TECHNISCHE UNIVERSITÄT MÜNCHEN

Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

Lehrstuhl für Technische Mikrobiologie

Lifestyle and functional properties of lactobacilli and bifidobacteria in water kefir

Viktor Philipp Lantbert Eckel

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

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender:	Prof. Dr. Siegfried Scherer
Prüfer der Dissertation:	1. Prof. Dr. Rudi F. Vogel
	2. Prof. Dr. Wilfried Schwab

Die Dissertation wurde am 13.05.2020 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 14.07.2020 angenommen.

Abbreviations

A7	25% apple juice at pH 7
Ab	*Acetobacter*
AF4-MALS	assymetric flow field flow fractionation coupled to multi angle light scattering
An	25% apple juice at native pH
ANI	average nucleotide identity
ANIb	average nucleotide identity based on BLAST+
B.	*Bifidobacterium*
BADGE	Blast Diagnostic Gene finder
BLAST	Basic Local Alignment Search Tool
BM	*Bifidobacterium* medium
CDM	chemically defined medium
CDM+AA	chemically defined medium with all canonic amino acids
CDM-AA	chemically defined medium without amino acids
dH_2O	distilled water
DIC	differential interference contrast
DNA	desoxyribonucleic acid
DSM	Deutsche Sammlung Mikroorganismen
DSMZ	Deutsche Sammlung Mikroorganismen und Zellkulturen
EPS	exopolysaccharide
F6P	fructose-6-phosphate
F6PPK	fructose-6-phosphate phosphoketolase
Gn	25% grape juice at native pH
G7	25% grape juice at pH 7
GOI	gene of interest
HePs	heteropolysaccharide
HoPs	homopolysaccharide
HPLC	high performance liquid chromatography
isDDH	*in silico* DNA-DNA hybridization

KEGG	Kyoto Enzyclopedia of Genes and Genomes
LAB	lactic acid bacteria
Lb.	*Lactobacillus*
Lc.	*Leuconostoc*
MALDI TOF	matrix assisted laser desorption and ionization coupled to time of flight
mMRS	modified deMan, Rogosa and Sharpe medium
MRS	deMan, Rogosa and Sharpe medium
NCBI	National Center for Biotechnology Information
OD	optical density
orf	open reading frame
PCR	polymerase chain reaction
PTS	phosphotransferase system
RAPD	randomly amplified polymorphic DNA
RAST	rapid annotation using subsystems technology
RNA	ribonucleic acid
RPM	revolutions per minute
rRNA	ribosomal RNA
S.	*Saccharomyces*
TMW	Technische Mikrobiologie Weihenstephan
TP	tryptone phytone medium
TPY	trypticase phytone yeast
UV	ultra violet light
wk	water kefir
W/V	weight by volume
W/W	weight by weight

Vorwort

Die vorliegende Arbeit entstand im Rahmen des durch das Bundesministerium für Wirtschaft und Energie über die die AiF-Forschungsvereinigung Wissenschaftsförderung der Deutschen Brauwirtschaft e.V. geförderten Projekts „Innovative Getränkezutaten“ (AiF 19180N).

Mein besonderer Dank gilt meinem Doktorvater Prof. Dr. Rudi F. Vogel. Danke für deinen Einsatz, dafür, dass du uns immer den Rücken freigehalten hast, und natürlich danke dafür, dass ich in einem derart angenehmen Umfeld; mit diesen Labors, Büros, Geräten und Kollegen, bei dir promovieren durfte.

Außerdem möchte ich mich bei Dr. Frank Jakob bedanken, für die Unterstützung, die fachliche Expertise und die vielen Diskussionen. Mein Dank gilt auch Prof. Dr. Matthias Ehrmann, für alle Unterstützung und Hilfe.

Mein Dank gilt auch Angela Seppeur, für die Übernahme aller Verwaltungsaufgaben und tatkräftiger Unterstützung, wo auch immer man sie brauchte.

Danke an meine Doktorandenkollegen, Julia Bechtner, Lara Eisenbach, Jonas Schmid, Alex Lauterbach und allen anderen, für die diversen Troubleshooting-sessions, die guten Stunden, die schlechten Stunden und alle Unterstützung die ich erfahren habe. Natürlich gilt mein Dank auch allen Technikern, besonders Monika Engel, Margarete Schreiber und Andrea Pape, dafür, dass sie den Laden am Laufen halten und für alle Hilfe im Labor.

Zusätzlich gilt mein Dank natürlich auch unseren Projektpartnern an der HSOWL, Prof. Jan Schneider, Sören Rossmann und Julian Huchtman für die angenehme Zusammenarbeit im Projekt und für das zur Verfügungstellen der Bilder. Danke auch an Prof. Dr. Ramon Torres-Ruiz am CALM für seine Hilfe bei den DIC-Aufnahmen. Danke an meine Studentinnen, Lisa-Marie Sattler und Swetlana Gerkhardt für die gute Zusammenarbeit.

Natürlich gilt mein Dank auch meiner Frau Dr. Manuela Hollering, für die Kommentare zum Manuskript und alle sonstige direkte und indirekte Unterstützung meiner Dissertation. Aber natürlich auch für alles Andere, die vielen Unternehmungen, für ihre Toleranz und dafür, dass sie mir alles ermöglicht. Danke Jonathan, für deine gute Laune und die Freude die du uns bereitest. Danke Mama und Papa, Klara und Theo dass ihr immer für mich da seid.

Index

1. Introduction

1.1. Fermented foods and beverages

The consumption of fermented foods most likely predates historic records (Campbell-Platt 1994). Archeological evidence of food fermentation exists from prehistoric times. Evidence of cheese making was uncovered from as early as the sixth millennium BC in northern Europe (Salque et al. 2012). Evidence of milk kefir production exists from 1980-1450 BC in China (Yang et al. 2014). In fact, archeological evidence exists for beer production and storage from as early as 13 000 years ago in a building complex uncovered in modern-day Israel, images of which can be seen in Figure 1 (Liu et al. 2018). In addition to archeological evidence, written records of beer production exist from ancient Egypt as early as 3100-2686 BC (Hornsey 2003) and from the Sumerian empire from approximately 6000 BC (Poelmans and Swinnen 2011).

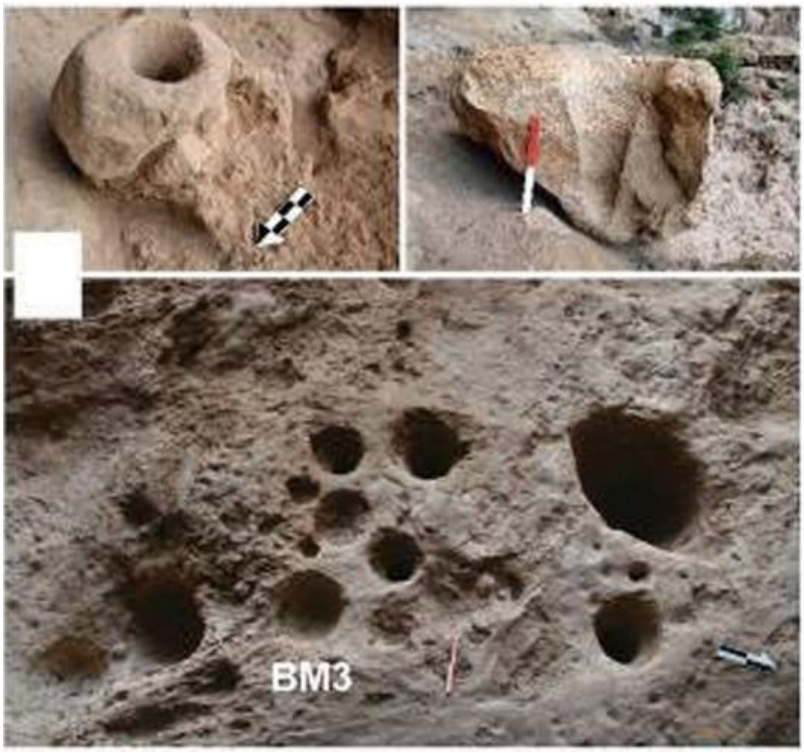

Figure 1. Boulder mortars (upper row) and bedrock mortars (lower row) thought to have contained fermented grains from a 13,000 year old excavation site in Israel. Figure adapted from Liu et al. (2018).

The fermentation of foods was likely and is still undertaken for three main reasons:

Firstly, fermented foods are typically more shelf stable than unfermented foods (Wood 2012). Lactic fermentation of e.g. cabbage produces sauerkraut, which has been traditionally produced in northern Europe and is much more shelf stable than the fresh vegetable, enabling the local

population to keep the vegetable for the winter time (Fleming and McFeeters 1981; Pederson and Albury 1969; Wood 2012).

Secondly, food fermentation is associated with an increase in nutritional value (Leroy et al. 2018; Reddy and Pierson 1994; Wood 2012). One example is the reduction of lactose content for the mainly lactose intolerant people of the Neolithic in the case of early cheese making (Burger et al. 2007; Itan et al. 2009; Salque et al. 2012). Other examples are the formation of aromatic compounds in sourdough bread that lead to higher organoleptic properties (Thiele et al. 2002)or the increase of palatability of less desirable meat parts as is the case for fermented meats (Leroy et al. 2018).

Thirdly, especially fermented beverages are historically valued due to their refreshing and intoxicating properties (Hornsey 2003). This stems mainly from their alcohol content, which is also related to shelf stability, as alcoholic fermentation preserves grape juice in the form of wine, making this well-tasting fruit available throughout the year (McGovern 2013). In fact, human use of the alcohol-producing yeast *Saccharomyces cerevisiae* has had such a strong effect on this organism, that its evolution seems to be closely linked to human history and its use for food fermentation (Legras et al. 2007).

It becomes clear, that historic reasons for fermentation are manyfold. In ancient times, food fermentations were most likely conducted by spontaneous fermentation or back-slopping (Campbell-Platt 1994). Today, production of fermented foods is in most cases achieved by the addition of starter cultures, ensuring a high level of control over the fermentation process (Durso and Hutkins 2003). While this practice works for systems of which the components have been fully understood and cultivated, there are still foods that are produced by back-slopping such as sourdough bread (Campbell-Platt 1994; Hammes and Gänzle 1998).

It is therefore not surprising, that early advances in microbiology are closely linked to yeast used for the brewing process, where efforts focused on gaining better control over the production process (Hansen 1881; Pasteur 1876). This indicates that fermenting foods and drinks is not only an important part of human nutrition and culture that predates history but it has also influenced the rapid development of modern science. However, independent of their history and current importance, fermented foods show great promise in the future, as they pose a natural way of producing desirable traits in foods, many of which yet remain to be elucidated (Hansen 2002; Paul Ross et al. 2002; Vijaya Kumar et al. 2015). This is also true for fermented drinks, which are anticipated to be highly important to the future functional food sector (Marsh et al. 2014). Not surprisingly, the demand for and interest in fermented foods has been growing

strongly in recent years, underlining their importance in our current and future nutrition (Granato et al. 2010; Johnson 2016; Villarreal-Soto et al. 2018).

1.2. LAB from foods

One main group of bacteria involved in food fermentations are lactic acid bacteria (LAB) (Campbell-Platt 1994). In fact, they perform the main bioconversion on fermented dairy, fermented meats and fermented vegetable products worldwide (Makarova et al. 2006a). LAB have been identified as one of the main agents involved in food fermentations early on in the research of food microbiology (Steinkraus et al. 2004; Stiles and Holzapfel 1997; Ward 1892). While early reports and definitions of LAB focused on morphological and physiological traits (Orla-Jensen 1919), todays definition is mainly founded on nucleic acids methods such as 16S RNA gene homology, DNA-DNA hybridization or genome to genome comparison (Klein et al. 1998; Makarova et al. 2006b; Schleifer 1987). It becomes clear that the definition of LAB and their taxonomic standing is constantly evolving, as even nomenclature changes and re-classifications of whole genera are currently being discussed (Salvetti et al. 2018; Stijn et al. 2019; Zheng et al. 2015). At this time, the genera *Lactococcus*, *Enterococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Leuconostoc*, and *Lactobacillus* are typically considered to be part of the LAB (Makarova et al. 2006a). The most important shared metabolic trait is that of production of lactic acid through fermentation. This fermentation can be divided in two pathways: the homofermentative pathway, in which lactic acid is the sole fermentation end-product and the heterofermentative pathway, in which lactic acid and/or acetic acid, CO_2 and ethanol are fermentation end products (Kandler 1983). This formation of lactic acid is one of the main reasons for their prevalence in foods, since by producing lactic and acetic acid and the associated low pH, they inhibit pathogens and increase shelf life of the fermented products (Adams and Hall 1988). However, it has become clear that LAB, in addition to acids and carbon dioxide, produce a phletora of antimicrobial substances like hydrogen peroxide, reuterin and bacteriocins (De Vuyst and Vandamme 1994; Vogel et al. 1999). These antimicrobial substances, together with antioxidant properties, immunomodulatory effects, colonization of the intestine and many more contribute to their positive health effect, as these organisms are often described as being probiotic (Ljungh and Wadstrom 2006; Mäyrä-Mäukinen et al. 1983; Perdigon et al. 1990). It becomes clear that LAB serve many purposes in food fermentations, some of which are summarized in Figure 2.

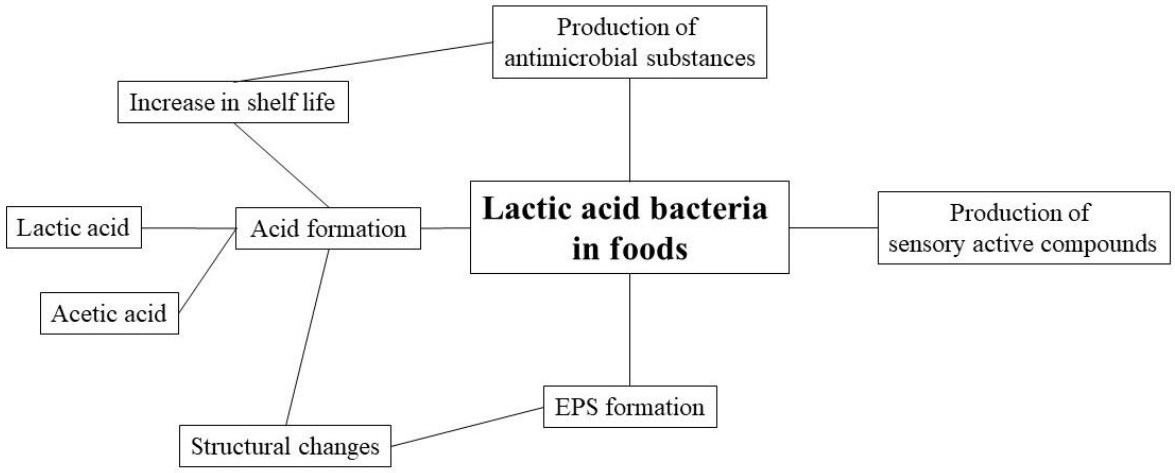

Figure 2. Various effects of LAB in food fermentation.

The source of LAB is often the raw material used for fermentation itself, while in the context of industrial production, they are of course often added as starter cultures (Buckenhüskes 1993). In food fermentation, LAB are the main agents responsible for fermentations as diverse as sauerkraut, sausage fermentation, coffee production and cheese fermentation (Aryana and Olson 2017; Lee et al. 2015; Lücke 1998; Pederson and Albury 1969).

In beverage fermentations, LAB cut both ways: they can be desirable or detrimental for a fermented food, often playing both parts in the same food. In wine, they serve both quality increasing and quality decreasing, by malolactic and lactic fermentation, respectively (Lonvaud-Funel 1999). In beer, they are mostly associated with spoilage (Ault 1965). Their intentional use in beer is limited to a few exceptions, like the production of Belgian-style Lambic ales or acidulated malt (Vriesekoop et al. 2012). In milk kefir and water kefir, LAB are the most abundant group of microorganisms and the most important for its fermentation (Arslan 2015; Gulitz et al. 2011).

1.3. Water kefir

Water kefir is a fermented beverage produced using traditional methods and fermented by a diverse consortium of microorganisms. As such, it has been in the scope of research early in the history of food microbiology (Pidoux 1989; Ward 1892). Even though this research was initiated over 120 years ago and our understanding of water kefir is constantly growing, the relationship of the organisms involved is still not fully understood (Laureys et al. 2018; Laureys et al. 2016; Xu et al. 2019a).

Water kefir is a fermented beverage based on the fermentation of a sucrose solution with added dried and fresh fruit, typically dried figs and a slice of lemon. Due to its components, water kefir has a low nitrogen content and high levels of sucrose before onset of fermentation (Laureys et al. 2018; Xu et al. 2019a). This results in several challenges to the microorganisms that take part in its fermentation, the most prominent being low content of amino acid and high osmotic pressure. Additional challenges include carbonation levels, low pH and alcohol content (Gulitz et al. 2011). Typically, water kefir fermentations are carried out in private households, as industrial fermentations are often found to be unstable (Laureys et al. 2017). The fermentation is initiated by back-slopping of slightly translucent water kefir grains (shown in Figure 3) consisting of exopolysaccharides (EPS) (Fels et al. 2018; Pidoux 1989; Pidoux et al. 1990).

Figure 3. Macroscopic image of three typical water kefir grains. Each grain is approximately 3-4 mm in diameter.

