in the liver, by means of cholesterol 7α-hydroxylases using plasma cholesterol (Andersson et al., 2002, Anttila et al., 2004, Ellegård and Andersson, 2007, Lia et al., 1997). Similar to those effects of the plant-based β-glucans were the effect with *L. brevis* TMW 1.2112, however, the mutant also showed CBC. In comparison *P. claussenii* spp. resulted significant lower CBC. Since the mutants showed also CBC effects it was

assumed that components of the peptidoglycan might have been interacting with the artificial micelles of the CBC analysis (Schlörmann et al., 2021) as such effects have already been described for LAB cells and cell lysates (Kamal et al., 2015, Tsai et al., 2014).

The chemopreventive potential was analyzed using an *in vitro* fermentation to analyze the pH reduction indicating SCFA (acetate, butyrate, or propionate) formation, reduction of the ammonia concentration, and growth inhibition in LT97 colon adenoma cells including induction of caspase-3 activity in those cells caused by β-glucan samples or cell preparations. Fermentations of mutant cell preparations (*L. brevis* TMW 1.2320 and *P. claussenii* TMW 2.2123) and LAB's β-glucans caused only none to slight pH reductions, respectively. Nevertheless, higher levels of SCFA with a shift towards propionate were determined with LAB's β-glucans, compared to mutant cell preparations. Unfortunately, high ammonia concentrations were also determined (Schlörmann et al., 2021). Ammonia is associated with negative effects in the colon, including tumor-promoting potential (Hughes et al., 2008, Windey et al., 2012). Nevertheless, SCFA formation and higher molar ratios of butyrate and propionate are associated with chemopreventive effects, with butyrate as a more effective SCFA (Hamer et al., 2008, Scharlau et al., 2009, Beyer-Sehlmeyer et al., 2003, Kautenburger et al., 2005). In comparison oats and barley β-glucans reduced the pH value, formed SCFA with a shift towards butyrate, and reduced the ammonia concentration. These differences are most likely the result of the low purity of isolated β-glucan samples from *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 which were contaminated with proteins and promoting proteolytic fermentation instead of saccharolytic fermentation (Hamer et al., 2008, Hughes et al., 2008, Windey et al., 2012, Louis and Flint, 2017). Furthermore, the enhanced induction of growth inhibition in LT97 colon adenoma cells was determined for the β-glucan samples. However, since the blank samples (without β-glucan or cell preparation) showed also strong effects, it was hypothesized that other fermentation products might possessed synergistic effects (Schlörmann et al., 2021). The 'suppressing agent' activity regarding growth inhibition in LT97 colon adenoma cells is mostly granted to butyrate (Kautenburger et al., 2005, Barnard and Warwick, 1993, Schlörmann et al., 2015), which was present in higher concentrations within fermented samples of oat and barley β-glucans (Schlörmann et al., 2021). Induction of the capsase-3 activity, a marker for apoptosis, can also lead, at least in part, to a reduction in LT97 colon adenoma cell numbers. Suppressing agents like butyrate are mediators of these chemopreventive effects since they act as histone deacetylase inhibitors (Hamer et al., 2008, Scharlau et al., 2009, Hinnebusch et al., 2002). Consistent with this, the highest induction of capsase-3 activity was determined in the samples with highest butyrate contents namely oat and barley β-glucans. Still, comparable effects were measured for LAB's β-glucans and cell preparations, whereby the induction using cell preparations was in part significantly lower (Schlörmann et al., 2021).

In addition, the modulation of the bacterial community during *in vitro* fermentation was analyzed to determine the prebiotic potential of the bacterial β-glucans. Therefore, Synergy1® an inulin-type oligosaccharide was used as the positive control. Several therapeutic, immunological, and prebiotic effects are associated with inulin such as bifidogenic effects (prebiotic), anticarcinogenic effect (reducing colon cancer risk), and enhancement of immune response (Wang et al., 2019, de Almeida Gualtieri et al., 2013). Most of the samples showed slight increases of *Bifidobacteriaceae* and only Synergy1® led

to a significant increase. Thus, no significant difference was detected between plant β-glucans, bacterial β-glucans and the cell preparations (Schlörmann et al., 2021).

The study of Schlörmann et al. (2021) analyzing health-promoting effects of β-glucans from *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 was limited due to low purity (≥ 23%) of the isolated EPS. These contaminations could be attributed to the capsular presence of the β-glucans, since larger amounts of proteins were detected within the β-glucan samples. In addition, the composition of the cultivation media might have an impact too. Investigations to reduce protein contaminations focused therefore on the role of the cultivation media and its initial pH value as well as posttreatments after the ethanol precipitation of the EPS. Table A 1 (IV. Appendix) includes the results of these analyses demonstrating the impact of the cultivation medium on the isolated β-glucans' purity. This effect was even observed visually from the coloration of the isolated polymer (Figure A 2, IV. Appendix). Moreover, attempts to reduce the protein contamination via enzymatic digestion resulted in even higher protein concentrations than without these treatments. On the other hand, posttreatments using perchloric acid and urea, for protein precipitation, improved the purity, however, also led to lower yields. Werning et al. (2014) described moderate losses of up to 20% to 38% β-glucan already during β-glucan precipitation using ethanol. These losses resulted from non-precipitable low molecular weight EPS (Werning, Pérez-Ramos, Fernández de Palencia, et al., 2014). Based on this, it would now be concluded that stronger purification processes of the β-glucans, might improve the effects in health beneficial studies. However, the following must be considered, the purification process of bacterial EPS, could affect its structure by the use of aggressive chemicals and physical forces (Goh et al., 2005, Mende et al., 2016), as has been done in the scope of this work (Bockwoldt et al., 2021, Bockwoldt et al., 2022, Schlörmann et al., 2022, Schlörmann et al., 2021). Furthermore, Thondre and Henry (2011) showed that lower molecular weight β-glucan indeed was not effective to lower the glycemic response. Further Lin et al. (2018) reported that higher CBC were observed with higher molecular weight β-glucans. Therefore, increased purification approaches of β-glucan through additional purification steps or use of additional chemicals could also result in β-glucans with reduced health-promoting effects.

In a follow-up study, therefore, more native conditions were selected, i.e., β-glucan enriched sourdoughs and sourdough breads. These sample were analyzed for chemopreventive potential and the modulation of the bacterial community. It was shown that comparable chemopreventive potentials and bacterial communities emanate from wheat and rye sourdoughs and breads, with and without β-glucan. This refers once again to the low yield of this polymer, which causes no measurable effects in the overall product analyzed by *in vitro* fermentation. Nevertheless, chemopreventive and prebiotic effects could also be identified for sourdoughs and breads fermented by *L. brevis* spp. and *P. claussenii* spp. (Schlörmann et al., 2022).

6.5 The biosynthesis and degradation of bacterial β-glucan - molecular mechanisms of *L. brevis* TMW 1.2112

Several pathways for β-glucan biosynthesis of *L. brevis* TMW 1.2112 were already postulated using different substrates e.g., maltose, glucose, or galactose. Since maltose was already described as the

favorable sugar for β-glucan biosynthesis (Fraunhofer et al., 2018b), the pathway using this substrate was analyzed by proteomic studies. In sum, the postulated metabolic pathway for maltose as the only carbohydrate source could be confirmed as illustrated in Figure 9, A (Bockwoldt et al., 2022). Previously enzymes involved in the β-glucan biosynthesis of *P. parvulus* 2.6 were described and included enzymes similar to those described for *L. brevis* TMW 1.2112 (Velasco et al., 2007). The study also reported that maltase and maltose phosphorylase activities were detectable regardless of the used carbon source, although higher activity levels were observed with maltose. Further, α-phosphoglucomutase was more active in β-glucan biosynthesis using a cell-free extract as β-phosphoglucomutase and the resulting α-glucose-1-phosphate was the intermediate for nucleotide-activated sugars synthesis and thus β-glucan biosynthesis (Velasco et al., 2007). Through proteomic studies in total three operons associated with the polymer biosynthesis were identified for *L. brevis* TMW 1.2112 including ORF of major facilitator superfamily (MFS) a maltose transporter MalT (AZI09_10325), maltose phosphorylases (MP) (AZI09_10320 and AZI09_04670), transcriptional regulator MalR (AZI09_10330), and two β-PGM (AZI09_04665 and AZI09_02330) (Figure 9, B).

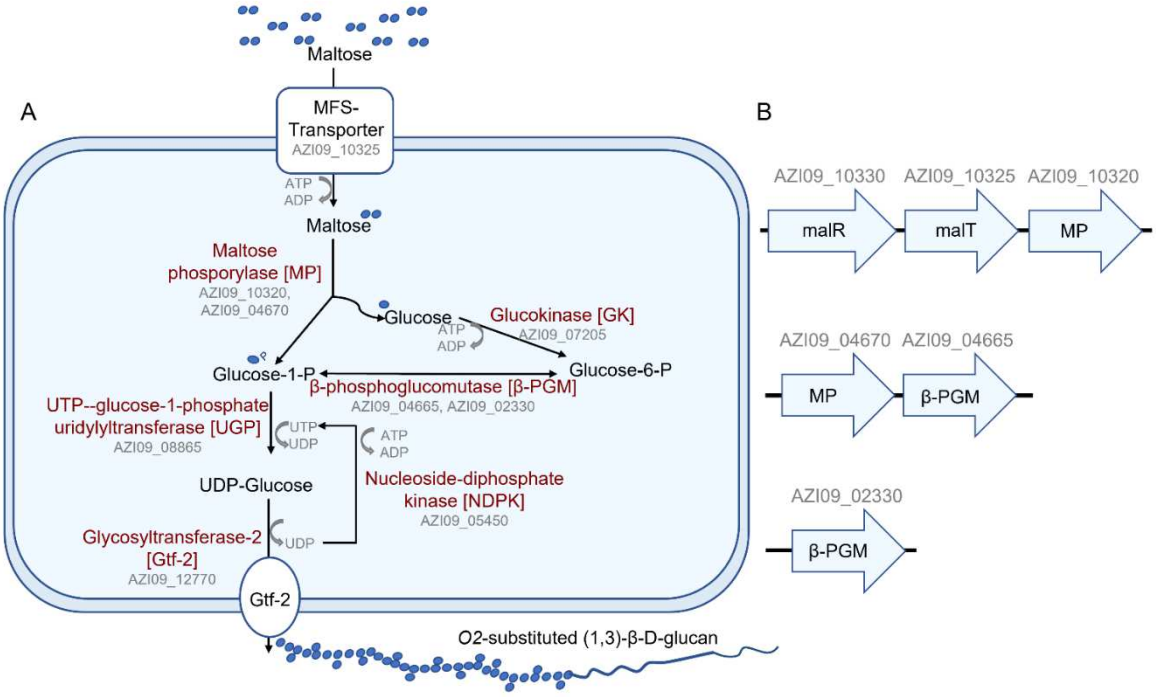

Figure 9. β-glucan biosynthesis of *L. brevis* TMW 1.2112.
A) Metabolic pathway of the β-glucan biosynthesis as suggested from genomic data and the proteomic analysis. B) Suggested maltose operons with malR = transcriptional regulator, MP = maltose phosphorylase, malT = MFS maltose transporter, and β-PGM = β-phosphoglucomutase (Bockwoldt et al., 2022).

The expression of two MP (AZI09_04670 and AZI09_10320) was detected during fermentation and further MP (AZI09_04670) correlated with changes of viscosity and D-glucose concentrations (Bockwoldt et al., 2022). Bockwoldt et al. (2021) previously reported an increase of the D-glucose amounts during sourdough fermentation using *L. brevis* spp. similar to the one observed in the study of Bockwoldt et al. (2022). This leads to the assumption that enzymes of those operons are probably also expressed during the sourdough fermentations further contributing to the *in situ* β-glucan formation.

Since low β-glucan concentrations were determined during sourdough fermentation (Bockwoldt et al., 2021), the findings of Velasco et al. (2007) with α-phosphoglucomutase and/or α-UDP-glucose pyrophosphorylase identified as the putative bottlenecks in β-glucan biosynthesis, this might help to improve β-glucan yields by using biotechnological approaches. However, the use of genetically modified LAB in food fermentation is rather unwanted as the authorization processes are complex, costly, and time-consuming ((EFSA), 2011).

The start of the β-glucan production during the exponential phase as observed with *L. brevis* TMW 1.2112 was already reported for *P. parvulus* spp. and *P. damnosus* lOEB8801 (Walling et al., 2005a, Velasco et al., 2006). Eventually, the expression of Gtf-2 of *L. brevis* TMW 1.2112 was constant regardless of cell number and growth phase, which is consistent with the characteristics of a constitutive enzyme (Bockwoldt et al., 2022). This characteristic was also observed with the previous named β-glucan forming LAB (Velasco et al., 2009, Velasco et al., 2006, Garai-Ibabe et al., 2010b). In addition, the culture broth viscosities and β-glucan concentrations of these LAB strains did not correlate (Bockwoldt et al., 2021, Bockwoldt et al., 2022, Fraunhofer et al., 2018a, Mårtensson et al., 2003, Pérez-Ramos et al., 2017). The Gtf-2 or β-1,3 glucan synthase (EC 2.4.1.34) was described as the key enzyme in β-(1,3)-D-glucan biosynthesis (Werning et al., 2006, Notararigo et al., 2014, Fraunhofer et al., 2018a, Walling et al., 2005b, Pittet et al., 2013). A study by Llamas-Arriba et al. (2018) demonstrated that the gene sequences of transmembrane β-1,3 glucan synthases are highly conserved, namely those from *Pediococcus* spp. i.a. *P. claussenii* TMW 2.340, *Oenococcus oeni* sp. and *Lactobacillus* spp. i.a. *L. brevis* TMW 1.2112. The high genetic similarity of the different LAB species might explain the similarity in β-glucan formation and characteristics.

As previously mentioned, is the presence of β-glucan capsules known for *L. brevis* spp., *P. parvulus* spp., *P. claussenii* sp., and *O. oeni* spp. (Llamas-Arriba et al., 2018, Dols-Lafargue et al., 2008, Garai-Ibabe et al., 2010a, Fraunhofer et al., 2018a, Dobson et al., 2002, Lonvaud-Funel et al., 1993, Dueñas-Chasco et al., 1997, Dueñas-Chasco et al., 1998). However, the attachment and involved structures or proteins linking the β-glucan capsules to the cell surface, covalently or loose, are rather unknown. The genome sequence of *L. brevis* TMW 1.2112 encodes for three LytR-Cps2A-Psr (LCP) family proteins, two of them, BrpA (Biofilm regulatory protein A) (AZI09_03640 and AZI09_03715), were constantly expressed (Bockwoldt et al., 2022). LCP proteins are described to be involved in linking polysaccharides to the peptidoglycan of Gram-positive bacteria. However, to date the role of LCP in *Lactobacilli* is not fully understood. Still, the capsule formation of other EPS from LAB has been described, involving proteins of the Wzy pathways which might interact with the LCP protein family (D'Abrosca et al., 2018, Bitoun et al., 2012, Bitoun et al., 2013, Whitfield et al., 2020). It was assumed, that the flippase (Wzx), polymerase (Wzy), and chain-length regulator (Wzz)) might be involved in the coupling of CPS to the cell surface and lipid-bound CPS precursors are used as substrates for LCP-enzymes which catalyze the ligation between CPS and the peptidoglycan layer (Whitfield et al., 2020, Islam and Lam, 2014). The genome sequence of *L. brevis* TMW 1.2112, for instance, does not contain any Wzy pathway genes, but it does encode a chain length determining protein (AZI09_03645) and an EPS biosynthetic protein (AZI09_03650). Furthermore, both proteins are encoded next to the expressed LCP protein (AZI09_03640). However, both proteins were not detected by MS (Bockwoldt et al., 2022).

Since the β-glucan was also detected in the supernatant of culture broths lacking the cell pellet, it must be at least partially released to the environment. This in fact could be due to an abortive Wzy polymerization step, incomplete ligation via LCP, absence of LCP function, or during growth when cell walls reorganize (Whitfield et al., 2020). The role of LCP and Wzy in capsule formation and binding processes to the cell surface in *L. brevis* TMW 1.2112 remain unclear. Despite this, these are interesting new insights in capsular structures of EPS from LAB.