The grains are also referred to as “ginger beer plant”, “Tibicos”, “Tibi” or ”Japanese beer seeds” (Kebler 1921; Lutz 1899b; Marsh et al. 2013; Ward 1892). Water kefir grains harbour a microbiological consortium including yeast, LAB, acetic acid bacteria and sometimes *Zymomonas* (Gulitz et al. 2013; Gulitz et al. 2011; Laureys and De Vuyst 2014; Marsh et al. 2013; Pidoux 1989). Parts of the consortium responsible for water kefir fermentation have been the focus of scientific research for over 100 years, like its yeast and *Lb. hilgardii* (Kebler 1921; Lutz 1899a; Pidoux 1989; Ward 1892). However, only recently has it been recognized that water kefir also harbours bifidobacteria as part of the fermentation consortium (Gulitz et al. 2013; Laureys et al. 2016). While LAB, especially *Lb. hilgardii* have been shown to be responsible for the production of the exopolysaccharides (Fels et al. 2018; Pidoux 1989; Waldherr et al. 2010), the role of other microorganisms in the consortium remains less clear

(Stadie et al. 2013; Xu et al. 2018). The cell counts on kefir grains have been reported to be in the range of 10^8 for LAB, 10^6-10^8 for acetic acid bacteria, 10^5-10^6 for bifidobacteria and 10^6-10^7 for yeast (Eckel et al. 2019b; Gulitz et al. 2013; Gulitz et al. 2011; Laureys and De Vuyst 2014).

Especially the interactions of the microbiota and the exact role of each species remains to be elucidated. Current studies regarding the interaction of the consortium revealed strong interactions between lactobacilli and yeasts (Bechtner et al. 2019; Stadie et al. 2013; Xu et al. 2019b). However, the exact ecological role of bifidobacteria for the water kefir consortium is still unknown (Eckel et al. 2019b).

Water kefir is a traditional source of bacterial EPS. The translucent water kefir grains are made up of dextrans, while the supernatant contains dextrans and fructans, all of which are formed *in situ* by the fermentation consortium (Fels et al. 2018; Pidoux et al. 1990). In fact, most LAB found in one water kefir produce some form of exopolysaccharide (Stadie 2013). These exopolysaccharides display different properties: while some are insoluble and form the kefir grain, others are soluble and are found in the supernatant, likely contributing to the naturally cloudy appearance of water keifr (Fels et al. 2018; Pidoux et al. 1990; Waldherr et al. 2010).

1.4. LAB from water kefir

Out of all microbial groups involved in the water kefir fermentation process, LAB are not only the most abundant, but have been described as the organisms responsible for the granule formation (Fels et al. 2018; Gulitz et al. 2013; Gulitz et al. 2011; Laureys and De Vuyst 2014; Pidoux et al. 1990). As outlined above, several genera are considered to be part of the LAB. In water kefir, the genera *Lactobacillus* and *Leuconostoc* are commonly found to be part of the consortium (Gulitz et al. 2013; Gulitz et al. 2011; Laureys et al. 2018; Pidoux 1989). Both *Leuconostoc* and *Lactobacillus* are non-motile, non spore-forming and gram staining positive aerotolerant anaerobes. While lactobacilli are typically rod shaped, *Leuconostoc* are coocoid to oval (Hugenholtz 1998).

Leuconostoc mesenteroides and *Lc. citreum* are often found species of *Leuconostoc* in water kefir fermentations (Gulitz et al. 2011; Laureys and De Vuyst 2014). While *Leuconostoc* species are desirable in many food fermentations, in sugar refineries they are feared spoilage organisms due to their slime formation through exopolysaccharide production (Cogan and Jordan 1994; McCleskey et al. 1947). The fact that these *Leuconostoc* species are often found as spoilers in sucrose production plants, underlines their adaptation to sucrose-rich

environments (Cogan and Jordan 1994). The role of *Leuconostoc* for the fermentation consortium is less clear, as its numbers are often low (Marsh et al. 2013).

Out of the lactobacilli, *Lb. hordei*, *Lb. hilgardii*, *Lb. nagelii* and *Lb. satsumensis* are typically found to occur in water kefir (Gulitz et al. 2011; Laureys and De Vuyst 2014). While the species and number of species of *Lactobacillus* encountered in water kefir is variable, *Lb. hilgardii* has been recognized as the species that produces the gelling polysaccharide responsible for the granule formation and is typically found in water kefir fermentations (Fels et al. 2018; Laureys and De Vuyst 2014; Pidoux et al. 1990; Waldherr et al. 2010). The enzyme responsible for the formation of the kefir grain dextran has been characterized in detail und its enzymatic properties have been characterized, while its products have been found to constitute the kefir grain (Fels et al. 2018; Waldherr et al. 2010). Additionally, *Lb. hilgardii* has been found to be part of the consortium by various culture dependent, culture independent, metagenomics, as well as polysaccharide profiling studies (Fels et al. 2018; Gulitz et al. 2013; Gulitz et al. 2011; Laureys and De Vuyst 2014; Pidoux 1989; Pidoux et al. 1990; Verce et al. 2019). Its importance for the consortium is thus established.

1.5. *Bifidobacterium* spp. in water kefir

In general, bifidobacteria are not traditionally regarded as part of the LAB (Stiles and Holzapfel 1997). However, whether or not bifidobacteria are part of the LAB also depends on the point in time and the author in question (Kandler 1983; Makarova et al. 2006a; Stiles and Holzapfel 1997). While the fermentation end-products of bifidobacteria are, similarly to LAB, lactic acid, acetic acid and ethanol, they can also produce formic acid and are taxonomically not related to the LAB *sensu stricto* (Scardovi 1986a). The first isolates of the genus were found in infant stool samples and they have not been regarded as autochthonous in foods, but have rather been added for their health benefits after isolation from different sources, typically the intestinal tract of healthy humans (Guyonnet et al. 2007; Leahy et al. 2005; Mitsuoka 1990; Scardovi 1986a; Tissier 1900).

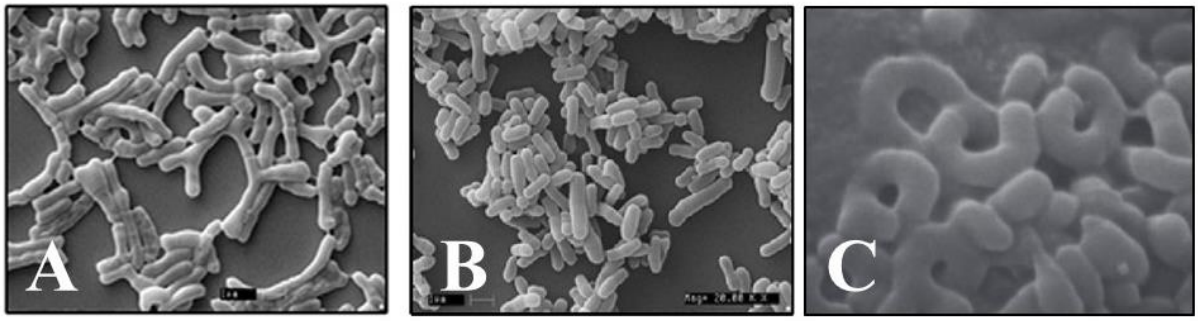

Figure 4. Different cell morphologies observed in the genus *Bifidobacterium*. A: Bifid and club-like shape displayed by *B. adolescentis* DSM 20083^T; **B**: rod shape of *B. animalis* subsp. *lactis* DSM 10140^T; **C**: ring-like morphology of *B. lemurum* DSM 28807^T. Figure modified from Modesto et al. (2015) and Dhanashree et al. (2017).

Bifidobacteria are strictly anaerobic or aerotolerant, non-motile, non-sporulating, non-gas-producing and Gram staining positive bacteria (Ventura et al. 2004). Their unusual, rod-, bifurcated or Y-, club like- and even ring-shaped cell morphology, is characteristic and gave rise to the genus name and is illustrated in Figure 4 (Dhanashree et al. 2017; Modesto et al. 2015; Tissier 1899; Tissier 1900). The presence of fructose-6-phosphate phosphoketolase (F6PPK) activity, the defining enzyme of the bifidus shunt, is regarded as an identifying trait of the family *Bifidobacteriacae*, of which they are a part (Biavati and Mattarelli 2006). This pathway is outlined in Figure 5. Originally, bifidobacteria were isolated from the infant gut and are now considered to be found in six ecological niches: human gastrointestinal tract (GIT), animal GIT, insect GIT, oral cavity, sewage and food (Tissier 1900; Ventura et al. 2004). Only in their more recent history have bifidobacteria been shown to be naturally occuring in fermented foods (Delcenserie et al. 2007; Gulitz et al. 2013; Laureys et al. 2016; Meile et al. 1997; Simpson et al. 2004; Watanabe et al. 2009). In the course of this research, they have also been shown to be a part of the microorganisms involved in water kefir fermentation (Eckel et al. 2019b; Gulitz et al. 2013; Laureys et al. 2016; Laureys and De Vuyst 2014). Originally, bifidobacteria in water kefir were first discovered through culture-independent methods (Gulitz et al. 2013). However, recently a novel strain has been isolated and cultured from water kefir and was described as a new species termed *Bifidobacterium aquikefiri* (Laureys et al. 2016).

Bifidobacteria are typically described as strictly anaerobic mesophiles, while the species from water kefir is aerotolerant with a comparatively low optimum growth temperature of 28 °C (Laureys et al. 2016; Scardovi 1986a). Taxonomic studies revealed this species to be closely related to several other bifidobacterial species found in food fermentations that were described

recently (Delcenserie et al. 2007; Laureys et al. 2016; Simpson et al. 2004; Watanabe et al. 2009).

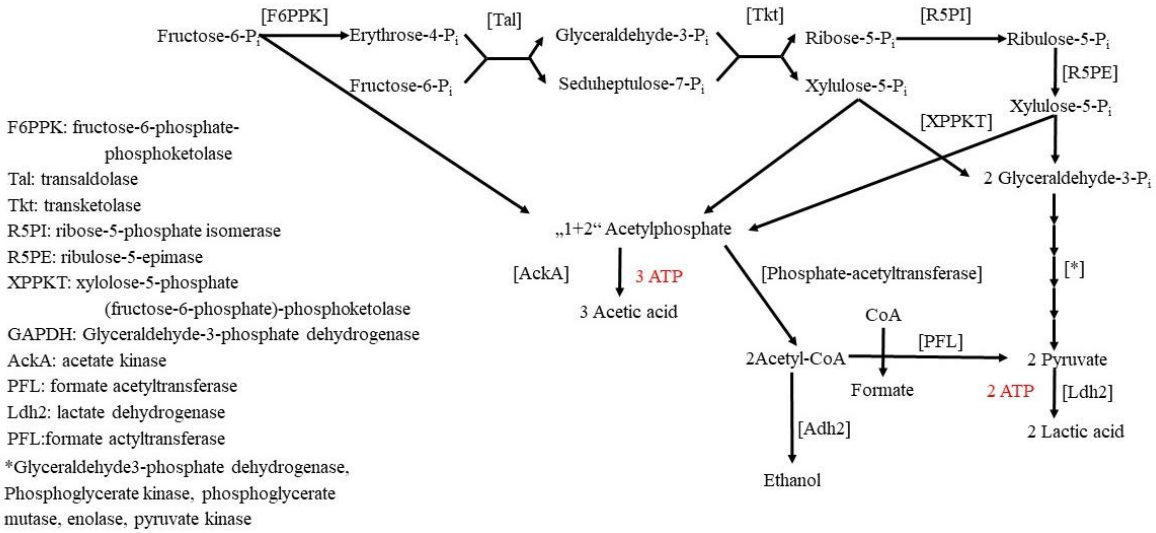

Figure 5. Schematic representation of the Bifidus shunt, the central carbohydrate metabolism pathway of bifidobacteria containing the defining enzyme F6PPK at the beginning of the pathway. Carbohydrates enter the pathway through conversion to fructose-6-Pi (e.g., galactose, ribulose, and glucose), glyceraldehyde-3-phosphate (e.g., fucose), ribose-5-phosphate (e.g., ribose) or xylulose-5-phosphate (e.g., xylose). The pathway from glyceraldehyde-3-phosphate to lactic acid is shortened for improved clarity. Figure adapted from Pokusaeva et al. (2011).

Culture dependent methods typically find only one species of *Bifidobacterium*, which is in contrast to culture-independent methods, which have shown that several species of bifidobacteria are sometimes present in the same water kefir (Gulitz et al. 2013; Laureys et al. 2016). While differential media for the enumeration of bifidobacteria from samples, which also contain LAB are described, they are typically not evaluated with respect to the presence of other groups of microorganisms like yeast and *Zymomonas* (Bunesova et al. 2015; Miranda et al. 2014; Roy 2001; Talwalkar and Kailasapathy 2004). Additionally, they are optimized for thermophilic probiotic strains used in the dairy industry that typically are not a part of the water kefir consortium (Bunesova et al. 2015; Roy 2001). The isolation of these organisms from water kefir has thus proven challenging, also due to long growth times reported (Gulitz et al. 2013; Laureys et al. 2016). These facts possibly explain the low number of culture-dependent studies concerning these organisms.

With regard to the role of these organisms in the consortium, several facts are worth to keep in mind. Bifidobacteria have been shown to be prototrophic for all amino acids before and are

known to produce several vitamins (Deguchi et al. 1985; Ferrario et al. 2015; LeBlanc et al. 2013; Verce et al. 2019). Therefore, bifidobacteria from water kefir suggest themselves as interesting candidates for cross feeding reactions in the water kefir consortium, which have recently been investigated (Stadie et al. 2013; Xu et al. 2019b). In addition, bifidobacteria produce acetic, formic and lactic acid as fermentation end-products of carbohydrate catabolism (Pokusaeva et al. 2011). They are thus likely involved in the production of organic acids over the course of the fermentation and therefore the organoleptic properties of the finished water kefir beverage. In any case, information on the metabolism and physiology of these bifidobacteria is limited and their role in the fermentation consortium is still unknown and speculative. Some exceptions are the knowledge on their acid production patterns from different substrates and the ability of *B. aquikefiri* to synthesize most amino acids *in silico*. (Laureys et al. 2016; Verce et al. 2019).

1.6. Other microorganisms involved in water kefir fermentation

In addition to LAB and bifidobacteria, the third most noteworthy group of bacteria in water kefir are acetic acid bacteria. They have been shown to occur in water kefir by culture dependent and independent studies (Franzetti et al. 1998; Gulitz et al. 2013; Gulitz et al. 2011). Acetic acid bacteria species commonly found in water kefir include *Acetobacter (Ab.) fabarum* and *Ab. orientalis*, *Gluconobacter (Gb.) albidus*, *Gb. oxydans* and others (Gulitz et al. 2013; Gulitz et al. 2011; Laureys and De Vuyst 2014). These organisms have been shown to produce fructans from sucrose, thus partly competing with LAB for this substrate (Jakob et al. 2012a). However, their role remains unclear and an essential role for the consortium is doubtful (Jakob 2014).

The last bacterial group worth mentioning is *Zymomonas*. *Zymomonas mobilis* has until now only been described as part of the water kefir consortium in one culture independent study (Marsh et al. 2013). *Zymomonas mobilis* is typically found in bacterial alcoholic fermentations like the Mexican “pulque” or African palm wine (Panesar et al. 2006).

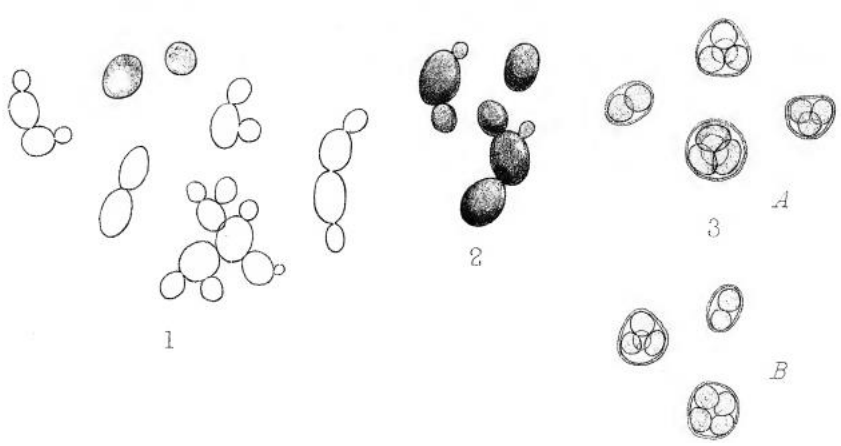

Figure 6. Historic depictions of yeast from water kefir. 1 shows a "characteristic" group of yeast occurring after 9 days of water kefir fermentation, **2** shows the same yeast after staining with Lugol's iodine, **3 A** and **B** show the same yeast after sporulation has been initiated (though incubation in pure gelatine at 22 °C for 4 days). Yeast were the first organisms that were described to be part of the water kefir microbiota. Figure adapted from Ward (1892).

Another group of microorganisms that plays a role in water kefir fermentation are yeast. Yeast are an important contributor to many food fermentations. They are typically found in alcoholic fermentations of sugar-rich media like beer wort or fruit juices (Schifferdecker et al. 2014; Walker and Stewart 2016). It is thus not surprising that several yeast have been recognized as part of the water kefir consortium early on (Figure 5) (Kebler 1921; Pidoux 1989; Ward 1892). Typical yeast species that are part of the water kefir fermentation consortium include *Dekkera bruxellensis*, *Hanseniaspora valbyensis*, *Saccharomyces cerevisiae*, and *Zygotorulaspora florentina* (Franzetti et al. 1998; Gulitz et al. 2011; Laureys and De Vuyst 2014; Pidoux 1989). Their role in the consortium is intriguing. It has been shown that some of the yeast species involved in water kefir fermentation, like *S. cerevisiae* and *Zygotorulaspora florentina* take part in cross-feeding reaction with the LAB of the water kefir consortium (Stadie et al. 2013; Xu et al. 2019b). In this context, yeast provide nutrients in the form of amino acids, vitamins and possibly other growth factors to enhance the growth of *Lactobacillus* species. It is therefore highly likely that even though yeast do not contribute to the kefir granule itself, they are an essential part of the consortium and facilitate the growth of LAB during water kefir fermentation (Xu et al. 2019b).

1.7. Exopolysaccharide formation in foods

Exopolysaccharide formation in foods is a common trait of LAB and has long been a research topic (De Vuyst and Degeest 1999; Han 1990; McCleskey et al. 1947; Monsan et al. 2001; Van Geel-Schutten et al. 1999). Exopolysaccharides are polysaccharides that are produced by bacteria outside of their cells, often with a typical "slimy" appearance (Figure 7 and 8). There is generally two types of exopolysaccharides when considering their monomeric constitution: homopolysaccharides (HoPs) and heteropolysaccharides (HePs) (De Vuyst and Degeest 1999; Monsan et al. 2001).