Adhesion processes of commensal lactobacilli were described to employ moonlighting proteins like GADPHs, EF-Tu, and enolases even contributing to probiotic effects (Wang et al., 2014, Ramiah et al., 2008, Hurmalainen et al., 2007, Siciliano et al., 2008). Members of these enzyme group and other known moonlighting proteins were detected in the secretome of *L. brevis* TMW 1.2112 at a very early stage (8 hours in fermentation) (Bockwoldt et al., 2022). Interestingly, these enzymes are commonly intracellular located and associated to glycolytic and metabolic pathways or as molecular chaperones (Singh and Bhalla, 2020). Moreover, four typical moonlighting genes (enolase (AZI09_08765), triose-phosphat isomerase (AZI09_08770), phosphoglycerate kinase (AZI09_08775) and glyceraldehyde-3-phosphate dehydrogenase (AZI09_08780)) were arranged within one operon (Bockwoldt et al., 2022). An example of such a moonlighting operon was previously reported for *Staphyloccocus aureus* (Hemmadi and Biswas, 2021). Moonlighting proteins are generally described to be highly conserved whether in pathogenic or probiotic bacteria (Wang et al., 2014). Assuming that those genes in *L. brevis* TMW 1.2112 are in fact moonlighting proteins, which might be involved in adhesion processes. Lastly, the actual role of these genes on the formation or stabilization of the β-glucan-cell-network or in adhesion processes to surfaces was not possible to resolve and needs further investigations

Previous studies described the decrease of EPS concentrations with increasing fermentation periods of LAB either through enzymatic activity or physical parameters (Cerning et al., 1992, Vuyst and de Ven, 1998, Dierksen et al., 1995, Zannini et al., 2016, Degeest et al., 2002). In contrast, LAB lacking hydrolytic enzymes to degrade the EPS formed by them were also described (Badel et al., 2011, Patel et al., 2012). As mentioned earlier in this work, *L. brevis* TMW 1.2112 was not able to degrade β-glucan itself according to ELISA analysis (Bockwoldt et al., 2022). However, enzymes described to be involved in the degradation of other EPS are glycohydrolases also called glycoside hydrolases (GH) such as α-D-glucosidase, β-D-glucosidase, α-D-galactosidase, or β-D-galactosidase (Degeest et al., 2002, Cerning et al., 1992). Enzymes from the more than 170 GH families are engaged in enzymatic hydrolysis of glycosidic bonds in carbohydrates and linkages to non-carbohydrate parts. These families are classified based on their amino acid sequences (Koshland Jr., 1953, Naumoff, 2011, Drula et al., 2022, Henrissat, 1991, Henrissat and Bairoch, 1993, Lombard et al., 2014). Enzymes which are described to be specifically engaged in β-glucan degradation are i.a. endo-β-glucanases, exo-β-glucanases, or β-glucosidases (Danielson et al., 2010, de la Cruz et al., 1995, Lopes et al., 2014, Rieder et al., 2015, Quéméner et al., 2007). For example, the GH 1 and the GH 3 families comprise exo-β-1,4-glucanase (EC 3.2.1.74); β-glycosidase (EC 3.2.1.-); glucan-1,4-β-glucosidase (EC 3.2.1. 74); exo-1,3-1,4-glucanase (EC 3.2.1.-); β-1,2-glucosidase (EC 3.2.1.-); β-1,3-glucosidase (EC 3.2.1.-); lichenase / endo-β-1,3-1,4-glucanase (EC 3.2.1.73) (Henrissat and Bairoch, 1993, Henrissat, 1991, Lombard et al., 2014, Drula et al., 2022). Fraunhofer (2018) assumed that the putative β-glucan degradation observed for

L. brevis TMW 1.2112 could be induced by chromosomal encoded β-endo-β-1,3-glucanase (AZI09_02135) of the GH 8 family and/or a β-1,3-glucosidase BglB (AZI09_02170) of the GH 3 family. Therefore, the enzymatic hydrolysis of isolated β-glucan samples from *L. brevis* TMW 1.2112 was performed using some of the potentially β-glucan-active enzymes described previously. In the first step, the solubility and thus the availability of the β-glucan was improved, which could be achieved by gelatinization (Danielson et al., 2010), ultrasound (Wang et al., 2010), or temperature (McCleary and Codd, 1991). The combination of 40 °C and ultrasound to dissolve isolated β-glucan samples resulted in only approx. 2.0% and using the gelatinization approach approx. 3.9% β-glucan could be determined. Further, the ultrasound treatment of water-insoluble β-glucan could dissolve impurities from the β-glucan network (Šandula et al., 1999) and lead to the depolymerization of biopolymers (Szu et al., 1986) which might have affected the analysis. In addition, enzymes used for this approach derived from other species such as *Trichoderma* sp. (endo-β-1,3-D-glucanase) with a low 1,3- and 1,4-β-D-glucan specificity (Llauberes et al., 1990). The higher β-glucan concentrations of the gelatinized samples were most likely due to the multiple step enzymatic digestion of the GEM assay employing a lyticase, exo-1,3-D-glucanase, and β-glucosidase. However, an increased resistance of bacterial β-(1,3)-D-glucan compared to other β-(1,3)-glucans towards exo-(1,3)-β-glucanase from *Trichoderma* sp. was already reported (Llauberes et al., 1990). The enzymatic degradation of the isolated β-glucan by means of commercially available enzymes resulted in approx. 4% theoretical β-glucan content. This indicated an incomplete and/or poor enzymatic hydrolysis of the isolated EPS.

Due to the unsatisfactory results with commercially available enzymes from other species, the β-glucosidase BglB from *L. brevis* TMW 1.2112 was heterologously expressed (Bockwoldt and Ehrmann, 2022). Beta-glucosidases could be assigned to both GH1 and GH 3 families. The β-glucosidases of both families retain the anomeric configuration of the substrates and often exhibit a $(\beta/\alpha)_8$-barrel structure (Naumoff, 2006, Rigden et al., 2003). GH 3 β-glucosidases can act as exo-enzymes and thus hydrolyze terminal, non-reducing β-D-glycosyl residues including β 1,2-: β 1,3-; β-1,4-; β-1,6-linkages. The hydrolytic activity would result in the release of β-D-glucose (Harvey et al., 2000, Cournoyer and Faure, 2003). BglB exhibited a molecular mass of 83.5 kDa and a strong preference for substrates with β-glycosidic linkages (Bockwoldt and Ehrmann, 2022). This preference and other kinetic properties were already reported for β-glucosidases of other *L. brevis* strains (Michlmayr et al., 2010a, Michlmayr et al., 2010b, Michlmayr et al., 2015, Zhong et al., 2016a, Zhong et al., 2016b) or other LAB like *O. oeni* spp. (Dong et al., 2014, Gagné et al., 2011, Mesas et al., 2012) and Bifidobacteria (Jung et al., 2012, Matsumoto et al., 2015, Michlmayr et al., 2015). The gene sequences of these LAB were used for phylogenetic studies, to gain knowledge about a connection between β-glucosidases and EPS formation and subsequently its degradation. However, the analysis demonstrated that the clustering of the β-glucosidases based on the species and not the ability to produce EPS (Bockwoldt and Ehrmann, 2022). Moreover, BglB as an intracellular enzyme was most likely an exo-acting enzyme hydrolyzing the terminal, non-reducing β-D-glycosyl residues of substrates. Due to this limited hydrolytic activities, only small amounts of D-glucose could be expected (Bissaro et al., 2015), as it was observed in this study (Bockwoldt and Ehrmann, 2022). In addition, it is reasonable to assume that multiple enzymes are involved in the degradation of extracellular high molecular weight β-glucan and that BglB cleaves smaller

molecules at later stages of the polymer degradation (Degeest et al., 2002, Pham et al., 2000). Finally, even though the onset of β-glucan degradation was observed with BglB which could have progressed with longer incubation times, *in vivo* analyses identifying all enzymes involved in the putative bacterial β-glucan degradation, is more appropriate, also considering solubility and structural integrity of the β-glucan. At least structural changes due to viscosity decreases with *L. brevis* TMW 1.2112 were observed even though the actual degradation could not be determined by means of ELISA (Bockwoldt et al., 2022). Consequently, this indicated some degradation or at least structural changes in the EPS, which might be initiated by so far unknown enzymes.

The hydrolytic activity towards β-glucan was observed under native conditions during different growth phases using *L. brevis* TMW 1.2112 in proteomic studies (Bockwoldt et al., 2022). Most strikingly, *L. brevis* TMW 1.2112 showed a continuous increase in β-glucan concentration during fermentation which did not decrease as observed with other β-glucan-forming LAB (Garai-Ibabe et al., 2010a, Walling et al., 2005a, Velasco et al., 2006). However, only *L. brevis* TMW 1.2112 cultures caused a significant decrease in viscosity after 4 days, while other LAB such as *Pediococcus* strains and *Pa. suebicus* caused further increase of the culture broth's viscosity even after 7 days or longer (Garai-Ibabe et al., 2010a, Dueñas et al., 2003). Nevertheless, the hypothesis of Fraunhofer (2018) regarding the enzymatic β-glucan degradation by *L. brevis* TMW 1.2112 β-endo-β-1,3-glucanase (AZI09_02135) or β-1,3-glucosidase BglB (AZI09_02170) could not be confirmed. The sequence of the endo-β-1,3-glucanase contained two stop-codon mutations which might have affected enzyme activity or its secretion since it was only detected after 4 days within cellular proteome samples. And the β-1,3-glucosidase (BglB) resulted an inverse correlation with the D-glucose concentration, which means it cannot be associated with an increase of D-glucose due to EPS degradation. In addition, the structural changes of the fermentation broth observed during progressing fermentation time could not be attributed to one or more of the previously identified 49 characterized GT and GH. In particular, the secretome, which contained several uncharacterized proteins and putative enzymes, may possess driving forces, and contributed to the viscosity decreases. Furthermore, changes in the β-glucan cell network during the late stationary and early death phases due to lysed cells could have induced the reduced viscosity (Fraunhofer, 2018, Bockwoldt et al., 2020). Autolysis, which is a strain-dependent process, preferentially induced under stress conditions or in late growth phases, is also known for LAB (Papadimitriou et al., 2016, Martínez et al., 2020). This, in addition, would correspond to the increasing numbers of proteins in the secretome samples after 7 days compared to 8 h (Bockwoldt et al., 2022).

Taken together, the decreasing viscosity and general changes of its heterogenous viscoelastic character of the culture broths could be the result of enzymatical and/or physical influences (Bockwoldt et al., 2022). These interactions could thus have hydrolyzed high molecular weight β-glucan to low molecular weight β-glucan, which could still be quantified by the immunological assay (ELISA) (Werning et al., 2014). At the same time, such a wide detection range of this assay could indicate for an overestimation of the polymer concentration and mask an actual degradation of the β-glucan. Besides, analysis of changes in the D-glucose concentration over time demonstrated, that it could not be associated with polymer degradation rather than with the polymer biosynthesis from phosphorolytic cleavage of maltose (Stolz et al., 1993, Wood and Rainbow, 1961, Bockwoldt et al., 2022, Bockwoldt et al., 2021). Here,

however, it would probably also be possible that the polymer would be degraded into oligomers and not the monomer D-glucose if it would have been degraded. In summary, the β-glucan could not be considered, at least exclusively, as a storage compound for later energy generation for *L. brevis* TMW 1.2112 and acts most likely as a protective shield towards ethanol, acidity, or hop bitter compounds as present in beer, the source of this strain (Fraunhofer, 2018, Suzuki, 2011, Stack et al., 2010, Lebeer et al., 2011).

In summary, the results of the enzymatic investigations and proteomics study provided new insights into the biosynthesis, degradation, and function of the bacterial *O2*-substituted (1,3)-β-glucan capsules of beer-spoiling LAB.

7 Conclusion

The present study demonstrated the persistence and adaptation of β-glucan forming LAB *L. brevis* and *P. claussenii*, isolated from spoiled beer, during wheat and rye sourdough fermentation. Bacterial β-glucan production is a typical feature of LAB isolates, which were most likely introduced in ethanol-containing beverages via insects. Of 23 LAB strains tested, 5 strains were able to adapt to the sourdough habitat and to produce appreciably high cell counts and β-glucan. However, it has been shown that the formation of β-glucan in sourdough is more of a metabolic burden rather than a selective advantage, as evidenced by the high rate of loss of this trait by the plasmid of *P. claussenii*. Although exogenous factors were able to significantly increase the yield, it was still too low with <1 g/kg bread. The β-glucan formation is coupled to the carbohydrate central metabolism and is therefore depending on cellular growth. Moreover, it is not only in competition with the energy generation of the cells but also with their cell wall biosynthesis. For this reason, the β-glucan formation is limited, and only biotechnological or metabolic engineering approaches might help to overcome the limitations.

Nevertheless, this work showed that it is possible to *in situ* introduce β-glucans into sourdough or baked goods by the use of selected strains. Moreover, the resulting sourdoughs obtain acceptable or even improved sensory and technological properties. Although the β-glucan amounts are not comparable to those present in β-glucan-containing cereals, they clearly influence the rheology of the doughs.

8 Summary

Nowadays, extracellular polysaccharides (EPS) synthesized by lactic acid bacteria (LAB) are increasingly used in the manufacturing processes of fermented foods. EPS possess not only structure-improving but also potentially health-promoting properties. Further, the addition of EPS to bakery products can delay starch degradation by means of increased water binding, thus reducing staling of the products. Until now, most of the described EPS in sourdough fermentation did not meet the criterion of clean label products and thus the absence of additives. However, this criterion could be met by using β glucan-forming LAB as sourdough starters. Wheat and rye are the most commonly used cereals in sourdough fermentations. However, they are also naturally deficient-β-glucan cereals. The enrichment of both cereals using bacterial β-glucan during sourdough fermentation, has been assumed to provide both technological as well as physiological benefits.

Previous studies have described the viscosity effects of β-glucan-forming LAB from beer, but so far, their applicability and use in sourdough fermentations has not been clarified. Therefore, their persistence and fermenting properties in wheat and rye doughs were analyzed first. Several of the previously described beer-spoiling bacteria *Levilactobacillus* (*L.*) *brevis* strains and *Pediococcus* (*P.*) *claussenii* proved to be suitable sourdough starters considering a clean label product since no additives for improved growth or EPS formation are necessary. For the optimization of the β-glucan yield in wheat and rye sourdoughs, the influence of different fermentation parameters was investigated, by using an immunological method to quantify the β-glucan amount. The fermentation temperature was a significant factor contributing to the increase in β glucan formation, with yields up to 600 mg/kg sourdough. Moreover, within 16 hours of fermentation, the start of the polymer degradation was observed, less by the producing strains themselves than by yeasts or endogenous flour enzymes. Furthermore, a genomic instability of *P. claussenii* TMW 2.340 led to strongly fluctuating β-glucan yields. In contrast, *L. brevis* TMW 1.2112 and TMW 1.2113 were found to be highly stable β-glucan-producing and persistent strains. To the extent of sensory analyses using an untrained panel, no significant differences were detected between wheat and rye sourdough breads with and without bacterial β-glucan. Nevertheless, positive technological effects were observed for β-glucan-containing sourdoughs. Since wheat sourdoughs with *L. brevis* TMW 1.2112 and TMW 1.2113 resulted in significant viscosity increase and rye sourdough breads with *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 exhibited increased water binding capacities.

The physiological properties of β-glucan-producing strains *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 as well as their corresponding isogenic β-glucan-negative strains were analyzed in an *in vitro* digestion. The isolated β-glucan as well as the cells themselves possessed cholesterol-lowering and apoptotic properties similar to those of oats and barley. However, contaminants in the β-glucan isolates negatively affected the analyses, which means that more extensive effects cannot be excluded. Further analyses of physiological properties of β-glucan enriched sourdoughs and sourdough bread samples revealed no difference from those without enrichment. Which suggests that the β-glucan concentrations in baked goods were too low for attributing any physiological effects.

In addition, molecular processes of β-glucan biosynthesis and degradation were studied, since for industrial applications in general, both yield and stability of polymers are critical parameters. Based on previous observations indicating the degradation of β glucan by *L. brevis* TMW 1.2112, proteomic analyses were performed. These analyses resulted, that in general, the use of β-glucan by *L. brevis* TMW 1.2112 as an energy storage compound could be mostly excluded, especially since no decrease in polymer concentration was measured and no degrading enzymes in the secretome could be identified. Therefore, it can be assumed that the capsular β-glucan has a protective function for the cells however, its role in attachment processes to surfaces cannot be excluded either. In previous studies, the glycosyltransferase 2 (Gtf-2) was described as a key enzyme for β-glucan synthesis. Proteomic analyses further identified other potential enzymes from the β-glucan synthesis pathway. These included maltose operons, which are most likely involved in β-glucan formation from maltose. The expression of Gtf-2 was constant regardless of cell number and growth phase, consistent with the characteristics of a constitutive enzyme. Also conspicuous was the secretion of several common intracellular proteins belonging to the group of moonlighting proteins. These secreted moonlighting proteins could also be assigned to an operon. Initial studies on these proteins from other LAB provided links to probiotic traits, such as adhesion processes to eukaryotic cells.

Within the scope of this study, the applicability and technological utility of β-glucan-forming lactic acid bacteria in sourdough fermentations was investigated and confirmed. This showed that despite low yields, positive technological effects occurred in sourdoughs and breads, however, these yields were not sufficient for measurable physiological effects of the food products. Furthermore, insights into intracellular mechanisms, such as the biosynthesis of the polymer and the putative benefit for the cell itself, could be provided. The knowledge gained on β-glucan formation and stability of lactic acid bacteria can be used as a basis for future product development and food fermentation processes.