HePs are produced through activated sugar moieties that are polymerized and afterwards exported (De Vuyst and Degeest 1999). HoPs are, with the exception of β-glucan, produced extracellularly and consist of only one type of monomer, either only glucose (called glucans) or fructose (called fructans) (Monsan et al. 2001). The synthesis of glucose containing HoPs is catalyzed by glucansucrases. These enzymes are transglycolases that utilize the energy stored in the glycosidic bond to form the new polysaccharide, while the second sugar moiety is freed (Monsan et al. 2001). Of the homopolysaccharides, dextrans are of particular interest in this study. Dextrans are glucans with backbones that are made up of α-(1→6)-linked glucose moieties (Buchholz and Monsan 2001). These backbones are of differing length and possess several branching points and can thus have different physical properties and of course molecular weight depending on their size and branching (Bovey 1959; Seymour et al. 1976). Dextrans are synthesized extracellularly by dextransucrases (EC 2.1.4.5), which employ the energy of the glycosidic bond in sucrose to fuel the transglycosylation and polymerization (Cerning 1990).

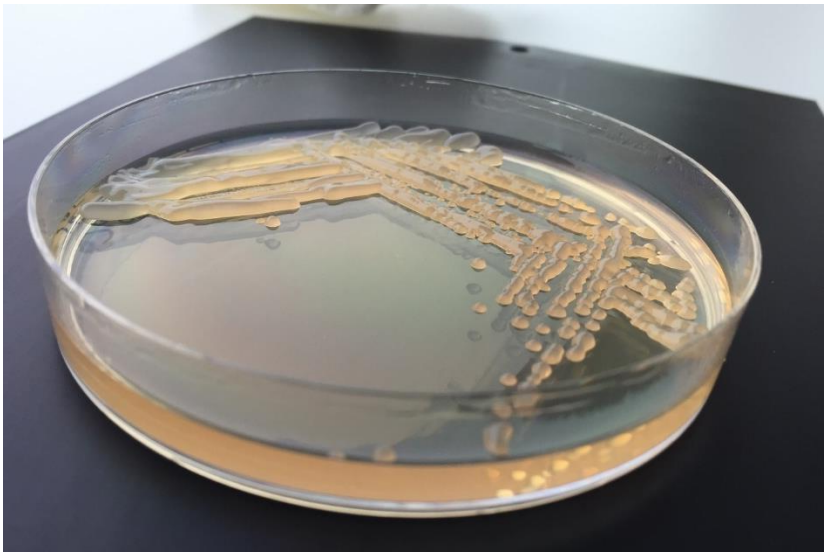

Figure 7. Visible homopolysaccharide formation by LAB from water kefir on sucrose-supplemented MRS agar. EPS formation is strong enough to create visible „slime" on top of the colonies.

For the bacterial cells, several physiological reasons for EPS production are discussed. A role in energy storage of the organism seems unlikely, since most EPS producing strains lack the enzyme for their degradation (Cerning 1990). More likely, EPS formation often mediates the interaction of the EPS producing strain and its environment (Zeidan et al. 2017). It has been shown that this may be achieved through a role in biofilm formation, the protection from harmful substances like ethanol, protection from dessication and protection from phage infection (Badel et al. 2011; Monsan et al. 2001; Sutherland 1979; Zannini et al. 2016). In addition, EPS have been shown to facilitate adhesion to e.g., the mucosa of the human intestine and therefore also mediate interaction with a host (Ryan et al. 2015). In host interaction, they have been described as possessing antitumor and immunomodulatory effects (De Vuyst and Degeest 1999; Hamada and Slade 1980; Ruas-Madiedo et al. 2002a). In water kefir, as mentioned before, *Lb. hilgardii* produces the insoluble dextran responsible for the formation of the kefir granule (Fels et al. 2018; Pidoux et al. 1990; Waldherr et al. 2010). *Lb. hilgardii* thus facilitates the passage and propagation of all microorganisms that adhere to this structure, since the water kefir grains are transferred from one fermentation to the next (Gulitz 2013).

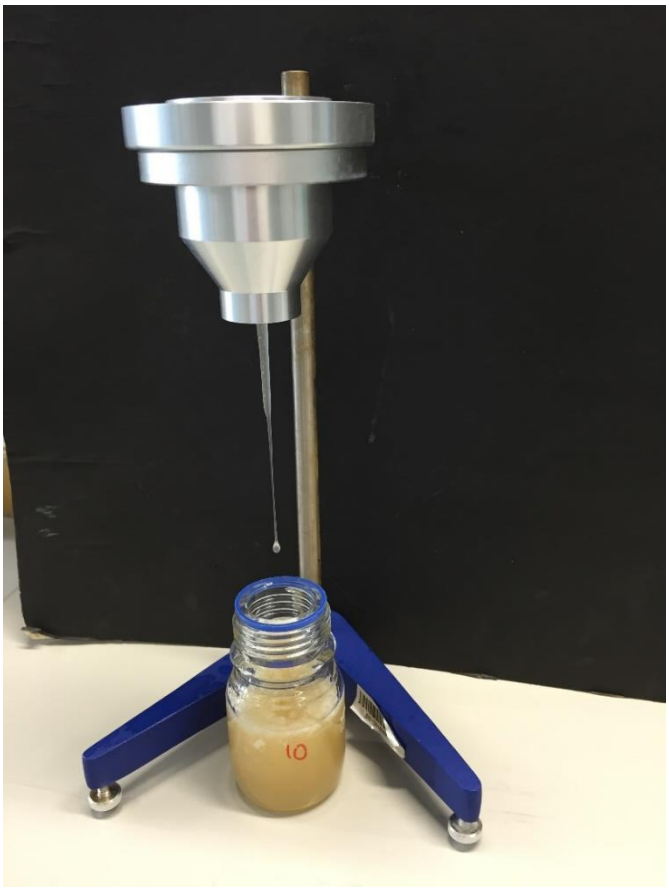

Figure 8. Strongly viscous behaviour of a fruit juice fermented with EPS producing strains. The undesireable ropiness becomes quite evident.

The utilization of EPS in foods, knowingly or unknowingly, typically takes place through *in situ* production of EPS using microorganisms. This *in situ* production has been reported to be preferable as no purification steps are necessary and an often higher level of functionality is achieved and may be preferable from a regulatory standpoint (Doleyres et al. 2005; Kaditzky and Vogel 2008; Ruas-Madiedo et al. 2002b). The positive traits of EPS in foods are traditionally employed in different dairy products like cheeses, yoghurt or milk kefir (Cerning 1990; Rimada and Abraham 2003). In dairy products, typically heteropolysaccharides are used to enhance rheological properties of the food products (Vuyst and Degeest 1999). In addition to heteropolysaccharides, bacterial homopolysaccharides are also of interest in different applications and foods. While their use may not be as widespread as that of heteropolysaccharides, bacterial homopolysaccharides are used for several purposes. The use of fructans in food ranges from baking over dairy to pet food and cosmetics applications (Booten et al. 1998; Jakob et al. 2012b; Ua-Arak et al. 2017b; Vincent et al. 2005). Dextrans from e.g., *Leuconostoc mesenteroides* are used for different purposes like analytical applications (e.g., molecular weight standards or carrier chemical for chromatography columns) (Monsan et al. 2001), baking applications (e. g. panettone) (Decock and Cappelle 2005; Rühmkorf et al. 2012a) or medical uses (e.g., blood plasma substitutes) (Naessens et al. 2005). An *in situ* production is in most cases discussed as the preferable way of production of EPS in foods, as the high cost and negative consumer image of additives can be averted (Moroni et al. 2009; Rühmkorf et al. 2012a).

1.8. Turbidity in beverages

Non-alcoholic refreshing beverages, mostly fruit juice based, are often turbid. This turbidity is typically caused by the addition of artificial cloud systems (Taherian et al. 2007). These cloud systems contain emulsions of oil in water, with typical oil droplet sizes between 0.2 to 5 µm (Dickinson 1994; Linke and Drusch 2016). The demand for this type of cloud systems is projected to grow world wide in the future due to their increasing use (Transparancy Market Research, 2017). However, these emulsions are thermodynamically unstable. The difference in density between the two phases results in a creaming of the lighter oil phase, as is the case with all emulsions (Tan 1998). This effect is founded on the fact that oil droplets in water behave like any other objects in water. According to Stokes law, they settle in the liquid and their settling velocity is faster with increasing density differences, larger droplet radii and lower viscosities of the continuous phase (McClements 2005; Stokes 1845). While they do not settle at the bottom, oil droplets of an emulsion cream at the top of a liquid due their lower density than water (McClements 2005). In beverages, the use of stabilizers is widespread to retard this

instability of emulsions (Cao et al. 1990). In many foods, high-molecular weight polysaccharides like xanthan, carrageenan and carboxmethylcellulose are added for this purpose (Cao et al. 1990).

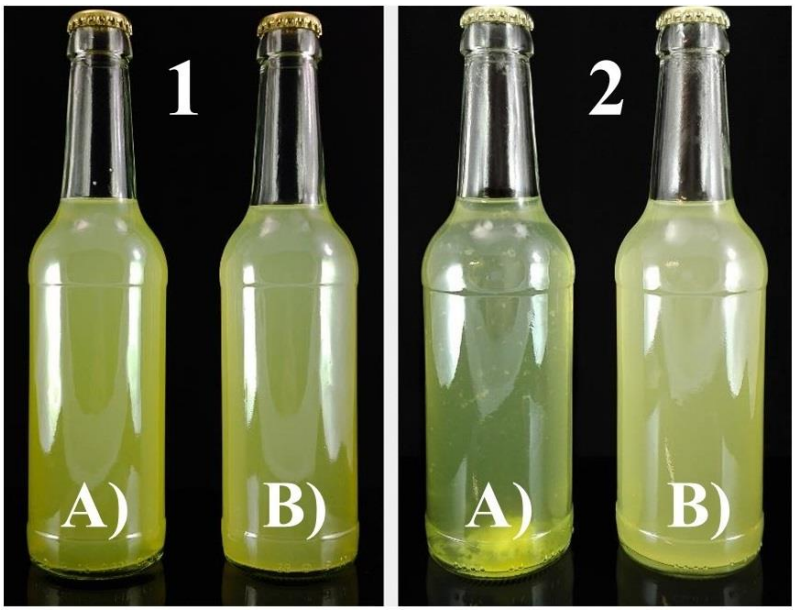

Figure 9. Stable and unstable turbid beverages. Panel 1 shows two beverages directly after preparation and **panel 2** after sufficient storage. Emulsion **A)** decayed and particles visibly separated, while emulsion **B)** was successfully stabilized over the duration of storage. Images reproduced with friendly permission of Julian Huchtmann, Hochschule Ostwestfalen-Lippe.

Of these polysaccharides, only the exopolysaccharide xanthan is produced by bacteria and is used as a stabilizer for cloud systems (Mirhosseini et al. 2008). Previous work on the properties of bacterial homopolysaccharides revealed that fructans (levans) with extremely high molecular weight that are derived from acetic acid bacteria and dextrans, e.g., those derived from fermentations using *Lactobacillus sakei*, show a turbidity on aqueous solution owing to their high molecular weight (> 10^8 g/mol), that causes light scattering (Jakob et al. 2013; Prechtl et al. 2018a; Ua-Arak et al. 2017a). The glucans, due to their stability under acidic conditions, therefore show great promise as an alternative to emulsion based cloud systems. They are produced by food-grade bacteria that are readily isolated and use food grade substrates for the production of the polysaccharide (Prechtl et al. 2018a; Ua-Arak et al. 2017a). Therefore, these polysaccharides could be used as cloud or turbidity-forming agents in beverages, under the prerequisite that they do not thicken liquids beyond palatability at the used concentrations. Additionally, it is important that no degradation or sedimentation effects occur upon storage. The use of these molecules would allow for less processing steps with less specialized

equipment in the production of turbid lemonades by omitting an emulsion preparation step and less use of stabilizers and costly cloud systems, as illustrated in Figure 9. This omission of additives and technological measures in exchange for natural ingredients and produced *in situ* is in the interest of consumers and therefore manufacturers, as consumers are interested in consuming less processed, more natural foods that have less artificial additives or chemicals added (Asioli et al. 2017). Water kefir, as elaborated before, proves to be a natural habitat for exopolysaccharide producing bacteria. Due to its naturally turbid occurrence, the organisms involved in water kefir fermentation show great promise in the development of turbidity forming beverage additive.

2. Motivation and aims of this work

Water kefir is a fermented beverage with a high diversity of microorganisms. Still, their exploitation for beverages is limited. Namely, the potential of their glucans for introducing naturally stable turbidity in beverages is unexplored and presents a target for technological and sensorical improvement of beverages. Furthermore, the adaptation and functional role of bifidobacteria in water kefir remains unknown. Since there is a growing demand for healthy, alcohol free beverages, this work should explore the functional potential of bacteria from water kefir for use in turbid, alcohol free beverage fermentations. We therefore postulate that:

- Water kefir contains exopolysaccharide-forming strains.
- Water kefir is a naturally cloudy beverage. This stable opacity is caused by specific exopolysaccharides.
- A relationship can be established between the molecular structure of glucans with their cloud-forming properties.
- These naturally cloudy exopolysaccharides can be used for beverage technological applications.
- Different bifidobacteria predicted from culture independent approaches can be isolated from water kefir and characterized with respect to adaptation, liefestyle and functionality.

From these hypotheses we derive the following experimental approach:

- The microbiota of water kefir should be re-evaluated with respect to functionally exploitable bacteria using culture-dependent methods.
- A focus should be on bifidobacteria and glucan-forming LAB.
- New isolates should be taxonomically classified and evaluated with respect to their application potential in alcohol free beverages.
- The adaptation, lifestyle and functional properties of bifidobacteria from water kefir should be characterized.
- The potential of isolates from water kefir to form exopolysaccharides should be explored.
- HoPs should be produced in substrates suitable for beverage technological use and their suitability for formation and stabilization of turbid beverages or beverage ingredients explored.

- Fermentation conditions for food grade production of HoPs should be optimized to increase yield, a goal should be 5 g/L, as derived from technological demands.
- Structure-function relationships of these molecules should be established for a selected EPS preparation.
- These fermentations should be evaluated with respect to the metabolites formed, to assess their applicability in beverage fermentations.

3. Material and methods

3.1. General microbiological methods

3.1.1. Strains

Table 1. Strain selection of LAB from water kefir.

Species		Strain number
Lactobacillus	*curvatus*	TMW 1.1624
Lactobacillus	*hilgardii*	TMW 1.828
Lactobacillus	*hordeii*	TMW 1.1817
Lactobacillus	*hilgardii*	TMW 1.1819
Lactobacillus	*hordeii*	TMW 1.1821
Lactobacillus	*hordeii*	TMW 1.1822
Lactobacillus	*nagelii*	TMW 1.1823
Lactobacillus	*nagelii*	TMW 1.1824
Lactobacillus	*nagelii*	TMW 1.1826
Lactobacillus	*nagelii*	TMW 1.1827
Lactobacillus	*hilgardii*	TMW 1.1828=TMW 1.2196
Lactobacillus	*satsumensis*	TMW 1.1829
Lactobacillus	*hordeii*	TMW 1.1907
Leuconostoc	*mesenteroides*	TMW 2.1073
Leuconostoc	*mesenteroides*	TMW 2.1075
Leuconostoc	*mesenteroides*	TMW 2.1076
Leuconostoc	*citreum*	TMW 2.1194
Leuconostoc	*mesenteroides*	TMW 2.1195

Table 2. *Bifidobacterium* strains used in this study.

Species		TMW strain number	Isogenic with
Bifidobacterium	*psychraerophilum*	2.1362^{T}	LMG 21775^{T}
Bifidobacterium	*crudilactis*	2.1369^{T}	LMG 23609^{T}
Bifidobacterium	*tibiigranuli*	21764	-
Bifidobacterium	*tibiigranuli.*	2.2057^{T}	DSM 108414^{T}
Bifidobacterium	*aquikefiri*	2.2058	CCUG 67145^{T}
Bifidobacterium	*aquikefiri*	2.2059	-
Bifidobacterium	*subtile*	2.2109^{T}	DSM 20096^{T}
Bifidobacterium	*indicum*	2.2110^{T}	DSM 20214^{T}
Bifidobacterium	*lemurum*	2.2111^{T}	DSM 28807^{T}
Bifidobacterium	*eulemuris*	2.2112^{T}	DSM 100216^{T}
Bifidobacterium	*breve*	2.447^{T}	DSM 20213^{T}
Bifidobacterium	*longum ssp. longum*	2.614^{T}	DSM 20219^{T}

All strains were retrieved from the in-house strain collection of the Lehrstuhl für Technische Mikrobiologie Weihenstephan (TMW). In case of proprietary strains, strains were isolated at the TMW and added to the strain collection in previous works. In case of non-proprietary or type strains, they were obtained from commercial strain collections as indicated. After obtaining the strains from the strain collection, cryo stocks were prepared. After preparation of cryo stocks, colonies were obtained by re-streaking from these cryo stocks on the appropriate medium. Single colonies were used to prepare liquid pre-cultures.

3.1.2. Strain culture

LAB strains were generally cultivated in a modified deMan, Rogosa and Sharpe medium (mMRS) according to Gulitz et al. (2011). Agar plates were incubated anaerobically (Anaerogen, Oxoid), while liquid cultures were prepared in 15 and 50 mL screw-top tubes filled to the top with a negligible atmospheric headspace. These pre-cultures were incubated statically at 30 °C in liquid mMRS medium for 48 h under anaerobic conditions. Stationary phase cells were used as starter culture for fermentations.