9 Zusammenfassung

Heutzutage werden in den Herstellungsprozessen fermentierter Lebensmittel vermehrt die von Milchsäurebakterien (MSB) synthetisierten extrazellulären Polysaccharide (EPS) eingesetzt. Diese Polymere besitzen neben strukturverbessernden auch potenziell gesundheitsfördernde Eigenschaften. Der Zusatz von EPS in Backwaren kann mittels erhöhter Wasserbindung die Stärkeretrogradation verzögern und so das Altbackenwerden der Produkte vermindern. In der Herstellung von Sauerteigprodukten wurden bisher ausführlich die EPS beschrieben, die am Ende nicht dem Kriterium eines Clean Label Produkts und somit dem Verzicht von Zusatzstoffen, entsprachen. Dieses Kriterium könnte jedoch durch die Nutzung von β-Glukanbildenden MSB als Sauerteigstarter erfüllt werden. Weizen und Roggen gehören zu den am häufigsten verwendeten Getreidesorten in Sauerteigfermentationen. Aber sie gehören auch von Natur aus zu den β-Glukan-ärmeren Getreidesorten. Die Anreicherung beider Getreidesorten mit bakteriellem β-Glukan während der Sauerteigfermentation, könnte technologisch wie auch physiologisch von Nutzen sein.

In früheren Studien wurden die Viskositätseffekte von β-Glukanbildenden MSB aus Bier beschrieben, bisher ist allerdings ihre Anwendbarkeit und Nutzen in Sauerteigfermentationen nicht geklärt. Zuerst wurde daher deren Durchsetzungsfähigkeit und fermentierenden Eigenschaften in Weizen- und Roggenteigen analysiert. Mehrere der zuvor beschriebenen bierverderbenden *Levilactobacillus* (*L.*) *brevis* und *Pediococcus* (*P.*) *claussenii* Stämme erwiesen sich als geeignete Sauerteigstarter unter Berücksichtigung eines Clean Label Produkts, da auf Zusatzstoffe verzichtet werden konnte. Für die Optimierung der β-Glukanausbeute in den Weizen- und Roggensauerteigen, wurde der Einfluss unterschiedlicher Fermentationsparameter untersucht. Mittels eines immunologischen Verfahrens konnte das β-Glukan spezifisch quantifiziert werden. Es zeigte sich, dass von den untersuchten Parametern die Fermentationstemperatur als maßgeblicher Faktor zur Erhöhung der β-Glukanbildung beiträgt, mit bis zu ca. 600 mg/kg Sauerteig. Außerdem wurde bereits ab einer 16-stündigen Fermentationsdauer die Abnahme der Polymerkonzentration und somit dessen Abbau beobachtet, welcher weniger durch die produzierenden Stämme selbst als durch z.B. Hefen oder den endogenen Mehlenzymen erfolgte. Des Weiteren konnte für *P. claussenii* TMW 2.340 eine genomische Instabilität festgestellt werden, die zu stark schwankenden β-Glukanausbeuten führte. Hingegen erwiesen sich *L. brevis* TMW 1.2112 und TMW 1.2113 als äußerst stabile, β-Glukanproduzierende und durchsetzungsfähige Stämme. Im Umfang von sensorischen Analysen mittels eines ungeschulten Panels wurden keine signifikanten Unterschiede zwischen Weizen- und Roggensauerteigbroten mit und ohne bakteriellem β-Glukan detektiert. Dennoch konnten für β-Glukan-haltige Sauerteige positive technologische Effekte beobachtet werden. Da zum einen Weizensauerteige, die mit β-Glukan von *L. brevis* TMW 1.2112 und TMW 1.2113 i*n situ* angereichert wurden, signifikante Viskositätserhöhung aufwiesen und zum anderen Roggensauerteigbrote von *L. brevis* TMW 1.2112 und *P. claussenii* TMW 2.340 ein erhöhte Wasserbindevermögen aufwiesen.

Die physiologischen Eigenschaften der beiden β-Glukan-bildenden Stämme *L. brevis* TMW 1.2112 und *P. claussenii* TMW 2.340 sowie deren isogenen β-Glukan-negativen Stämme wurden in einem *in vitro*-

Verdau analysiert. Das isolierte β-Glukan wie auch die Zellen selbst verfügten über cholesterinsenkende und apoptotische Eigenschaften, ähnlich dem von Hafer und Gerste. Allerdings erschwerten Verunreinigungen der β-Glukanisolate die Analysen, wodurch umfangreichere physiologische Effekte nicht ausgeschlossen werden können. Weitere Analysen physiologischer Eigenschaften von β-Glukan angereicherten Sauerteigen und Sauerteigbroten, ergaben keinen Unterschied zu denen ohne Anreicherung oder der positiv Kontrolle Synergy®. Was darauf deutet, dass die in Backwaren gebildeten β-Glukankonzentration zu gering waren, um eindeutige physiologische Effekte bestimmen zu können.
Faktoren welche die Synthese sowie den Abbau des β-Glukans von MSB beeinflussen, wurden analysiert, da für zukünftige industrielle Anwendungen, sowohl die Ausbeute wie auch Stabilität des EPS relevant sein könnten. Aufgrund vorheriger Beobachtungen des mutmaßlichen Abbaus von β-Glukan durch *L. brevis* TMW 1.2112 wurden proteomische Analysen durchgeführt. Allgemein konnte die Nutzung des β-Glukans als Energiespeicher ausgeschlossen werden, da zumal keine Abnahme der Polymerkonzentration gemessen wurde und die Expressionen bekannter abbauender Enzyme im Sekretom fehlten. Daher kann angenommen werden, dass das kapsuläre β-Glukan eine schützende Funktion für die Zellen übernimmt aber auch dessen Mitwirkung für Anheftungsprozesse an Oberflächen kann nicht ausgeschlossen werden. In früheren Studien wurde die Glycosyltransferase 2 (Gtf-2) als das Schlüsselenzym für die β-Glukan Synthese beschrieben. Durch die proteomische Analysen konnten noch weitere potenzielle Enzyme aus dem β-Glukansyntheseweg identifiziert werden. Hierzu gehörten u.a. mehrere Maltoseoperons, welche bei der β-Glukanbildung aus Maltose mutmaßlich involviert sind. Die Expression von Gtf-2 war unabhängig von der Zellzahl und der Wachstumsphase konstant, und entspricht somit den Merkmalen eines konstitutiven Enzyms. Auffällig war außerdem die Sekretion von üblicherweise intrazellulären Proteinen, auch schon während der exponentiellen Wachstumsphase, welche zur Gruppe der Moonlighting Proteine gehören. Die vier sekretierten Moonlighting Proteine konnten zudem einem einzigen Operon zugewiesen werden. Erste Studien zu diesen Proteinen aus MSB stellten Verbindungen zu probiotischen Merkmalen dar, wie den Adhäsionsprozessen an eukaryotische Zellen.

Im Umfang dieser Studie wurde die Anwendbarkeit und der technologische Nutzen von β-Glukanbildenden Milchsäurebakterien in Sauerteigfermentationen untersucht und bestätigt. Hierbei zeigte sich, dass sich trotz niedriger β-Glukan Ausbeuten positive technologische Effekte in den Teigen und Broten einstellten, diese Ausbeuten jedoch nicht für messbare physiologische Effekte ausreichten. Außerdem konnten Einblicke in die intrazellulären Mechanismen, wie der Biosynthese des Polymers und dem vermeintlichen Nutzen für die Zelle selbst gegeben werden. Die gewonnen Erkenntnisse zur β-Glukan-Bildung und dessen Stabilität in Milchsäurebakterien können als Grundlage für zukünftige Produktentwicklungen und Lebensmittelfermentationsprozesse genutzt werden.

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II. Register of figures

III. Register of tables

IV. Appendix

Part I - Persistence and β-glucan formation of beer-spoiling lactic acid bacteria in wheat and rye sourdoughs

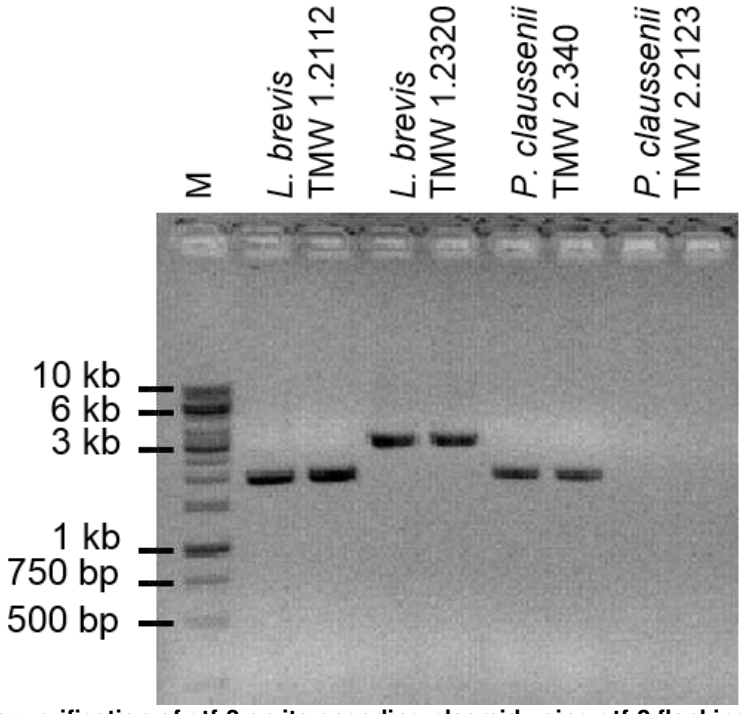

Figure S 1. Molecular verification of gtf-2 on its encoding plasmid using gtf-2 flanking specific primers. The PCR product of the wild-type strains *L. brevis* TMW 1.2112 and *P. claussenii* TMW 2.340 has a size of 1,942 bp, while for the mutant strain *L. brevis* TMW 1.2320 (Δ*gtf-2*) an amplicon of 2,991 bp is obtained because of a transposon (IS30) insertion. In case of *P. claussenii* TMW 2.2123 (Δ*gtf-2*) no PCR product is obtained because of the loss of the *gtf2*-encoding plasmid. The PCR was performed using the Taq Core Kit (MP Biomedicals, Santa Ana, USA) and the following primers F: 5'-GAAGGTAAACGCGGTTCTGA-3' and R: 5'-GGATTAAAGCGCTGTGTCCTTC-3'.

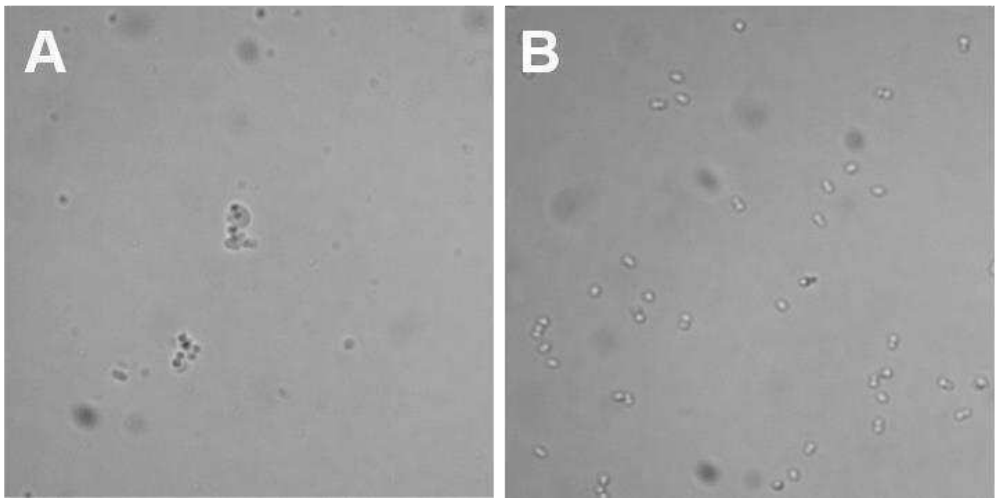

Figure S 2. Agglutination test of *P. claussenii* TMW 2.340 (wt) and *P. claussenii* TMW 2.2123 (Δgtf-2). A) *P. claussenii* (wt) isolated from wheat sourdough and B) *P. claussenii* (Δgtf-2) isolated from wheat sourdough.

Part II - β-Glucan Production by *Levilactobacillus brevis* and *Pediococcus claussenii* for In Situ Enriched Rye and Wheat Sourdough Breads

Table S 1. Results of wheat sourdough analyses: cell count, pH values, MALDI-TOF MS and ratio of EPS positive colonies.

			L. brevis TMW 1.2112		*L. brevis* TMW 1.2320		*P. claussenii* TMW 2.340		*P. claussenii* TMW 2.2123	
			Fermentation time [h]							
	Temperature	Inoculum	0	24	0	24	0	24	0	24
Cell count [CFU/g]	25 °C	1 x	$6.6 \cdot 10^5$	$9.0 \cdot 10^8$	$7.5 \cdot 10^5$	$4.1 \cdot 10^8$	$2.8 \cdot 10^6$	$8.4 \cdot 10^8$	$3.0 \cdot 10^6$	$6.6 \cdot 10^8$
	28 °C	1 x	$2.3 \cdot 10^6$	$1.0 \cdot 10^9$	$2.0 \cdot 10^6$	$1.0 \cdot 10^9$	$4.0 \cdot 10^6$	$6.4 \cdot 10^8$	$3.3 \cdot 10^6$	$6.9 \cdot 10^8$
	35 °C	1 x	$1.5 \cdot 10^6$	$1.2 \cdot 10^9$	$1.5 \cdot 10^6$	$1.3 \cdot 10^9$	$3.2 \cdot 10^6$	$3.3 \cdot 10^8$	$3.7 \cdot 10^6$	$4.2 \cdot 10^8$
	28 °C	1/2 x	$8.6 \cdot 10^5$	$6.5 \cdot 10^8$	$6.8 \cdot 10^5$	$8.4 \cdot 10^8$	$1.3 \cdot 10^6$	$6.7 \cdot 10^8$	$1.2 \cdot 10^6$	$7.4 \cdot 10^8$
	28 °C	2 x	$3.0 \cdot 10^6$	$6.6 \cdot 10^8$	$3.6 \cdot 10^6$	$7.2 \cdot 10^8$	$6.5 \cdot 10^6$	$8.1 \cdot 10^8$	$5.7 \cdot 10^6$	$6.6 \cdot 10^8$
	28 °C	1 x (Coculture)	$1.5 \cdot 10^5$	$1.7 \cdot 10^9$	$1.6 \cdot 10^5$	$1.6 \cdot 10^9$	$2.3 \cdot 10^5$	$1.1 \cdot 10^9$	$2.7 \cdot 10^5$	$1.6 \cdot 10^9$
pH [-]	25 °C	1 x	5.98 ± 0.01	3.94 ± 0.02	5.99 ± 0.01	3.92 ± 0.02	6.02 ± 0.01	3.76 ± 0.01	6.04 ± 0.01	3.74 ± 0.01
	28 °C	1 x	6.02 ± 0.01	3.69 ± 0.01	6.01 ± 0.01	3.69 ± 0.01	5.95 ± 0.04	3.67 ± 0.01	5.97 ± 0.05	3.68 ± 0.01
	35 °C	1 x	5.97 ± 0.02	3.50 ± 0.01	5.96 ± 0.01	3.51 ± 0.01	5.99 ± 0.02	3.62 ± 0.02	5.99 ± 0.01	3.59 ± 0.01
	28 °C	1/2 x	5.98 ± 0.00	3.72 ± 0.01	5.97 ± 0.01	3.71 ± 0.01	5.97 ± 0.01	3.68 ± 0.01	5.97 ± 0.01	3.68 ± 0.01
	28 °C	2 x	5.96 ± 0.01	3.70 ± 0.02	5.97 ± 0.02	3.70 ± 0.01	5.95 ± 0.01	3.65 ± 0.00	5.96 ± 0.01	3.64 ± 0.01
	28 °C	1 x (Coculture)	5.99 ± 0.01	3.57 ± 0.01	5.96 ± 0.01	3.56 ± 0.01	5.95 ± 0.01	3.56 ± 0.01	5.95 ± 0.01	3.55 ± 0.01
Inoculated species identity [%]	25 °C	1 x	93	100	94	100	100	98	100	100
	28 °C	1 x	100	100	91	100	94	100	72	100
	35 °C	1 x	99	99	92	100	100	100	100	100
	28 °C	1/2 x	100	100	100	100	100	100	99	100
	28 °C	2 x	100	100	100	100	100	100	99	100
	28 °C	1 x (Coculture)	45	26	38	21	50	61	60	21
EPS positive CFU [%]	25 °C	1 x	100	100	0	0	99	96	0	0
	28 °C	1 x	100	100	0	0	39	42	0	0
	35 °C	1 x	100	100	0	0	97	95	0	0
	28 °C	1/2 x	100	100	0	0	48	41	0	0
	28 °C	2 x	100	100	0	0	57	36	0	0
	28 °C	1 x (Coculture)	100	100	0	0	32	8	0	0

Table S 2. Results of rye sourdough analyses: cell count, pH values, MALDI-TOF MS and ratio of EPS positive colonies.