Bifidobacteria were cultured on Deutsche Sammlung Mikroorganismen und Zellkulturen (DSMZ) medium 58. Strains of *B. aquikefiri* were incubated at 30 °C for 6 days, strains of *B. tibiigranuli* at 30 °C for 3 days and *B. animalis* subsp. *lactis* BB12® was incubated at 37 °C for 2 days. Agar plates were incubated anaerobically (Anaerogen, Oxoid) and liquid cultures were

prepared in 15 and 50 mL screw-top tubes filled to the top with a negligible atmospheric headspace.

3.1.3. Media for microbiological culture

All media were pepared using dry ingredients and autoclaved at 121 °C for 15 min using a VX 150 autoclave (Systec, Linden, Germany). Sugars were autoclaved separately and added after cooling below 80 °C. For solid media, 1.5% w/v of Agar-Agar (Carl Roth GmbH, Karlsruhe, Germany) were added. MRS, M144, TP and TPY agar medium were prepared according to literature, partly with slight modifidactions (De Man et al. 1960; Gulitz et al. 2013; Laureys et al. 2016; Scardovi 1986b). The contents are given in the following tables (Table 3-8). Cultures were incubated under anaerobic conditions. All cell morphologies and morphological characteristics of colonies of strains TMW 2.1764 and TMW 2.2057^T were evaluated after 3 days of anaerobic growth at 30 °C on BM agar medium.

Table 3. Contents of mMRS medium.

Compound	Final concentration
Glucose	20.0 g/L
Peptone from soy	10.0 g/L
Yeast extract	6.0 g/L
Sodium acetate * 3 H_2O	5.0 g/L
K_2HPO_4 * 3 H_2O	2.5 g/L
Ammonium citrate monohydrate	2.0 g/L
Tween 80	1.0 g/L
$MgSO_4$ * 7 H_2O	0.2 g/L
$MnSO_4$ * H_2O	0.038 g/L

Table 4. Contents of BM (DSMZ No. 58 medium).

Compound	Final concentration
Tryptone/peptone from casein	10.0 g/L
Yeast extract	5.0 g/L
Meat extract	5.0 g/L
Peptone from soy	5.0 g/L
Glucose	10.0 g/L
K_2HPO_4 * 3 H_2O	2.0 g/L
$MgSO_4$ * 7 H_2O	0.2 g/L
$MnSO_4$ * H_2O	0.05 g/L
Tween 80	1.0 mL/L
NaCl	5.0 g/L
L-Cysteinehydrochloride * H_2O	0.5 g/L
Salt solution	40 mL/L

Table 5. Contents of salt soulution for BM.

Compound	Final concentration
$NaHCO_3$	10.0 g/L
NaCl	2.0 g/L
K_2HPO_4 * 3 H_2O	1.0 g/L
KH_2PO_4	1.0 g/L
$MgSO_4$ * 7 H_2O	0.5 g/L
$CaCl_2$ * 2 H_2O	0.25 g/L

Table 6. Contents of M144 medium.

Compound	Final concentration
Special peptone	23.0 g/L
Glucose	5.0 g/L
NaCl	5.0 g/L
Starch	1.0 g/L
L-Cysteinehydrochloride * H_2O	0.3 g/L

Table 7. Contents of TP medium.

Compound	Final concentration
Tryptone/peptone from casein	10.0 g/L
Peptone from soy	10.0 g/L
Yeast extract	6.0 g/L
NaCl	5.0 g/L
$K_2HPO_4 * 3\ H_2O$	2.5 g/L
Glucose	2.0 g/L
Raftilose	2.0 g/L

Table 8. Contents of TPY medium.

Compound	Final concentration
Tryptone/peptone from casein	10.0 g/L
Peptone from soy	5.0 g/L
Glucose	5.0 g/L
Yeast extract	2.5 g/L
$K_2HPO_4 * 3\ H_2O$	2.0 g/L
Tween 80	1.0 g/L
L-Cysteinhydrochloride $* H_2O$	0.5 g/L
$MgCl_2 * 6\ H_2O$	0.5 g/L
$ZnSO_4 * 7\ H_2O$	0.25 g/L
$CaCl_2$	0.15 g/L
$FeCl_2$	traces

3.1.4. Determination of cell counts

For cell count determination, 100 µL of liquid sample were used and the sample was serially diluted in 0.9% NaCl solution. In case water kefir grains, 10 g of grains were washed using 1 L of sterile 0.9% NaCl in a sterile metal sieve. 10 g of washed grains were homogenized in 90 mL of 0.9% NaCl using a homogenator for 60 s. Dilutions were plated in triplicates using glass beads and were counted visually using a plate counter.

3.1.5. Propagation of water kefir

Water kefir was propagated using standard household methods. Water kefir grains were washed with water for 60 s. Approximately 200 g of washed kefir grains were used to inoculate the fermentation medium which was prepared from 80 g of sucrose dissolved in 1 L of tap water, 1 dried fig and a slice of lemon. Both fruits were organic. Water kefir fermentation was carried out for 72 h before sampling or propagation.

3.1.6. Isolation of bifidobacteria from water kefir

To isolate bifidobacteria from water kefir, serial dilutions of water kefir grain homogenate were plated on a modified *Bifidobacterium* selective medium. Incubation was carried out anaerobically for 12 days at 25 °C. The modified *Bifidobacterium* selective medium was based on the work of Miranda et al. (2014). The content of the medium can be found below in Table 9, the major modification was the addition of nystatin to inhibit yeast growth.

Table 9. Contents of BSM.

Compound	Final concentration
Sodium propionate	15 g/L
Casein enzymic hydrolysate	10 g/L
Raffinose	5 g/L
$K_2HPO_4 * 3\ H_2O$	4.8 g/L
$(NH_4)_2SO_4$	3 g/L
KH_2PO_4	3 g/L
Yeast extract	1 g/L
$MgSO_4 * 7\ H_2O$	0.2 g/L
Agar	15 g/L

The medium was prepared according to standard procedure. After cooling to 50 °C, 500 mg/L of sterile filtered L-cysteine hydrochloride monohydrate solution, 500 mg/L of kanamycin, 1000 mg/L of mupirocin and 30 mg/L of nystatin were added.

3.1.7. Species identification using MALDI-TOF MS

Species were identified based on their sub-proteome using a MALDI-TOF (Microflex LT spectrometer, Bruker Corporation, Billerica, MA, USA) and the manufacturers and an in-house database (Kern et al. 2014). For measurements, single colonies were obtained using the standard growth parameters for each strain. One single colony was removed from the agar plate and smeared on the measuring area of a stainless steel target. The colony material was subsequently overlaid with 1 µL of formic acid (70%, Sigma Aldrich, Germany) for cell disruption. After drying, 1 µL of α-cyano-4-hydroxy cinnamic acid matrix solution (Bruker Daltonics, Germany) were added and similarly dried. Subsequently, the target was inserted into the mass spectrometer and spectra were measured using a nitrogen laster (λ = 337 nm) in linear positive detection mode. 240 mass spectra were recorded in a molecular mass range of 2 – 20 kDa and their average determined as described by Usbeck et al. (2013) and subsequently used for identification. The mass spectrometer was controlled using Biotyper Automation Control 3.0 (Bruker Daltonics, Germany).

3.1.8. Peptidoglycan structure and cellular fatty acid determination

For taxonomic purposes, peptidoglycan structure and cellular fatty acids were analyzed. These analyses were conducted by the identification service of the DSMZ (Braunschweig, Germany). For peptidoglycan structure determination, the method described by Schumann (2011) was used. Fatty acid methyl esters were determined using the slightly modified methods of Miller (1982) and Kuykendall et al. (1988) with 40 mg of cells scraped from solid cultures by saponification followed by methylation and extraction. After extraction, fatty acid methyl esters were separated using a Sherlock Microbial Identification System (MIDI, Microbial ID, Newark, DE 19711 U.S.A.). Integration of peaks was carried out automatically using the MIDI, names of fatty acids and their percentages were determined with the MIS Standard Software (Microbial ID).

3.1.9. Determination of growth conditions for culture of bifidobacteria

To determine the growth limits and optima, growth of strains TMW 2.1764 and TMW 2.2057^T and reference strains was assessed on different media. For this purpose, solid media were prepared using standard methods and streaks from fresh exponential growth liquid cultures were prepared on these media. Growth was assessed visually after 2, 4 and 10 days of growth depending on the species. MRS, DSMZ medium no. 58 *Bifidobacterium* medium (BM), M144,

TP and TPY agar medium were used at 20, 25, 30, 35, 37 and 40 °C under anaerobic atmosphere (Oxoid Anaerogen, Thermo Scientific, Schwerte, Germany).

3.1.10. Microscopic imaging

Differential interference contrast microscopic imaging (DIC) was carried out with an Olympus BX61 microscope with an F-view II digital CCD camera and 100 x immersion objective. The images obtained were used directly without further processing.

3.1.11. Gram staining

Gram staining was conducted using a modified version of the method described by Gram (1884). Cells were resuspended in dH_2O and applied to a microscopic slide on which it was fixed using heat. Subsequently, samples were dipped in crystal violet solution (ethanolic, 10% w/v) and then washed using dH_2O. After washing, samples dipped in Lugol's iodine solution (5% (w/v) of potassium iodine and iodine at a mass ratio of 2:2) for 1 minute and once again washed. De-staining was done using 96% ethanol until no dye could be extracted from the microscopic slide. Subsequently, 2% Safranin T solution (aq.) was used for counter staining. After a final washing step in dH_2O, light microscopic evaluation was carried out using a 100 x immersion objective. *Photobacterium iliopiscarium* TMW 1.992 was used as negative control, *Lactobacillus hilgardii* TMW 1.828 was used as positive control.

3.1.12. Fermentation patterns using API 50 CH

The API 50 CH test was used for determination of carbohydrate usage by novel bifidobacteria. Since it is developed for LAB, it uses a half-strength MRS medium for their culture. We therefore employed a half strength BM medium for determination of bifidobaterial carbohydrate usage patterns. The half strength BM medium was prepared without carbohydrate and with an addition of 0.17 g/L Bromcresol purple as a pH indicator.

For inoculation, a pre-culture was doubly washed in API CH BM by cell harvest (5000 x g; 5 min) and resuspension in API 50 CH BM. This washed pre-culture was used for inoculation of the test strips according to the manufacturer's guidelines. After inoculation, the wells were overlaid with paraffin oil to generate anoxic conditions. The carbohydrate fermentation patterns were evaluated at 24, 48, 72 and 96 hours post inoculation, while the 96 hour values were used for evaluation.

3.1.13. Fermentation patterns using API rapid ID 32 A

The API 50 CH test was additionally used for determination of carbohydrate usage and selected enzyme activities by novel bifidobacteria. The test was used according to the manufacturer's instructions.

3.1.14. Determination of fructose-6-phosphate phosphoketolase

Fructose-6-phosphate phosphoketolase is the key enzyme of the bifidus shunt, its detection is therefore crucial for the assignment of species to the family bifidobacteriacae (Scardovi 1986a). Its detection was carried out using the method by Scardovi (1986a) and modified by Orban and Patterson (2000). There, F6P is transferred to acetylphosphate, which subsequently forms a chromogenically active iron chelate complex. For F6PPK detection, 2 mL pre-cultures were harvested and washed thrice with solution 1 (pH 6.5; 36.0 g/L Na_2HPO_4; 13.5 g/L KH_2PO_4; 1.0 g/L cysteine-HCl) by centrifugation (10 000 x g; 10 min, 4 °C) and resuspension in the same volume of solution 1. After the final washing step, the supernatant was removed. Lysis was carried out by addition of 200 µL solution 1 and 40 µL solution 8 (4.5 mg/mL hexadecyltrimethylammoniumbromide) and incubation at RT for 10 min. 50 µL of solution 2 (6.0 mg/mL NaF; 10.0 mg/mL sodium iodoacetate) and 50 µL of solution 7 (80 mg/mL D-fructose-6-phosphate) were added. This suspension was incubated at 37 °C for 90 min in order to force fermentation of fructose-6-phosphate by the freed intracellular enzymes. After successful conversion, the reaction was stopped by addition of 300 µL of solution 3 (150 mg/mL hydroxylaminhydrochloride) and incubation at room temperature for 10 min. Finally, 200 µL each of solution 4 (150 mg/mL trichloroacetic acid), 5 (4 mol/L HCl) and 6 (50 mg/mL iron chloride hexahydrate; 0.1 mol/L HCl) were added for the chromogenic reaction, which was evident shortly after by occurrence of a reddish-violet colour. As a negative control, *Lactobacillus hilgardii* TMW 1.828 was used.

3.1.15. Catalase activity

To assess presence of catalase, the method described by Smibert and Krieg (1981) was used. For this purpose, cell mass from a single colony was applied to a microscopic slide and overlaid with 3% H_2O_2 solution. Presence of catalase was displayed by vigorous gas production.

3.1.16. Oxidase test

Oxidase was assessed using oxidase test discs (Oxidase-Test; Sigma-Aldrich) that employ the method described by Gaby and Hadley (1957). These discs use a mechanism in which N,N-dimethyl-p-phenylendiaminoxalate and α-naphthol are oxidized to indolephenole blue by

bacterial oxidase. The presence of oxidase is therefore indicated by the development of a blue colour after 2 minutes of incubation at 30 °C.

3.1.17. Growth in the presence and absence of oxygen

To determine the growth in presence and absence of oxygen, an experiement modified from the method described by MacFaddin (1972) was used. BM agar medium supplemented with 0.5 g L-1 sodium thioglycolate was stab inoculated and incubated aerobically at 30 °C in biological triplicates. The addition of thioglycolate resulted in a gradient of oxygen content in the agar. Growth was assessed visually each day for one week to gain a thorough insight into oxygen (in)sensitivity.

3.1.18. Test for bacterial motility

Strain motility was assessed using a soft agar stab method. In this test, a culture was stab inoculated into BM medium containing only 0.5% w/v agar. The agar was inoculated in biological triplicates and incubated anaerobically at 30 °C. Growth was assessed visually on a dayly basis for 7 days. If growth after 7 days was restricted to the stab canal, strains were considered non-motile.

3.1.19. Determination of amino acid auxotrophy

To determine amino acid auxotrophy patterns, a method described by Petry et al. (2000), Cronin et al. (2012) and Ferrario et al. (2015) was used. First, the media were prepared. For this purpose, a base medium without amino acids called CDM-AA was prepared (see Table 10). CDM-AA contained all components but the amino acids. The amino acids were then weighed and added in the appropriate concentrations to the base medium and the pH was checked and the medium sterile filtered thereafter. The respective concentrations used in the medium are given below. The media were prepared in 50 mL batches, the pH then adjusted individually to pH 6.8 and each batch was subsequently sterile filtered using syringe filters with 0.2 µm pore size.

Table 10. Contents of CDM-AA.

Compound	Final concentration
Glucose	30 g/L
Sodium acetate	4.0 g/L
Triammonium citrate	1.0 g/L
KH_2PO_4	2.0 g/L
K_2HPO_4	2.0 g/L
$MgSO_4$ * 7 H_2O	0.5 g/L
$MnSO_4$ * H_2O	0.05 g/L
$FeSO_4$ * 7 H_2O	0.02 g/L
$CaCl_2$	0.2 g/L
Orotic acid	0.5 g/L
Guanine	50 mg/L
Xanthine	40 mg/L
Adenine	20 mg/L
p-Aminobenzoic acid	0.5 mg/L
Folic acid	0.5 mg/L
Biotin	1.0 mg/L
Vitamin B12	1.0 mg/L
Ca-pantothenate	2.0 mg/L
Pyridoxal	2.0 mg/L
Riboflavin	2.0 mg/L
Nicotinic acid	2.0 mg/L
Thiamine	4.0 mg/L

Table 11. Amino acid concentrations in CDM+AA.

Compound	Final concentration
Cysteine	0.4 g/L
Aspartic acid	0.3 g/L
Glutamic acid	0.3 g/L
Asparagine	0.3 g/L
Glutamine	0.3 g/L
Alanine	0.2 g/L
Arginine	0.2 g/L
Glycine	0.2 g/L
Histidine	0.2 g/L
Isoleucine	0.2 g/L
Leucine	0.2 g/L
Lysine	0.2 g/L
Methionine	0.2 g/L
Phenylalanine	0.2 g/L
Proline	0.2 g/L
Serine	0.2 g/L
Threonine	0.2 g/L
Tryptophane	0.2 g/L
Tyrosine	0.2 g/L
Valine	0.2 g/L

Using this medium, growth of bacterial isolates was determined in 96-well plates. As controls, BM medium, CDM with all amino acids (CDM+AA) and CDM-AA on each 96-well plate to ensure proper inoculation and pre-culture viability. The plates additionally contained CDM containing all but one amino acid to determine the requirements of these bacteria for the amino acids. Pre-cultures were prepared using the standard procedure and then washed twice. One washing step consisted of centrifugation of the pre-culture (5 min, 5000 x g, 4 °C) and subsequently resuspending the harvested cells in CDM-AA. Each well contained 250 µL of medium that was inoculated from twice washed pre-cultures. Inoculation was done to an initial optical density at 590 nm (OD_{590}) of 0.1 in biological triplicates. Following inoculation, each well was covered with 50 µL of sterile paraffin oil for the generation of anoxic conditions. 96-well plates were shaken for 20 s using double-orbital shaking at 500 RPM prior to OD

measurements. All cultures were incubated at 30 °C for *B. tibiigranuli* and 28 °C for *B. aquikefiri*. *B. animalis* subsp. *lactis* was incubated at 37 °C. Initial OD was recorded after inoculation to ensure proper inoculation and homogenization by shaking. The final OD was recorded after 2, 4 and 6 days for *B. animalis* subsp. *lactis*, *B. tibiigranuli* and *B. aquikefiri*, respectively.

3.1.20. Optimization of CDM for growth of BB12®

Since *B. animalis* subsp *lactis* BB12® showed no growth in CDM+AA, an attempt was undertaken to optimize the CDM for this strain. Therefore, the pH of CDM+AA was adjusted to pH 5.5, 6.0, 6.5 and 7.0 using HCl and NaOH (1 mol/L each). Additionally, lactose was used as a carbon source instead of glucose. Finally, an addition of adenosine, cytosine, uracil (50 mg/L each) and ascorbic acid (0.1 g/L and 1 g/L) was investigated, as well as a combination of all these 4 compounds.

3.2. EPS production and treatment

3.2.1. Screening for EPS production in selected strains

In order to evaluate the EPS production by the selected strains a screening on solid medium was conducted. For this purpose, an MRS solid medium was prepared as per usual. In contrast to the standard medium, the carbon source glucose was exchanged for 80 g/L sucrose, as described by Stadie (2013). After incubation at 30 °C for 48 h, an EPS formation score from 0 to 3 was given for each isolate, with 0 indicating no visible EPS formation and 3 indicating strong visible EPS formation.

3.2.2. Fermentation of fruit juice based media

Fermentation of fruit juice based media were conducted using clear commercial apple or grape juice (Wolfra, Erding, Germany, 2019). The juices were used pure or appropriately diluted (as indicated in the respective figures) and supplemented with 40 g/L sucrose as fermentation substrate for EPS formation. Dilute grape (G) and apple (A) juices were used at their native pH (An, Gn) as well as with a pH adjusted to pH 7 (A7, G7). All juice based media were sterile filtered after preparation using a CytoOne 0.2 µm bottle top filter (Starlab international GmbH, Hamburg, Germany). The fermentation media were inoculated to an OD_{590} of 0.1 from pre-cultures that were prepared as described above. Small-scale fermentations were carried out in 15 and 50 mL screw-top tubes filled with the nominal volume of fermentation medium. 2 L laboratory-grade screw-top glass bottles were used for large-scale fermentations. 2 L

fermentations were divided after fermentation: 0.5 L were used for stability testing. These 0.5 L were cooled to 4 °C and cells were removed by centrifugation at 10 000 x g, 10 min at 4 °C in two 500 mL laboratory centrifuge beakers. After decanting the supernatant, the supernatant was centrifuged again using the same conditions to enhance cell removal. The remaining 1.5 L were split in four parts. All were transferred to 0.5 L centrifuge beakers, that were afterwards cooled to 4 °C. After cooling, cells were also removed by centrifugation (10 000 x g, 10 min at 4 °C). The supernatant from this step was used for EPS purification.

3.2.3. Dextran purification

Liquid samples containing EPS were precipitated using 2 volumes of denatured ethanol (chilled to -20 °C). After precipitation overnight at 4 °C, the precipitate was harvested by centrifugation (10 min, 10 000 x g, 4 °C) and resuspended in water. In some cases, 1 h of shaking (200 RPM, 20 °C) was used to resuspend the samples. The resuspended EPS was transferred into dialysis tubes (MWCO 3500 Da, Membra-Cell™, Serva, Germany) which were closed using clamps. Closed tubes were transferred into dialysis vessels in which dialysis was conducted using dH_2O. Dialysis took place at 4 °C for 48 h. After dialysis, samples were removed from the tubes and frozen at -20 °C. After freezing, samples were freeze-dried usind a freeze dryer (FreezeZone™, Labconco, US) and stored at room temperature until analysis. Quantification of these samples was performed gravimetrically.

3.2.4. Heat treatment of raw fermentates and purified EPS solutions

To simulate beverage technological heat treatment and microbial inactivation, fermentates and EPS solutions heat treated on a lab scale. For heat treatment, 0.5 L samples were transferred to a VX 150 autoclave (Systec, Linden, Germany) in laboratory grade screw-top glass bottles and the autoclave was used to generate the heating protocol. An appropriate water filled reference bottle was used for temperature reference. The temperature program was set to hold at 85 °C for 3 min, similarly to a heat treatment described for a pure grape juice (Zhao 2012). After the samples were cooled 80 °C, the autoclave was opened and the bottles were removed from and cooled to room temperature using ambient cooling. Laboratory scale heat treatment was conducted using1.5 mL ragent tubes and a heating block with the same treatment regime but prompt removal after 3 minutes of heating.

3.2.5. Stability of dextran solutions towards long-term storage

To determine the stability of dextrans towards long term storage, raw fermentates and isolated dextrans resuspended in buffer were stored over the course of 3 months. Isolated dextran was

dissolved at the same concentration as it was present in the fermentates it was obtained from. These fermentations were 2 L fermentations (6.54 g/L for A7 at 20 °C and 9.90 g/L for A7 at 30 °C). They were subsequently resuspended in phosphate-citrate buffer pH 3 prepared according to McIlvaine (1921). After these solutions were heat treated to guarantee microbial stability, dextran containing solutions were stored statically in the dark at room temperature. Sampling was conducted weekly. During sampling, screw-top bottles were removed with minimal disturbance. Then, sampling was conducted under sterile conditions and care was taken to sample from the upper 2 cm of the liquid in the screw-top bottle using a pipette. OD_{400} was determined weekly using a standard laboratory spectrophotometer.

3.3. Analytical methods

3.3.1. HPLC analysis

For HPLC analysis, samples were prepared as follows: all samples (fermentation broth, etc.) were centrifuged to remove cells and particulate (10 000 x g, 10 min, 4 °C). After centrifugation, samples were sterile filtered using a 0.2 µm syringe filter (Phenomenex, USA). To determine sugar and organic acid concentrations, an HPLC system (Dionex Ultimate 3000, Thermo FisherScientific, USA) coupled to Shodex refractive index (RI) detector (Showa Denko Shodex, Germany) was used. For analysis, 20 µL of sample were injected. Possible dilutions were carried out using ultr pure water to stay within the range of the calibration curve.

A Rezex™ RPM Pb2+ (Phenomenex, Germany) column at a flow-rate of 0.6 mL/min (85 °C) was used for sugar quantification. Filtered (0.2 µm) deionized water was used as eluent.

A Rezex™ ROA H+ column (Phenomenex, Germany) at a flow-rate of 0.7 mL/min (85 °C) was used for separation of organic acids. Filtered 2.5 mM H_2SO_4 was used as eluent for organic acid separation. External standards were used for the preparation of calibration curves, typically in the range of 1-150 mM. Chromeleon™ (v. 6.8; Dionex, Germany) software was used to evaluate the standard curve and quantify all analytes.

3.3.2. Determination of extinction coefficients of the isolated dextrans

The extinction coefficient of turbid dextrans is one of their defining characters used for calculations in the ASTRA software. Therefore, extinction coefficients of the isolated dextrans were determined in 96-well microtiter plates using a plate reader. For this purpose, 5 solutions of 0.5, 1, 2, 3 and 4 mg/mL were prepared in 50 mM $NaNO_3$. These solutions were re-hydrated over night to ensure proper hydratization. 250 µL of each solution was added to the plate in triplicates and the extinction of these solutions were measured. The resulting extinction at

different concentrations was used for the calculation of the extinction coefficient using the law of Lambert Beer through calculating a slope.

3.3.3. Structural analysis of produced dextrans by asymmetric flow field-flow fractionation (AF4) coupled to multi-angle light scattering (MALS)

In order to gain insights into the molecular weight and structure of dextrans, purified dextrans were separated by AF4 (Wyatt Technology, Germany) and analyzed using a MALS (Dawn Heleos II, Wyatt Technology, Germany) and UV detector (Dionex Ultimate 3000, Thermo Fisher Scientific, USA). Dextrans were produced and purified as described above. Isolated dextrans were resuspended in 50 mM $NaNO_3$ to a concentration of 1 mg/mL and incubated over night (4 °C) to ensure proper hydratization. 100 µL of this sample were then injected into the separation channel for analysis. A modified separation method based on the work by Ua-Arak et al. (2017a) was used. 2 mL/min injection flow and 1 mL/min elution flow were used. The gradient cross flow was set to decrease from 3 mL/min to 0.1 mL/min within 10 min and kept at 0.1 mL/min for the following 30 min. All separations were carried out using a 10 kDa regenerated cellulose membrane (Superon GmbH, Germany). 50 mM $NaNO_3$ (aq.) was used as the eluent solution. Experimental values were used for extinction coefficients for each dextran sample. dn/dc values for dextran were set to 0.1423 mL/g according to Yuryev et al. (2007). All recorded data was analyzed using the manufacturers software (ASTRA v 6.1, Wyatt Technology, Germany).

3.3.4. Determination of viscosities

100 mL of MRS medium with a pH of 7 and 40 g/L sucrose as the primary carbon source (sucMRS) was inoculated from standard pre-cultures to a pre-fermentation OD_{600} of 0.1. Fermentation was carried out at 20 °C for 48 h. After that, the flow times of untreated fermentate were determined using a DIN 53211 flow-cup. Actual times were determined using a chronograph. All determinations were carried out in triplicates.

3.4. Molecular biological methods

3.4.1. RAPD PCR

To discriminate strains, RAPD PCR was used. Two types of primers were used for, namely M13V (5'GTT TTC CCA GTC ACG AC-3') according to the method described by Ehrmann et al. (2003) and BOXA1R (5' CTA CGG CAA GGC GAC GCT GCT CAC G 3') according to the method described by Versalovic et al. (1994).

In short, each PCR reaction (25 µL) contained 25 pmol primer, 0.2 mM of each deoxyribonucleoside triphosphate, reaction buffer containing 3.5 mM $MgCl_2$, 0.75 U Taq polymerase and 1 µL of DNA solution. DNA concentration was kept approximately constant, with 50-100 ng being used. All PCRs were carried out by using a Primus 96plus cycler. The PCR program was denaturation at 94 °C for 45 s, followed by 32 cycles of 94 °C for 1 min, 60 °C for 2 min, 72 °C for 3 min. Resulting PCR products were separated on 1.4% w/v agarose gels electrophoretically. After separation, they were stained with dimidium bromide. UPGMA cluster analyses were performed using BioNumcerics Version 6.50.

3.4.2. 16S rRNA sequencing

16S rRNA genes were amplified using 27f and 1392r as described by (Lane 1991). After ensuring purity using gel electrophoresis, samples were sequenced using the sanger sequencing service of Eurofins Genomics (Ebersberg, Germany) using two samples: one with the forward and one with the reverse primer. All raw chromatograms obtained from the sequencing service were evaluated by hand using MEGA 7 and the resulting sequences were used for analysis. Full sequences were contructed by aligning both sequencing results. All results were later cross-checked using the data derived from whole genome sequencing.

3.4.3. Genome sequencing of selected strains

For whole genome sequencing, cultures were grown anaerobically at 30 °C for 72 h in MRS in case of bifidobacteria and for 48 h in case of LAB. They were then harvested and DNA was isolated using an E.Z.N.A® Bacterial DNA Kit (Omega Bio-Tek Inc., Norcross, GA, USA). Genome sequencing was carried out using a PCR-free library preparation on a MiSeq sequencing platform (Illumina, Inc., San Diego, US-CA). Processing and assembly was done using SPAdes V3.9.0 (Bankevich et al. 2012) according to Huptas et al. (2016). For annotation, the NCBI Prokaryotic Genome Annotation Pipeline was used (Haft et al. 2018; Tatusova et al. 2016). A list of all genome sequences generated in this work can be found in Table 12. Additionally, several genomic sequences were obtained from public databases. Their accession numbers can be found in Table 13.

3.5. Bioinformatic methods

3.5.1. Generation of phylogenetic trees

For the generation of phylogenetic trees, the sequences were analyzed using MEGA 7 (Kumar et al. 2016). Additionally, reference sequences were downloaded from the NCBI database. 16S rRNA gene sequences of selected type strains were then aligned using the CLUSTAL_W algorithm described by Larkin et al. (2007) and currently implemented in MEGA 7. A phylogenetic tree was generated using the neighbour-joining method (Saitou and Nei 1987), the minimum-evolution method (Rzhetsky and Nei 1993) and the maximum likelihood method (Tamura and Nei 1993). Bootstrapping values are based on 1000 replicates, as described by Felsenstein (1985).

3.5.2. Generation of concatenated marker gene sequences

To gain further insight into the phylogeny of strains, a concatenated sequence was constructed using MEGA7. For this purpose, the sequence of six housekeeping genes (*clpC*, *dnaB*, *dnaG*, *dnaJ*, *hsp60* and *rpoB)* of TMW 2.2057^{T}, TMW 2.1764 and related reference strains were derived from the genome annotations as well as the work of Jian et al. (2001), Ventura et al. (2004) and Kim et al. (2010) for the reference organisms. In total, the sequence comprised 1917 shared positions. All sequences were concatenated in MEGA 7 (Kumar et al. 2016) and further aligned using the CLUSTAL_W algorithm (Larkin et al. 2007) implemented in the same program. A phylogenetic tree was then constructed using the neighbour-joining method (Saitou and Nei 1987) and the maximum likelihood method according to the model published by Tamura and Nei (1993) as described above. Bootstrapping values were again based on 1000 replicates.

3.5.3. List of all genomic sequences used in this study

Lists of all genomic sequences that were established or used in this work are given in the following tables (tables 12 and 13).

Table 12. Genomic sequences obtained in this work.

Species	Strain	Accession number
B. tibiigranuli	TMW 2.2057^{T}	QLZA00000000
B. tibiigranuli	TMW 2.1764	QDAG00000000
Lb. hordei	TMW 1.1907	PDDD00000000
Lb. hilgardii	TMW 1.2196	PPFW00000000
Lb. hilgardii	TMW 1.828	NSMC00000000

Table 13. Genomic sequences used in this work and obtained from public repositories.

Species	Strain	Accession number
B. animalis subsp. lactis	BB12	CP001853
B. aquikefiri	LMG 28769^T	MWXA00000000.1
B. breve	DSM 20213^T	NZ_AP012324
B. eulemuris	DSM 100216^T	NZ_MWWZ00000000
B. indicum	DSM 20214^T	NZ_CP006018
B. lemurum	DSM 28807^T	NZ_BDIS00000000
B. longum	NCC 2705	AE014295
B. longum subsp. longum	DSM 20219^T	FNRW00000000
B. pullorum	DSM 20433	NZ_JDUI00000000
B. subtile	DSM 20096^T	NZ_JGZR00000000
Lb. hordei	TMW 1.1822	P018176.1

Nucleotide sequences that were used in this study to determine presence or absence of dextranase, fructanase or dextransucrase sequences in genomes are given in the appendix section, tables A2 to A4.

3.5.4. Taxonomic values derived from the genomes

For taxonomic purposes, average nucleotide identity (ANI) values as well as *in silico* DDH (isDDH) were calculated for taxonomic purposes. Both values were calculated for the closest relatives considering 16S rRNA gene comparison. The values were calculated using the average nucleotide identity based on BLAST+ (ANIb) algorithm implemented within the JspeciesWS web (Goris et al. 2007; Richter et al. 2016) and the GGDC 2.1 service (Meier-Kolthoff et al. 2013) for isDDH.

3.5.5. Genomic analysis

For evaluation of genomic content after annotation through the NCBI GAP, the whole genome sequences of *B. aquikefiri* CCUG 67145^T (MWXA00000000), *B. tibiigranuli* TMW 2.2057^T and TMW 2.1764 (QLZA00000000 and QDAG00000000), *B. longum* NCC 2075 (AE014295), *B. breve* DSM 20213^T (NZ_AP012324) and *B. animalis* subsp. *lactis* BB12® (CP001853) were also analyzed and annotation was carried out using the RAST and SEED algorithms (Aziz et al. 2008; Overbeek et al. 2014). The tool PSORTb (Version 3.0.2, http://www.psort.org/psortp) was used to predict the subcellular localization of the genes (Gardy et al. 2004; Yu et al. 2010). To determine biochemical pathways, a reference derived from KEGG (http://www.genome.jp/kegg/pathway.html) was used to determine genes involved in the

respective pathways. After this, NCBI and RAST annotations were checked for the annotations referring to the gene of interest (GOI). If RAST and NCBI annotations matched, the orf was confirmed as the GOI. This was done by submitting it into the NCBI conserved domain search and affirming the presence of the conserved domain (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). If the annotations were different, the orf in question was submitted to BLAST and the BLAST output was used to clarify the orf function. If the GOI was not present in neither annotation, a BLAST query using the genome in question and the GOI from closely related bifidobacteria was conducted. The resulting gene was again subjected to a BLAST query to determine its function. If this search using a GOI sequence from a closely related species did not yield a result, the gene was presumed to be absent from the genome. To reconstruct the carbohydrate catabolism in bifidobacteria, the review by Pokusaeva et al. (2011) was used in order to identify all relevant genes and as a reference. For amino acid synthesis and vitamin synthesis pathways, the KEGG mapper (https://www.genome.jp/kegg/pathway.html) and BioCyc Database Collection (https://biocyc.org/) were used as references.

3.5.6. Genomic BADGE calculations

To determine genes shared by a group of organisms derived from water kefir and a group of organisms comprising known probiotic bifidobacteria, the Blast Diagnostic Gene finder (BADGE) was used with standard settings (Behr et al. 2016). The sequences of *B. aquikefiri* CCUG 67145^{T}, *B. tibiigranuli* TMW 2.2057^{T} (=DSM 108414^{T} = LMG 31086^{T}) and TMW 2.1764 were referred to as the Water Kefir group, and the whole genome sequences of *B. longum* NCC 2075, *B. breve* DSM 20213^{T} and *B. animalis* subsp. *lactis* BB12® were used as the probiotic group. This was done to gain insights into the adaptations shared by the bifidobacteria from water kefir.