			L. brevis TMW 1.2112		*L. brevis* TMW 1.2320		*P. claussenii* TMW 2.340		*P. claussenii* TMW 2.2123	
			Fermentation time [h]							
	Temperature	Inoculum	0	24	0	24	0	24	0	24
Cell count [CFU/g]	25 °C	1 x	$5.6 \cdot 10^5$	$3.3 \cdot 10^9$	$3.4 \cdot 10^5$	$1.2 \cdot 10^9$	$2.9 \cdot 10^6$	$1.2 \cdot 10^9$	$2.6 \cdot 10^6$	$1.2 \cdot 10^9$
	28 °C	1 x	$2.3 \cdot 10^6$	$3.4 \cdot 10^9$	$1.2 \cdot 10^6$	$3.0 \cdot 10^9$	$1.7 \cdot 10^6$	$1.4 \cdot 10^9$	$2.8 \cdot 10^6$	$1.9 \cdot 10^9$
	35 °C	1 x	$1.6 \cdot 10^6$	$2.3 \cdot 10^9$	$1.3 \cdot 10^6$	$2.0 \cdot 10^9$	$3.3 \cdot 10^6$	$7.0 \cdot 10^8$	$3.0 \cdot 10^6$	$8.8 \cdot 10^8$
	28 °C	1/2 x	$8.0 \cdot 10^5$	$2.6 \cdot 10^9$	$7.2 \cdot 10^5$	$2.4 \cdot 10^9$	$1.6 \cdot 10^6$	$1.4 \cdot 10^9$	$1.1 \cdot 10^6$	$1.4 \cdot 10^9$
	28 °C	2 x	$3.3 \cdot 10^6$	$4.0 \cdot 10^9$	$2.7 \cdot 10^6$	$2.1 \cdot 10^9$	$6.6 \cdot 10^6$	$7.9 \cdot 10^8$	$4.3 \cdot 10^6$	$1.0 \cdot 10^9$
	28 °C	1 x (Coculture)	$1.3 \cdot 10^5$	$2.4 \cdot 10^9$	$1.7 \cdot 10^5$	$1.9 \cdot 10^9$	$2.9 \cdot 10^5$	$2.2 \cdot 10^9$	$3.1 \cdot 10^5$	$1.7 \cdot 10^9$
pH [-]	25 °C	1 x	5.78 ± 0.01	3.79 ± 0.01	5.80 ± 0.01	3.78 ± 0.01	5.81 ± 0.03	3.92 ± 0.01	5.81 ± 0.01	3.86 ± 0.01
	28 °C	1 x	5.86 ± 0.01	3.68 ± 0.01	5.83 ±0.02	3.67 ± 0.01	5.77 ± 0.05	3.77 ± 0.02	5.79 ± 0.03	3.76 ± 0.02
	35 °C	1 x	5.78 ± 0.01	3.58 ± 0.01	5.80 ± 0.01	3.58 ± 0.01	5.81 ± 0.01	3.75 ± 0.01	5.80 ± 0.02	3.70 ± 0.01
	28 °C	1/2 x	5.76 ± 0.01	3.70 ± 0.01	5.75 ± 0.01	3.71 ± 0.01	5.75 ± 0.01	3.81 ± 0.00	5.74 ± 0.01	3.80 ± 0.01
	28 °C	2 x	5.73 ± 0.02	3.69 ± 0.01	5.74 ± 0.01	3.68 ± 0.01	5.72 ± 0.01	3.75 ± 0.01	5.86 ± 0.01	3.70 ± 0.00
	28 °C	1 x (Coculture)	5.74 ± 0.01	3.61 ± 0.01	5.75 ± 0.00	3.61 ± 0.01	5.74 ± 0.01	3.60 ± 0.01	5.72 ± 0.02	3.61 ± 0.01
Inoculated species identity [%]	25 °C	1 x	100	100	100	100	98	99	99	100
	28 °C	1 x	96	100	96	100	96	100	92	100
	35 °C	1 x	100	100	98	93	99	99	96	100
	28 °C	1/2 x	99	100	98	100	96	100	97	100
	28 °C	2 x	100	100	100	100	99	100	98	97
	28 °C	1 x (Coculture)	48	48	47	40	66	70	65	21
EPS positive CFU [%]	25 °C	1 x	100	100	0	0	97	98	0	0
	28 °C	1 x	100	100	0	0	16	38	0	0
	35 °C	1 x	100	100	0	0	98	95	0	0
	28 °C	1/2 x	100	100	0	0	50	34	0	0
	28 °C	2 x	100	100	0	0	48	42	0	0
	28 °C	1 x (Coculture)	100	100	0	0	27	5	0	0

Part IV - Proteomic analysis reveals enzymes for β-D-glucan formation and degradation in *Levilactobacillus brevis* TMW 1.2112

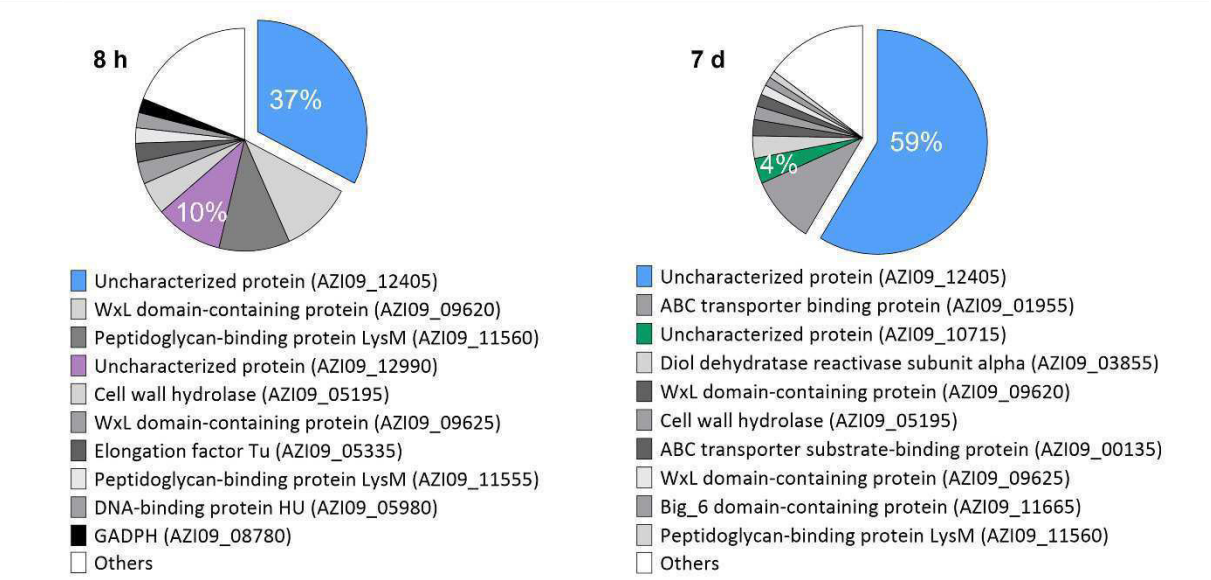

Figure S 3. Relative proportion of proteins in the samples generated from iBAQ intensity values.

Additional appendices to this publication can be found at the following link and are not listed here due to their very large size.

Link to supplementary materials: https://www.mdpi.com/article/10.3390/ijms23063393/s1.