4. Results

4.1. Isolation and identification of bifidobacteria from water kefir

In order to find isolates of bifidobacteria from water kefir, the microbiota of a typical household water kefir fermentation was investigated. To isolate bifidobacteria, a selective medium was employed. Dilutions of water kefir grains were plated on this medium and incubated at 25 °C for 12 days. After that, the bifidobacterial cell count of water kefir grains was determined as 2.5×10^6 cfu mL^{-1} by plate counting. The subsequent identification of the isolates using the database-dependent MALDI-TOF MS analysis failed. However, RAPD PCR using M13V primers revealed all isolates to possess the same fingerprint, suggesting them to belong to the same species. Upon generation of new MALDI-TOF MS reference spectra re-identification was possible with this technology. The spectra of TMW 2.2057^T were matched to the spectra recorded initially with scores higher than the cut-off for species-level identification, suggesting good quality spectra with no previous match in the database. Subsequently, the 16S rRNA gene was amplified using PCR and sequenced. 16S rRNA gene comparison revealed that the sequence of TMW 2.2057^T was identical to the sequence of TMW 2.1764, an isolate obtained during a routine microbiota analysis from standard MRS plates (3 days anaerobic incubation at 30 °C) in January 2016. The 16S rRNA sequences of both strains were submitted to the NCBI nucleotide database and are available under accession numbers MK461560 and MK988442. To differentiate the strains, RAPD PCR was used. Indeed, strains TMW 2.2057^T and TMW 2.1764 show different RAPD PCR fingerprints when M13V and BOXA1 primers are used, as indicated in Figure 10. They are thus two strains of the same, previously undescribed species of *Bifidobacterium*. When considering database entries, the closest relation considering 16S rRNA identity was found towards *B. subtile* DSM 20096^T (98.35% identity) when only cultured samples were considered. The 16S rRNA sequence showed 99% identity to an “Uncultured bacterium clone 6-12W5“ (accession no.: KC179058.1). That sample was taken from ”an activated sludge reactor” used for treatment of synthetic wastewater in 2013.

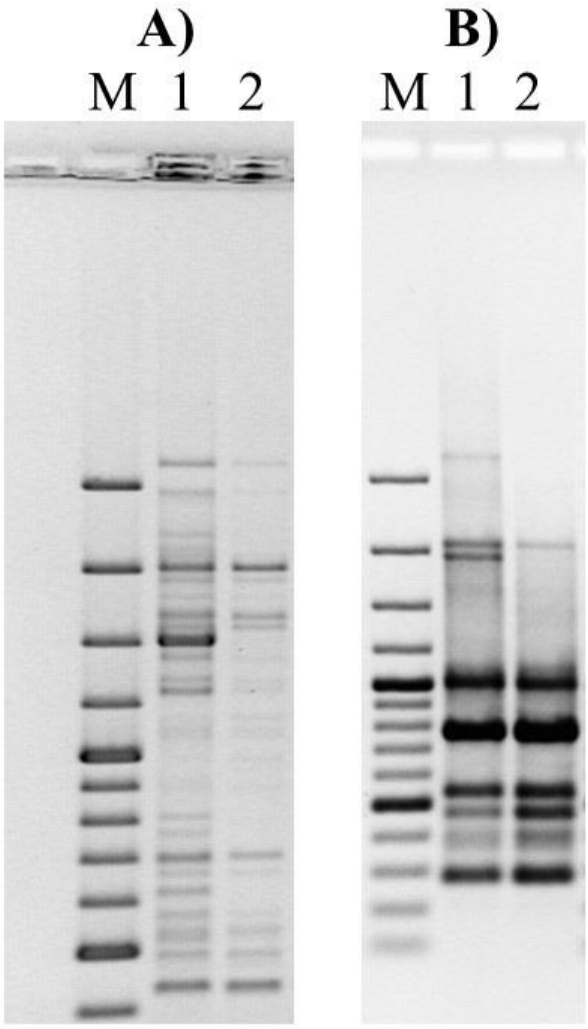

Figure 10. RAPD PCR fragments generated using M13V primer (A) and BOXA1R primer (B). Fragments were separated by gel electrophoresis and negatives are shown after staining. Lane 1 shows PCR products from TMW 2.2057 ^{T} and lane 2 those of TMW 2.1764, lane M shows 100 bp plus DNA Ladder (Thermo Fisher Scientific). Figure modified from Eckel et al. (2019b).

4.2. Phylogenetic placement of bifidobacterial isolates from water kefir

The full length 16S rRNA gene sequences were entered into MEGA 7, together with other type strain sequences of the genus *Bifidobacterium*. The sequences were then aligned and a phylogenetic tree based on 16S rRNA genes was constructed (Figure 11). This phylogenetic tree showed our novel strains to be phylogenetically related to a cluster of bifidobacteria with members which were also isolated from food sources (*B. aquikefiri*, *B. crudilactis*, *B. psychraerophilum*) and have been described as having growth optima between 25-30 °C (Delcenserie et al. 2007; Laureys et al. 2016; Watanabe et al. 2009). The topology of this tree

is confirmed, when different algorithms are used for its calculation (see appendix Figure A1 and Figure A2).

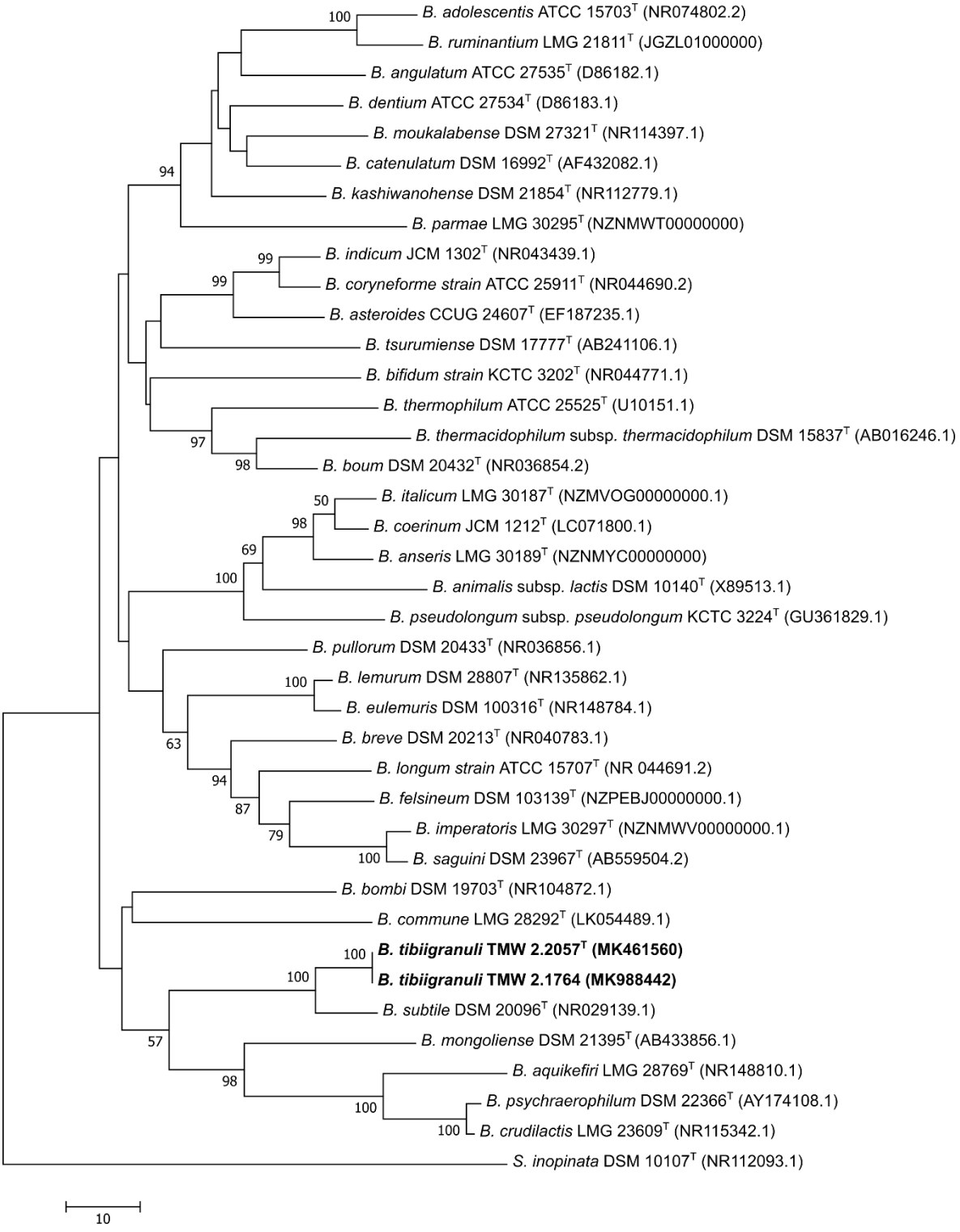

Figure 11. Phylogenetic tree derived from 16S rRNA gene sequences that shows the relationship of novel strains to closely related species of the genus *Bifidobacterium*. The tree was constructed using the Neighbor-Joining method. Bootstrap test (1000 replicates) percentages are shown at the branch nodes, values below 50% were omitted. The 16S rRNA

sequence of *Scardovia inopinata* was used as an outgroup. Base differences per sequence are indicated by the bar (Eckel et al. 2019b).For more rigorous phylogenetic identification and placement, a phylogenetic tree based on a concatenated marker sequence has often been employed (Mattarelli et al. 2014). Therefore, whole genome sequencing of strains TMW 2.2057^T and 2.1764 was undertaken and the whole genome sequences deposited in the public databases under accession numbers QLZA00000000 and QDAG00000000, respectively. To generate a concatenated marker gene tree, the sequences of *clpC*, *dnaB*, *dnaG*, *dnaJ*, *hsp60* and *rpoB* were obtained from the genomes and concatenated for our novel isolates. Such sequences were likewise prepared for several closely related species of the genus. These sequences were then aligned and a phylogenetic tree constructed as for the 16S rRNA sequences. The resulting phylogenetic tree using the neighbor-joining method is shown in Figure 12, also revealing *B. subtile* DSM 20096^T as the closest phylogenetic neighbour of TMW 2.2057^T and TMW 2.1764. This topology is confirmed when using different algorithms (see Figure A3).

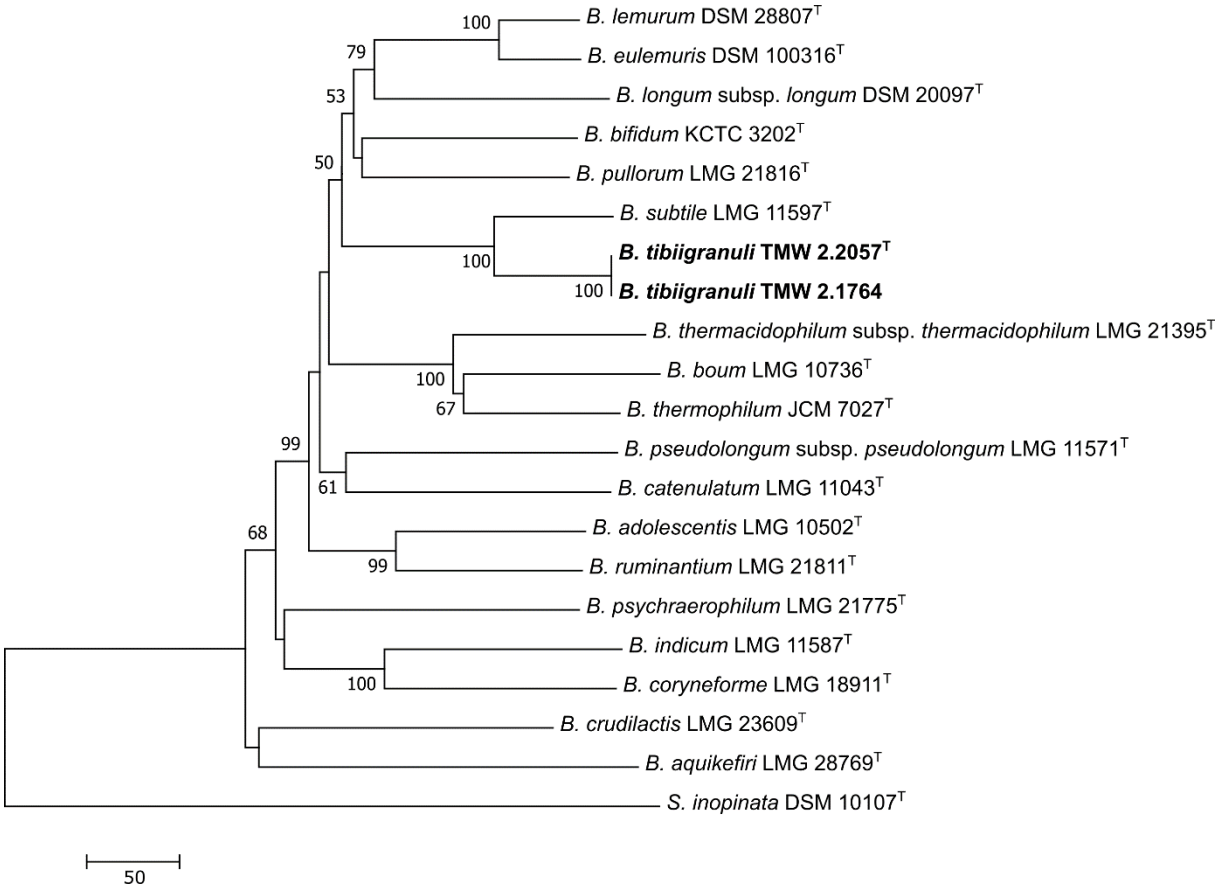

Figure 12. Phylogenetic tree derived from concatenated partial sequences of *hsp60, rpoB, clpC, dnaG* and *dnaB* that shows the relationship of the novel strains to other selected *Bifidobacterium* species. The tree was constructed using the Neighbor-Joining method. Bootstrap test (1000 replicates) percentages are shown at the branch nodes, values below 50% were omitted. The concatenated sequence of *Scardovia inopinata* was used as an outgroup. Base differences per sequence are indicated by the bar (Eckel et al. 2019b).

For additional taxonomic placement and more rigorous species delineation, isDDH and ANIb values were calculated. These values are commonly consulted for taxonomic species delineation and are based on the whole genome sequences in comparison to selected reference genomes. These values, as well as general data concerning the sequenced genomes are given in Table 14.

Table 14. Genomic features of novel bifidobacterial isolates and closely realted species**.** Data from Eckel et al. (2019b).

Strain	*B. tibiigranuli* TMW 22057^{T}	*B. tibiigranuli* TMW 21764	*B. subtile* DSM 20096^{T}	*B. aquikefiri* LMG 28769^{T}	*B. psychraerophilum* DSM 22366^{T}	*B. crudilactis* LMG 23609^{T}
Accession number	QLZA00000000	QDAG00000000	NZ_JGZR00000000	MWXA00000000.1	JGZI01000000	JHAL01000000
GC content	59.82%	60.34%	60.90%	52.30%	58.75%	56.40%
Contigs	132	61	27	18	11	6
Length [mbp]	2.817	2.762	2.79	2.408	2.615	2.362
Genes	2340	2324	2335	2000	2184	1938
Coverage	256	267	123	96	72	20
ANIb	-	99.99%	87.91%	69.95%	71.44%	71.34%
In silico DDH	-	99.90%	35.80%	22.10%	22.00%	21.60%
16S identity	-	100.00%	98.35%	94.19%	93.85%	93.81%

4.2.1. Investigation of physiologic and morphologic properties of novel *Bifidobacterium* isolates

In addition, several physiologic and morphologic features of strains TMW 2.2057^{T} and TMW 2.1764 were determined. Both strains were found to be non-motile, Gram staining positive, F6PPK positive, catalase negative and oxidase negative. After 72 h of anaerobic growth at 30 °C on BM medium, microscopy showed the cells to be irregular rods of 2-3 µm length and <1µm diameter, as shown in Figure 13. The fermentation end products when fermenting glucose in BM medium were determined using HPLC. The products, lactic acid and acetic acid, are produced at a ratio of 1:1.75. Colonies of TMW 2.1764 and TMW 2.2057^{T} were determined to be opaque white, 1-2 mm in diameter, convex with smooth edges.

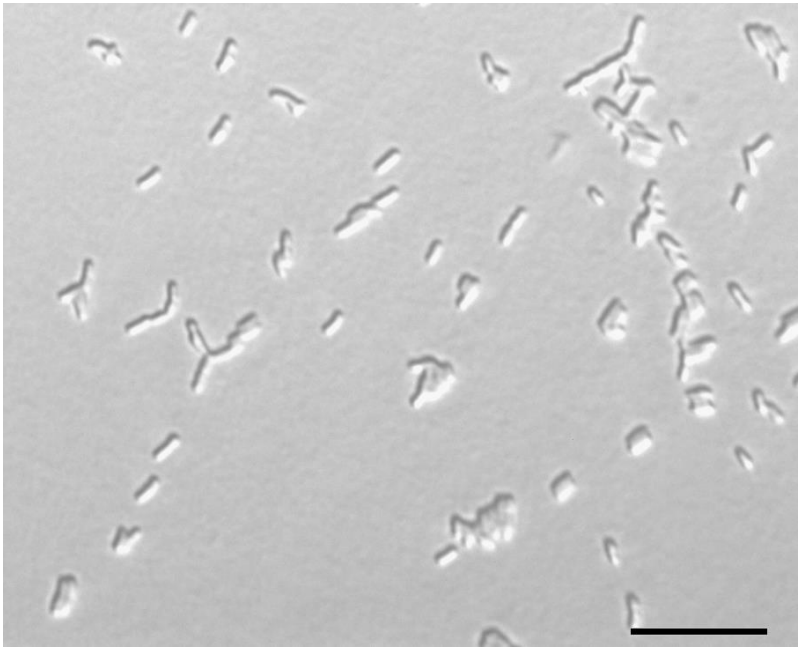

Figure 13. Image of TMW 2.2057^{T} obtained from DIC microscopy, scale bar is 10 µm (Eckel et al. 2019b).

The peptidoglycan structure was analyzed and as A4α L-Lys – D-Asp (Type A11.31 according to DSMZ), which is identical to that of *B. subtile* DSM 20096^{T} and *B. pullorum* DSM 20433^{T}, two close relatives (Mattarelli et al. 2017; Schleifer and Kandler 1972; Schumann 2011). The cellular fatty acid composition of stationary phase cells after 72 h of growth in BM was analyzed and revealed that major fatty acids were 16:0, 18:1 ω9c and fatty acids summed as feature 7 (19:0 Cyclo ω10c/19ω6). All results from this analysis can be found in Table 15.

Table 15. Cellular fatty acid composition of TMW 2.2057^{T}**.** Data from Eckel et al. (2019b).

Peak Name	Percent
10:0	0.1
12:0	0.6
14:0	7.1
16:1 ω9c	1.2
Sum In Feature 3	0.7
16:0	31.6
17:0 CYCLO	0.3
18:1 ω9c	26.7
18:1 ω7c	3.3
18:0	2.6
19:1 ISO I	0.2
19:0 ISO	0.4
Sum in Feature 7	25.4

Additionally, an analysis of the physiological characteristics of the isolates was conducted. A summary of all growth characteristics as well as of the enzymatic and fermentative properties of the organisms can be found Table 16, where only differential characteristics are shown that delineate the novel isolates from isolates of known species. A complete list inlucing non-differential characteristics can be found in the appendix (Table A1), also including experimental data for less-related bifidobacteria that is not shown here.

Table 16. Differential physiologic characteristics of *B. tibiigranuli* and selected closely related bifidobacteria. A modified version of his table was published in (Eckel et al. 2019b).

Strain	**1**†	**2**†	**3**‡	**4***	**5***	**6**
Growth						
Temperature (°C)	15-40	15-40	15-40	4-37	4-42	4-45
Optimal temperature (°C)	30	30	30-37	28	37	
pH	4.0-8.5	4.0-8.5	4.0-8.5	4.0-8.0	4.5-n.d.	4.7-n.d.
Optimal pH	6.5-7.0	6.5-7.0	6.5-7.0	n.d.	n.d.	
Enzyme activity						
β-Galactosidase	-	-	-	+	+	+
Alkaline phosphatase	+	+	v	-	-	v
α-Fucosidase	-	-	-	-	+	-
Alkaline phosphatase	+	+	v	-	-	v
Production of acid from*						
L-Arabinose	-	-	-	+	+	-
D-Xylose	-	-	-	-	+	-
D-Mannose	-	-	v	+	-	-
L-Sorbose	+	+	v	-	+	n.d.
Inositol	-	v	-	-	-	n.d.
D-Mannitol	+	+	-	w	-	-
D-Sorbitol	+	+	+	-	+	-
Methyl-αD-Glucopyranoside	+	+	+	+	+	v
N-Acetylglucosamine	-	-	-	w	-	-
Amygdalin	-	-	-	w	+	-
Arbutin	+	+	-	-	+	-
Salicin	-	-	+	-	+	-
D-Cellobiose	-	-	-	-	+	+
D-Maltose	+	+	+	+	-	+
D-Lactose	-	-	-	-	-	+
D-Trehalose	+	+	-	-	+	-
D-Melezitose	+	+	+	-	+	-
Gentiobiose	-	-	v	+	+	-
D-Lyxose	v	-	-	-	-	+
Potassium gluconate	+	+	v	+	+	+

Strains: 1, TMW 2.2057^T; 2, TMW 2.1764; *3, B. subtile DSM 20096^T*; *4, B. aquikefiri LMG 28769^T*; *5, B. psychraerophilum* DSM *22366^T*; *6, B. crudilactis LMG 23609^T. The physiological traits were determined to be present (+), weak (w), absent (-) and variable, indicating different reactions for 3 biological triplicates (v).*

†assessed at 30 °C.

‡assessed at 37 °C.

*Data taken from Modesto et al. (2015), Delcenserie et al. (2007) and Simpson et al. (2004).

The occurence of bifidobacteria in different water kefirs (Gulitz et al. 2013; Laureys et al. 2016) raises the question if and how these organisms are involved in the granule formation. This would be possible directly through exopolysaccharide formation or degradation in water kefir. Therefore, the genomes of TMW 2.2057^T and TMW 2.1764 were evaluated *in silico* regarding the presence or absence of putative dextransucrases, fructansucrases, dextranases and fructanases using the blast.n and blast.p algorithms. However, no dextranases, fructanases, dextransucrases or fructansucrases were identified in the genomes of *B. tibiigranuli*. This suggests that our novel strains possess no enzymes directly participating in the synthesis or degradation of water kefir grains.

4.3. Determination of unique and potential functional properties of bifidobacteria from water kefir

Bifidobacteria have long been considered mesophilic anaerobes (Scardovi 1986a). The emergence of new, aerotolerant and cold-tolerant isolates, especially in water kefir raises the question of what sets these organisms apart from other bifidobacteria. Answering this question might shed light on their ecological role as well as their applicability as pure cultures in food fermentation and ways of producing these organisms on a commercial scale.

4.3.1. Initial genomic investigation

The fact that these organisms have been genome sequenced enables us to predict their physiology from their genomes. To get an initial understanding of the genomes in question, we generated an overview of their genomic features. This overview includes the genome size, number of encoded genes, GC content and more and is shown in Table 17. The table contains the data of three water kefir strains together with a well-characterized group of probiotic bifidobacteria, that are mesophile anaerobes and are included for reference.

Table 17. General genomic features of bifidobacteria from water kefir and three probiotic strains. Modified from Eckel and Vogel (2020).

Species	*B. tibigranuli*	*B. tibigranuli*	*B. aquikefiri*	*B. animalis subsp. lactis*	*B. longum*	*B. breve*
Strain	TMW 22057^{T}	TMW 21764	LMG 28769^{T}	BB-12®	NCC 2705	DSM 20213^{T}
Accession number	QLZA00000000	QDAG00000000	MWXA00000000.1	CP001853	AE014295	NZ_AP012324
GC content	59.8%	60.3%	52.3%	60.5%	60.1%	58.8%
Contigs	132	61	18	1	1	1
Length [mbp]	2.817	2.762	2.408	1.934	2.257	2.269
Genes	2340	2324	2000	1.629	1.797	1.989
Coverage	256	267	96	11	8	8.9
ANIb	-	99.99%	69.95%	73.10%	74.15%	73.89%
16S identity	-	100.00%	94.19%	92.83%	94.98%	95.47%

When considering genome sizes of the organisms in question, the genomes in the probiotic group are generally smaller. *B. animalis subsp. lactis* BB12® has the smallest genome of 1.9 MBp, followed by *B. longum* NCC 2705 (2.3 Mbp) and *B. breve* DSM 20213^T (2.3 Mbp). In the water kefir group, *B. aquikefiri* has a larger genome (2.4 MBp) and *B. tibigranulii* has the largest genome size of all genomes considered (2.8 MBp). This is similarly the case for the number of genes, where the organisms of the probiotic group have the lowest number of encoded genes and the water kefir organisms have the highest number of genes as is highlighted in Figure 14 We additionally determined the cellular localization of these genes. However, no drastic differences between all organisms was observed, as can be seen in Figure 14.

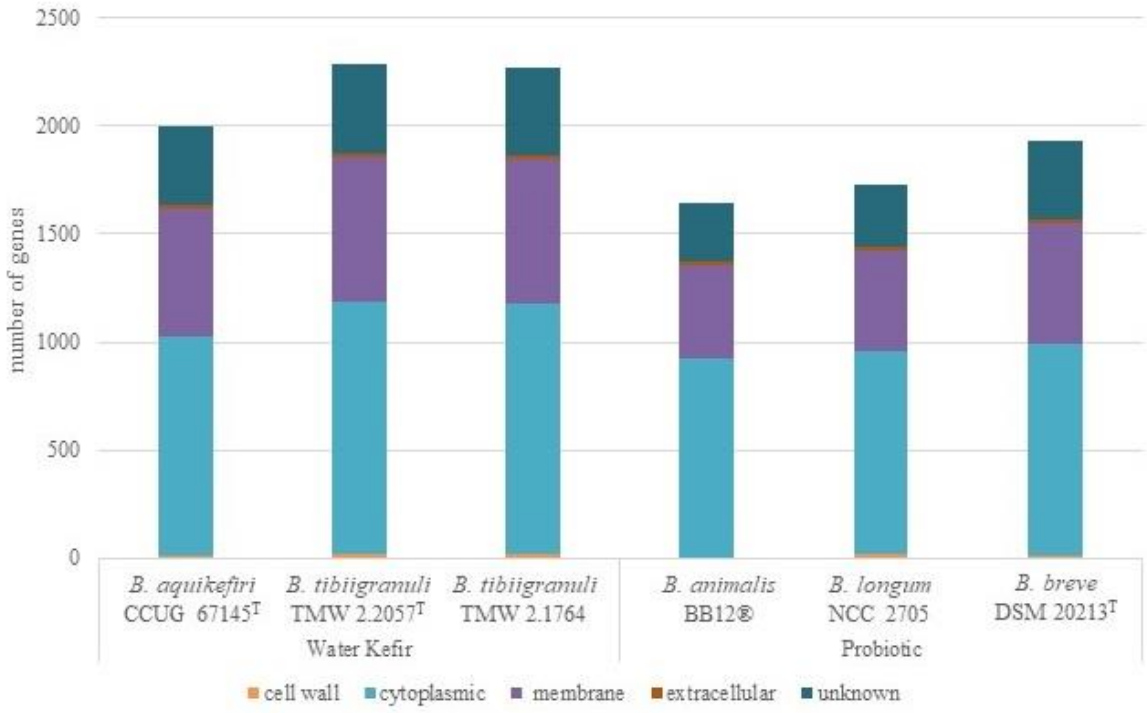

Figure 14. Predicted cellular localization of all genes from the genomes of bifidobacteria from water kefir and three representative probiotics. Data based on the genomic annotation of the six genomes and associated Psortb data. Figure modified from Eckel and Vogel (2020).

4.3.2. Analysis of genes unique to bifidobacteria from water kefir using BADGE

To gain insight into the genomic differences between the genomes of the bifidobacteria isolated from water kefir and the probiotic, further comparative genomic investigations were conducted. Therefore, we separated the genomes into two groups: The first group, the "water kefir" group containing the genomes of the three strains derived from water kefir (*B. tibiigranuli* TMW 2.2057^T and TMW 2.1764 as well as *B. aquikefiri* CCUG 67145^T). The second group is the probiotic group that includes the genomes of *B. animalis* subsp. *lactis* BB12®, *B. longum* NCC 2705 and *B. breve* DSM 20213^T. It was used as a control group that represents mesophilic probiotic *Bifidobacteriacae*. BB12® was chosen as it is considered to be the most well-studied probiotic *Bifidobacterium* strain and is known for its widespread use in foods, while both *B. longum* NCC 2705 and *B. breve* DSM 20213^T were chosen since they are two well known representatives of two species of probiotic bifidobacteria that are genomically well characterized (Bottacini et al. 2014; Jungersen et al. 2014; Schell et al. 2002). To determine the genes unique to bifidobacteria from water kefir, the BADGE algorithm was used. This algorithm identifies genes unique to a group of genomes when compared to another group of genomes. The algorithm generates a list of genes, that can be used as diagnostic marker genes (DMG) (Behr et al. 2016). These genes additionally represent a type of nice-specific core genome of these organisms, which can reveal the genes shared by a group. Genes shared by a group could be considered typical and play an important role in the lifestyle of this group of organisms. In our context, an investigation of such a set of genes unique to and shared by the organisms from water kefir may be used to gain insight into the unique physiological properties that enables these strains to prosper in the extremely specialized habitat of water kefir. Additionally, it can help identify the genomic adaptations necessary for colonization of water kefir. This comparison of the three genomes of the water kefir isolates with the three genomes of the probiotic group resulted in a list of 143 DMGs. These genes are shared by the water kefir group and not found the probiotic group (see appendix Table A5).

In a first step, the SEED algorithm was used to gain insight into the general metabolic categories that these genes are assigned to. The occurence of the categories among the DMGs classified as in-category genes are depicted in Figure 15. Due to the nature of these genes (exclusive and shared by the water kefir isolates) this data is identical for all three water kefir strains. The investigation revealed that the most prominent category found in the BADGE output is amino

acids and derivates (26%), the second most abundant is carbohydrates (24%), followed by membrane transport (13%).

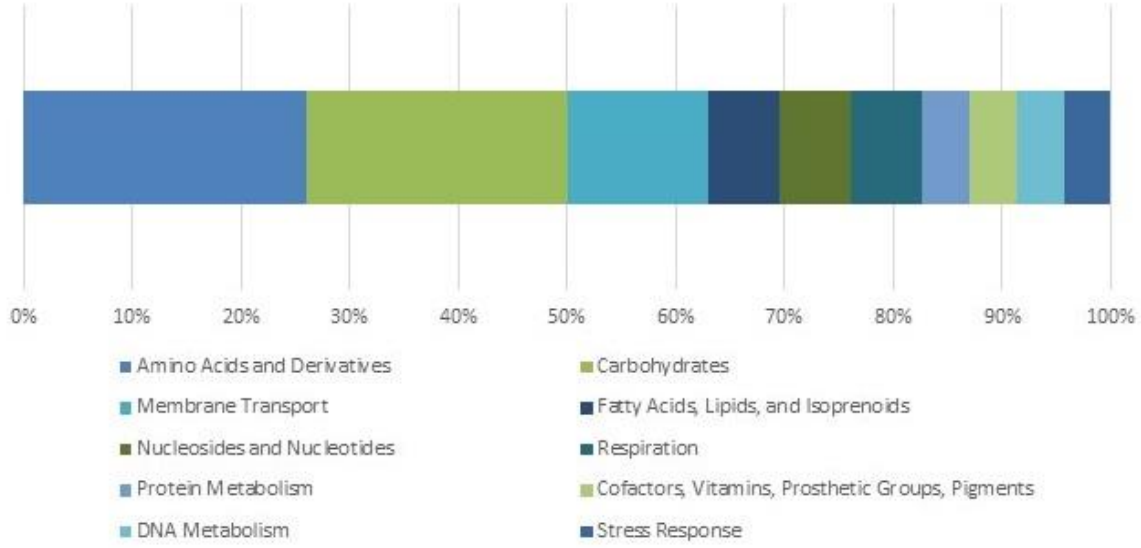

Figure 15. Distribution of "in subsystem" genes derived from BADGE over the seed categories. Figure modified from Eckel and Vogel (2020).

The list of genes in the amino acid group comprises several groups of genes. Especially noteworthy is the predominance of aspartate and asparagine metabolism related genes, like asparaginase, aspartate racemase, three "aspartate aminotransferase family" proteins, and asparagine synthase (glutamine-hydrolyzing), which are all part of the aspartate and asparagine synthesis.

In addition to proline racemase, D-proline reductase (dithiol), protein PrdB and D-proline reductase (dithiol), proprotein (PrdA) are enzymes that are encoded by genes found in the amino acid group of the water kefir genomes. Another gene encoded by orfs of this category is formimidoylglutamate deiminase, whichis part of the histidine metabolism (Martí-Arbona et al. 2006).

The aforementioned abundance of asparagine related genes is especially interesting to note. Asparagine synthase, asparaginase, and asparagine permease were identified as being exclusively present in the water kefir group. Together with a glutamine ABC-transporter which is also found among the water kefir DMGs, these genes can mediate glutamine and asparagine

uptake and subsequent conversion to aspartate, leading to ammonia production inside the cell. This metabolic pathway formed by these genes is shown in Figure 16.

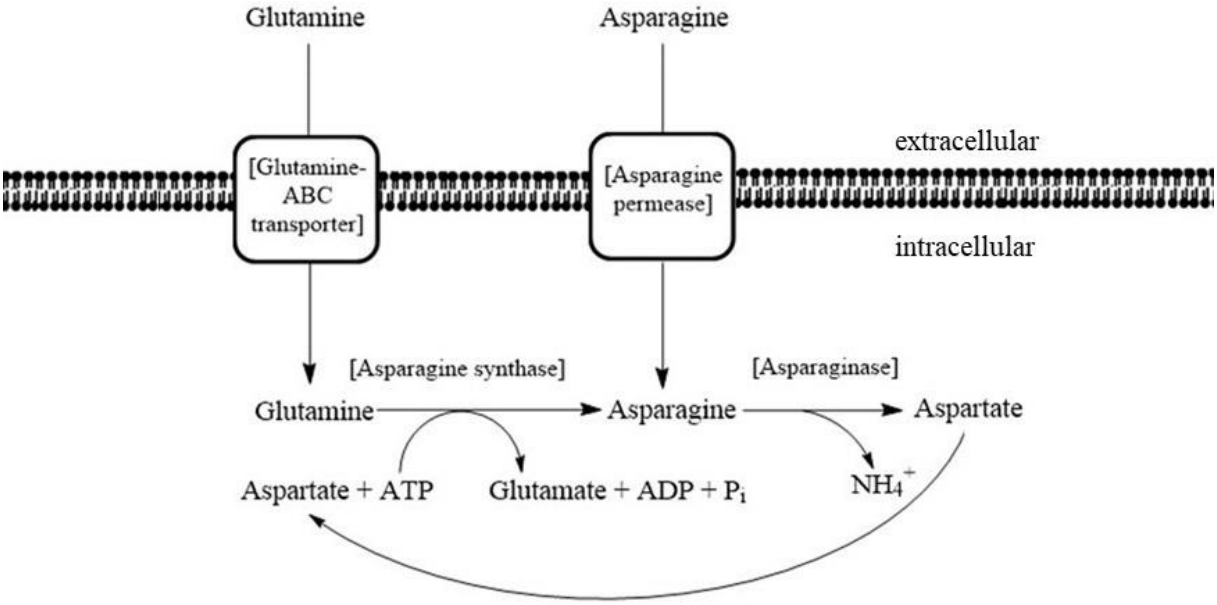

Figure 16. Glutamine and asparagine import and subsequent conversion to glutamate and ammonia. All enzymes shown are only encoded in the water kefir group and were obtained from the BADGE output. Figure modified from Eckel and Vogel (2020).

DMGs categorized as carbohydrate related comprise genes encoding a trehalose-phosphatase, an aminotransferase, an aspartate aminotransferase family protein and a glycyl-radical enzyme activating protein and its glycyl radical enzyme, a formate C-acetyltransferase/glycerol dehydratase family glycyl radical enzyme, an (S)-acetoin forming diacetyl reductase, a sugar ABC transporter permease domain, and a pyruvate oxidase. On top of this, several genes of unknown function are encoded: two decarboxylating 6-phosphogluconate dehydrogenases, a dehydrogenase and an NAD(P)-dependent oxidoreductase of unknown function.