Part of Discussion

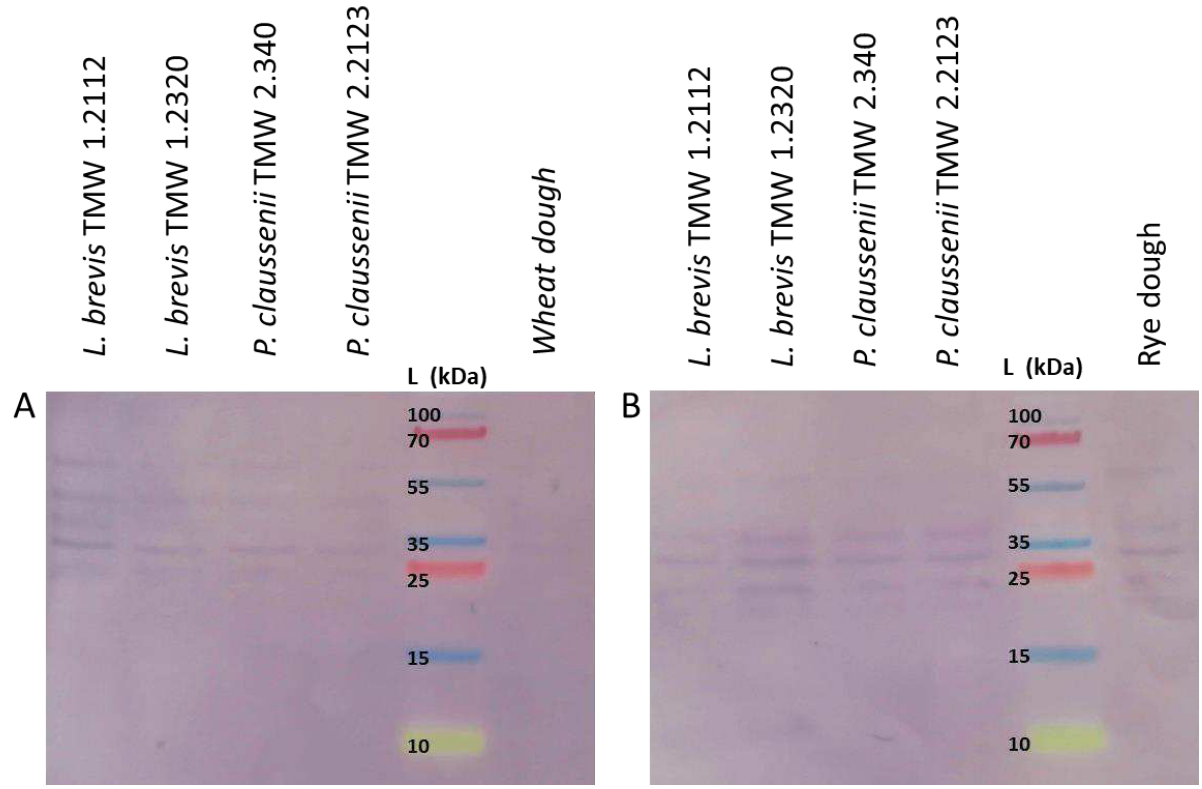

Figure A 1. Western-Blot of wheat (W) and rye (R) sourdough samples - analyzing cross-reactions with ELISA antiboody.

Table A 1. Results of bacterial β-glucan isolation using different media, strains, and posttreatments - yields and protein contaminations.

Strain	Medium	Initial pH [-]	Posttreatment	Yield freeze dried [g/L]	Std. dev. [g/L]	Protein [%]	Std. dev. [%]
L. brevis TMW 1.2112	mMRS	4,3	-	0.375	0,033	61.11	1.35
L. brevis TMW 1.2112	mMRS	6,2	-	0.548	0,014	66.86	0.79
L. brevis TMW 1.2112	CDM	5,5	-	0.066	0,013	20.75	1.23
L. brevis TMW 1.2112	SDM	5,5	-	0.220	0,010	50.97	2.60
L. brevis TMW 1.2112	SDM	5,5	Perchloric acid	0.044	0,025	27.96	1.94
L. brevis TMW 1.2112	SDM	5,5	Urea	0.091	0,021	36.50	3.08
L. brevis TMW 1.2112	SDM	5,5	Lysozyme, RNAse und Proteinase K	0.427	0,033	62.22	1.23
P. claussenii TMW 2.340	SDM	5,5	Lysozyme, RNAse und Proteinase K	0.522	0,033	55.91	1.83

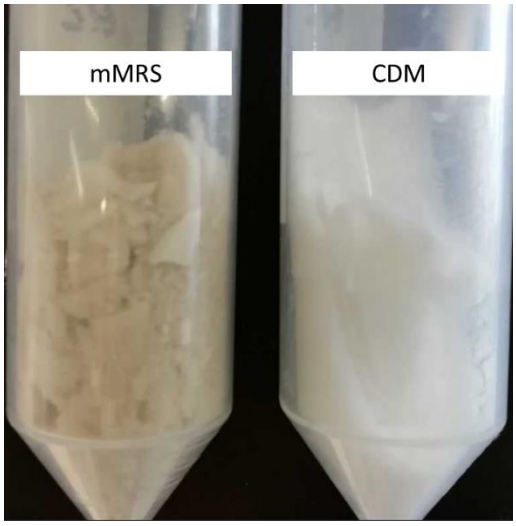

Figure A 2. Interference of the culture medium with the purity of isolated *L. brevis* TMW 1.2112 β-glucan. Beta-glucan isolated from mMRS indicated a higher contamination optically due to the brown coloration compared to the isolate from CDM medium which was white.

V. Publications and student theses

Peer-reviewed journals (Underlined names indicate the first author)

Bockwoldt, J.A.; Stahl, L.; Ehrmann, M.A.; Vogel, R.F.; Jakob, F. "Persistence and beta-glucan formation of beer-spoiling lactic acid bacteria in wheat and rye sourdoughs". Food Microbiology 2020, 91, 103539. https://doi.org/ 10.1016/j.fm.2020.103539

Bockwoldt, J.A.; Fellermeier, J.; Steffens, E.; Vogel, R.F.; Ehrmann, M.A. "β-Glucan Production by *Levilactobacillus brevis* and *Pediococcus claussenii* for In Situ Enriched Rye and Wheat Sourdough Breads". Foods 2021, 10, 547. https://doi.org/10.3390/foods10030547

Schlörmann, W.; **Bockwoldt, J.A.**; Mayr, M.F.; Lorkowski, S.; Dawczynski, C.; Rohn, S.; Ehrmann, M.A.; Glei, M. "Fermentation profile, cholesterol-reducing properties and chemopreventive potential of β-glucans from *Levilactobacillus brevis* and *Pediococcus claussenii* - a comparative study with β-glucans from different sources". Food & Function 2021, 12, 10615-10631. https://doi.org/10.1039/D1FO02175C

Bockwoldt, J.A.; Meng, C.; Ludwig, C. Kupetz, M.; Ehrmann, M.A. "Proteomic analysis reveals enzymes for β-D-glucan formation and degradation in *Levilactobacillus brevis* TMW 1.2112". International Journal of Molecular Science 2022, 23, 3393. https://doi.org/10.3390/ijms23063393

Schlörmann, W.; **Bockwoldt, J.A.**; Hübner, S.M.; Wittwer, E.; Reiners, S.; Lorkowski, S.; Dawczynski, C.; Ehrmann, M.A.; Glei, M. "Use of the β-glucan-producing lactic acid bacteria strains *Levilactobacillus brevis* and *Pediococcus claussenii* for sourdough fermentation - Chemical characterization and chemopreventive potential of in situ-enriched wheat and rye sourdoughs and breads". Nutrients 2022, 14, 1510. https://doi.org/10.3390/nu14071510

Bockwoldt, J.A. and Ehrmann, M.A. "Characterisation of recombinant GH 3 β-glucosidase from β-glucan producing *Levilactobacillus brevis* TMW 1.2112". Antonie van Leeuwenhoek 2022, https://doi.org/10.1007/s10482-022-01751-7

Conference contributions (Underlined names indicate the first author)

Wittwer, E.; Mayr, M.F.; **Bockwoldt, J.A.**; Rohn, S.; Schlörmann, W.; Glei, M. "Charakterisierung des Fermentationsprofils verschiedener β-Glucane", 58. Wissenschaftlichen Kongress der Deutschen Gesellschaft für Ernährung e.V., 2021, online

Bockwoldt, J.A.; Vogel, R.F.; Ehrmann, M.A. "Glycoside hydrolases from β-Glucan producing *Levilactobacillus brevis*: cloning, expression, and characterization", 13th International Symposium on Lactic Acid Bacteria, 2021, online

Bockwoldt, J.A.; Stahl, L.: Fellermeier, J.; Ehrmann, M.A. Optimizing the β-glucan formation of lactic acid bacteria in sourdoughs, 2nd International Conference on Microbial Food & Feed Ingredients, 2021, Copenhagen, Denmark

Schlörmann, W.; **Bockwoldt, J.A.**; Hübner, S.M.; Wittwer, E.; Lorkowski, S; Glei, M. "Gesundheitliches Potential von Weizen- und Roggenbroten nach Sauerteigfermentation mit den β-Glukan-produzierenden Milchsäurebakterien *Levilactobacillus brevis* und *Pediococcus claussenii*", 59. Wissenschaftlichen Kongress der Deutschen Gesellschaft für Ernährung e.V., 2022, online

Student theses

Jessica Hagl, “The influence of stress parameters in growth and β-glucan formation of beer-spoiling lactic acid bacteria”, Bachelor thesis, 2020

Johanna Fellermeier, “Increase of the β-glucan formation by lactic acid bacteria in sourdough”, Master thesis, 2020

Helen Chau, “Methodenoptimierung der β-Glukanisolation von *Levilactobacillus brevis*“, Internship, 2020

Emma Steffens, “Backversuche von Weizen- und Roggensauerteigen mit β-Glukanbildenden Milchsäurebakterien“, Internship 2021

Daniel Wanken, “Fermentation von bakteriellem *O2*-substituierten (1,3)-β-D-Glukan durch Bifidobakterien“, Internship 2021

Felix Ulmer, “Vorkommen und Nutzung von β-Glukanen in Lebensmitteln“, Literature research project 2020

Luisa Murer, “Methoden zur Isolierung und Quantifizierung von Milchsäurebakterien produzierten Exopolysacchariden“, Literature research project, 2021