The list of membrane transport associated genes comprises ABC transporter subdomains and a transcriptional regulator.

Due to the fact that the in-subgroup genes represent only a small part of the genes derived from the BADGE-output, we further investigated the BADGE output and identified genes of interest that are only present in the water kefir group but not categorized as in-subgroup. For example,

otsA, a trehalose-6-phosphate synthase is encoded by genes present in the water kefir group. While the operon is incomplete (otsB is missing), a putative treC, a trehalose phoshphate hydrolase, is encoded in the genomes. This possibly gives these organisms the enzymatic tools to sythesize trehalose-6-phosphate.

Two other noteworthy genes, CydC and CydD, were identified in the water kefir group. Both are involved in oxygen tolerance. In addition to these, genes encoding ruberythrin, superoxide dismutase, pyruvate oxidase sbxB, glutathione peroxidase and peroxide stress protein YaaA are found in the water kefir group, all of which are oxidative stress related.

4.3.3. Genomic investigations of the water kefir group independent of BADGE

The phenotypic sugar utilization was already determined in a previous chapter, however, the genomic background of these observations remains to be elucidated. An investigation of the genomes yielded genes encoding all enzymes of the bifidus shunt and for the formation of pyruvate in all 3 strains. Through these, the strains are predicted as capable of forming ethanol, formate, acetic acid and lactate from fructose through the intermediates acetyl phosphate, acetyl-CoA and pyruvate and subsequent conversion to the respective acids. Through the presence of ribokinase, a ribose phosphotransferase system (PTS) and the fructose utilization and uptake mechanisms outlined in Figure 17, these strains are able to produce acid from fructose. Other sugars can be utilized through formation of fructose-6-P_i, as shown in Figure 17. This figure similarly outlines how fructose can be utilized by the bifidobacteria of the water kefir group: through a fructose PTS transporter and additionally through a putative fructose ABC transporter.

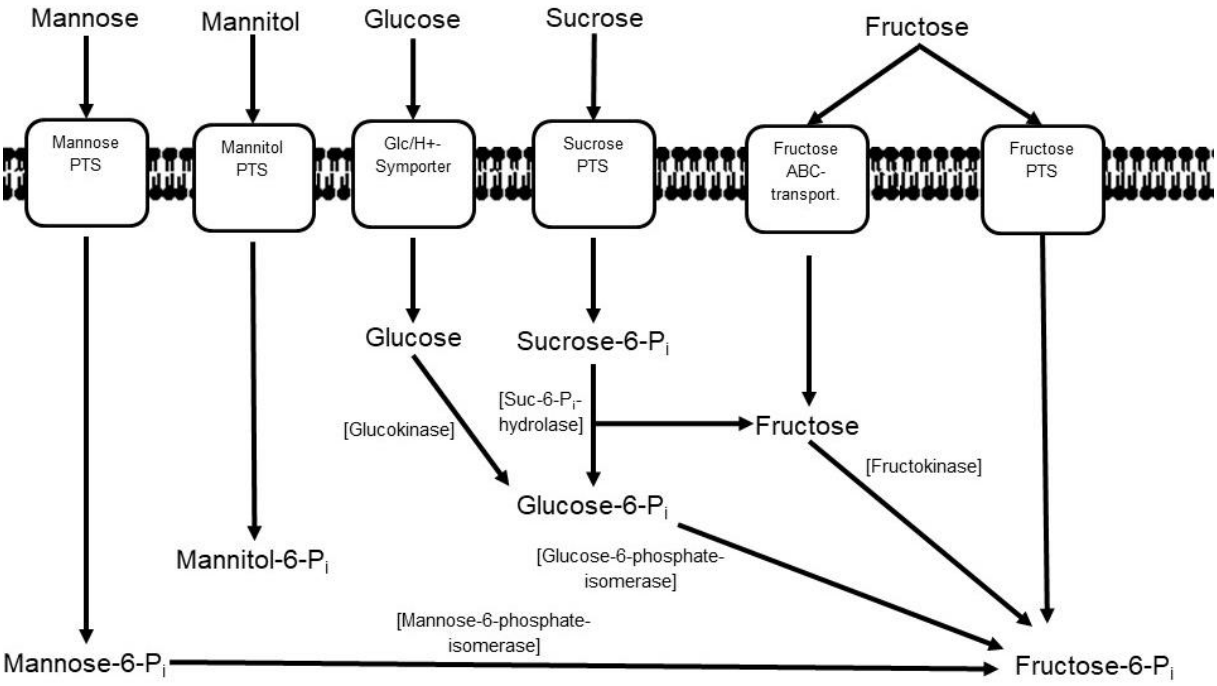

Figure 17. Carbohydrate utilization and uptake as predicted from genomic data for all bifidobacteria from water kefir. All carbohydrates are converted to fructose-6-phosphate, which then enters the bifidus shunt. All genes and transporters are based on orfs derived from the genomic annotation. Figure modified from Eckel and Vogel (2020).

The ScrT (sucrose specific permease), which is described as a sucrose uptake mechanism for several other bifidobacteria (Reid and Abratt 2005; Trindade et al. 2003) is not encoded in the investigated genomes. However, a sucrose PTS transporter and a corresponding sucrose-6-phosphate hydrolase is encoded which presents a different uptake mechanism for sucrose. The imported sucrose is then converted by intracellular hydrolysis by a sucrose-6-phosphate hydrolase that is encoded in all genomes. Furthermore, a mannitol PTS was encoded in all genomes of the water kefir group. This is interesting to note, since no mannitol phosphate dehydrogenase gene nor a candidate gene for the reaction of mannitol-phosphate to Fructose-6-phosphate can be derived from the genomes. Additionally, no sugar alcohol or polyol dehydrogenases, typical candidate genes for this function (Lee et al. 2008; Lee and O'Sullivan 2010), can be derived from the genomic annotation or through blast searches. *B. aquikefiri*, however, does have a gene encoding a SIS superfamily protein in close genomic proximity. The exact prediction of its functionality, however, is not possible based on this data.

Concerning the amino acids synthesis pathways, full pathways for the synthesis of each canonic amino acid are present in the genomes of the water kefir group. This includes the synthesis pathways for asparagine and glutamine for which no tRNA synthase is encoded. This fact implies that these organisms use tRNA amidotransferases to synthesize correctly charged asn and gln tRNA, a common pathway in bifidobacteria. These amidotransferases are in fact encoded in the genomes of the water kefir group. This fact is especially noteworthy as it seems counter-intuitive that these organisms on the one hand synthesize these amino acids, while on the other hand they likely do not use them for protein biosynthesis.

Bifidobacteria from foods are often regarded with a notion of positive health effects. The most obvious positive health effect a microorganism can have in food fermentations is the synthesis of vitamins that are subsequently consumed through the food and serve to enhance the health of a consumer (LeBlanc et al. 2013). Several *Bifidobacterium* species have been shown to be capable of vitamin synthesis (Deguchi et al. 1985; Milani et al. 2014; Noda et al. 1994). Therefore, the genomes were evaluated of the water kefir bifidobacteria with regard to vitamin synthesis pathways. The biotin synthesis pathway is absent in the water kefir group, and as a consequence BioY (substrate specific component of ECF transporter) is encoded in all 3 water kefir genomes. The fact that BioY is encoded in the genomes is therefore unsurprising, considering the importance of biotin for bacterial proliferation. Riboflavin production by the three water kefir bifidobacteria seems unlikely, since this pathway is largely incomplete. Folate synthesis pathway is similarly incomplete. The chorismate branch lacks two enzymes (2.6.1.85 and 2.5.1.15), while the second branch is completely absent. Pyridoxine synthesis is incomplete in all investigated strains. The nicotinic acid synthesis pathway is also incomplete. *B. aquikefiri* only lacks EC 1.4.1.21 for full NAD synthesis. In contrast, the *B. tibiigranuli* strains lack several genes in all involved synthesis pathways. This suggests that the water kefir bifidobacteria are incapable to synthesize typical vitamins that are of importance in human health and which could be of bacterial origin.

Since the predominant group of genes derived from the BADGE output was amino acid synthesis and protein metabolism related, the investigation of physiologic amino acid requirements of the water kefir group isolates was crucial. However, amino acid prototrophy and auxotrophy determination in BB12® failed, since it did not grow in the chemically defined medium. Therefore, an attempt at optimization of the medium for BB12® was undertaken. For this purpose, CDM+AA was adjusted to different pH values (pH 5.5, 6.0, 6.5, 6.8 and 7.0) to investigate a pH-dependence of the growth of BB12® in CDM. The carbon source in CDM+AA

was changed from glucose to lactose, a sugar often employed for the culture of intestinal bifidobacteria. Lastly, an addition of adenosine, cytosine, thymine, uracil and ascorbate to CDM+AA was investigated. However, none of these modifications resulted in the growth of BB12® in CDM, as can be seen in Figure 18.

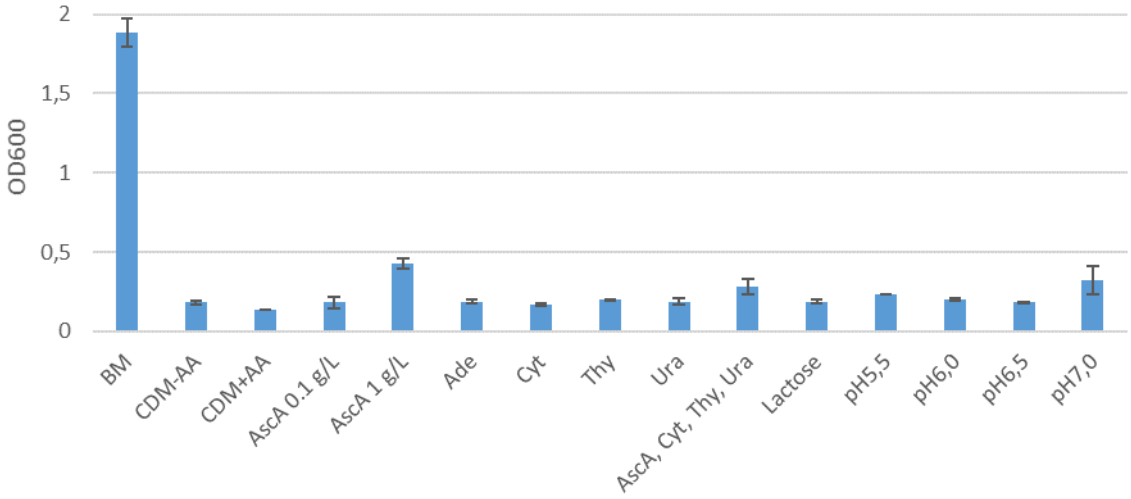

Figure 18. Optical density of *Bifidobacterium animalis* subsp. *lactis* BB12® in CDM with different variations after 48 h of growth at 37 °C. Full medium positive control (BM) and two negative controls are shown on the left (CDM+AA and CDM-AA). The following colums show data for CDM+AA with additions of ascorbic acid (AscA), Adenosine (Ade), Cytosine (Cyt), Uracil (Ura) (50 mg/L each) and a combination of all of these substances (50mg/L each and 1 g/L AscA). In addition, CDM+AA with lactose as the carbon source and CDM+AA with different pH values are shown.

The investigation of amino acid auxotrophy in the water kefir group revealed the ability of all three water kefir isolates to be fully prototrophic for all canonic amino acids. This is illustrated in Figure 19.

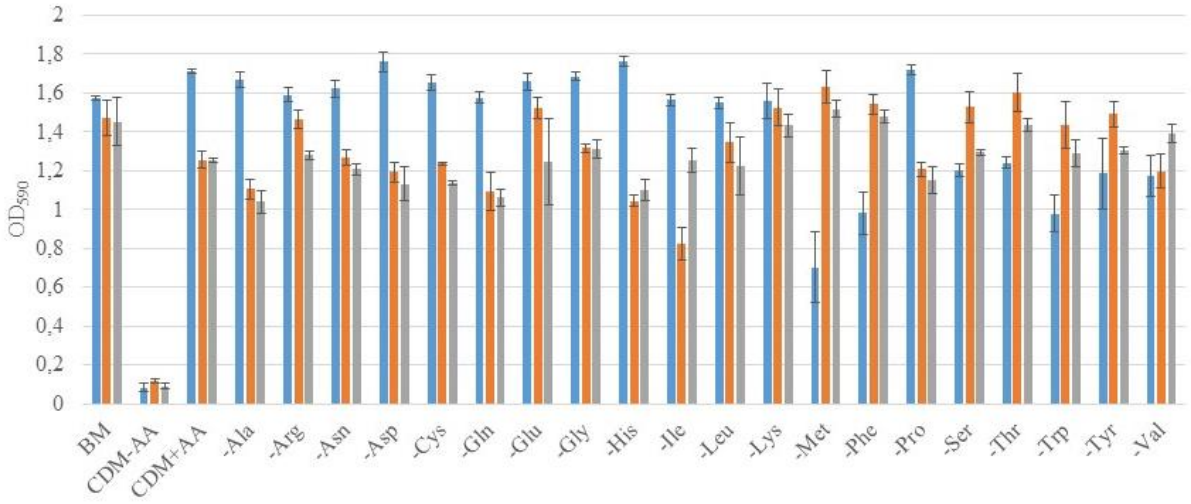

Figure 19. Terminal optical density reached by the water kefir bifidobacteria in CDM. Blue collumns denote *B. aquikefiri* CCUG 67145^{T}, orange columns denote *B. tibiigranuli* TMW 2.2057^{T}, and grey columns denote *B. tibiigranuli* TMW 2.1764. Error bars indicate standard deviaton of three biological replicates. BM shows the full medium control, CDM-AA shows CDM without amino acids, CDM+AA shows CDM with all canonic amino acids, a "–" followed by the three letter amino acid code indicates CDM+AA lacking the indicated amino acid. Figure modified from Eckel and Vogel (2020).

These findings of Figure 19 indicate that all 3 strains from water kefir are prototrophic for all amino acids and that the mutations reported by Verce et al. (2019) do not impair the ability of *B. aquikefiri* for full amino acid synthesis. In fact, an investigation of the genomic sequences of all 3 water kefir organisms reveal an untruncated asparagine kinase gene in each strain with no nonsense mutation, resulting in the *in silico* ability for full amino acid synthesis.

4.3.4. EPS screening of *B. tibiigranuli* and *B. aquikefiri*

A screening of *B. tibiigranuli* TMW 2.2057^{T} and 2.1764 as well as *B. aquikefiri* revealed no slimy nor ropy phenotype when grown on BM or BM supplemented with 80 g/L sucrose. They thus likely do not produce large amounts of HoPs and were therefore not included in further experiments concerning glucan production for use in beverages.

4.4. Production of cloud-forming beverage additives using LAB from water kefir

As discussed before, acetic acid bacteria, *Zymomonas*, bifidobacteria and LAB are the four groups of bacteria typically being found as part of the water kefir microbiota. The goal of this study was to identify bacteria producing exopolysaccharides with beverage technological prospects for non-alcoholic beverage fermentations. Since *Zymomonas* is a strong ethanol producer, it was excluded from the investigation regarding the suitability of bacterial isolates from water kefir for non-alcoholic beverage fermentations. The bifidobacteria that were recovered in the previous analysis were, even though they were taxonomically identified, not included in the investigation. This is due to the fact that they did not produce significant amount of slime or ropiness when grown in the presence of sugar, as shown in the previous chapter. Since acetic acid bacteria are aerobes, their growth requirements are hard to realize in typical (anaerobic) beverage fermentation plants. On top of this, production of acetic acid is unwanted in beverages. Due to these two reasons, the acetic acid bacteria were likewise excluded from the analysis. This exclusion process leaves the LAB as a likely source of EPS producing bacteria from water kefir that are suited for fermentation of non alcoholic beverages. Due to the lower stability of fructans towards acidic hydrolysis, which would be expected in the context of low pH as typically encountered in beverages, the investigation focused on identifying glucan producing strains. These glucans need to be water soluble and naturally opaque, resulting in a stable turbidity after fermentation, independent of the content of bacterial cells.

4.4.1. Initial strain selection for beverage fermentations

Since the LAB content of water kefir has been studied with sufficient detail in the past, the strain selection was limited to isolates described in previous studies (Gulitz et al. 2013; Gulitz et al. 2011). Therefore, 18 strains were selected from the culture collection of the Lehrstuhl für Technische Mikrobiologie that were either already reported to produce EPS or likely to produce EPS, originated from water kefir and typically prominent in the water kefir microbiota. These strains were selected from the genera *Leuconostoc* and *Lactobacillus*, as these genera have been shown to be important in EPS formation in water kefir. Strain *Lb. curvatus* TMW 1.624 is a previously investigated polysaccharide-forming strain that was included as a reference due to its well documented EPS formation characteristics (Rühmkorf et al. 2013; Rühmkorf et al. 2012b). The initial strain selection can be found in Table 1, section 3.1.1.

After streaking the strains on solid medium, 2 strains showed only very little growth after 48 h, characterized by almost invisble colonies on agar and no visible growth after inoculation of liquid media. These strains were TMW 1.1826 and TMW 1.1827. Since this work aims at an industrial applicability of the strains involved, these strains were removed from the selection due to their low growth and therefore poor performance during pre-culture. The remaining 16 strains were used for the EPS production screenings.

4.4.2. EPS screening of LAB isolates

In order to get first insights into the HoPs production capabilities of the remaining bacterial isolates from water kefir, a screening of their HoPs formation properties on solid medium was conducted. For this purpose, bacterial isolates were streaked on MRS + sucrose as described by Stadie (2013) and an HoPs formation score was given for each isolate. Due to unsatisfactory HoPs production after 24 h, an incubation time of 48 h was chosen, corresponding well to the standard incubation conditions used for the culture of these organisms. The results are reported in Table 18. Exemplary results of the HoPs formation are shown in Figure 20, highlighting the EPS formation phenotypes. It becomes clear that several of the selected strains show strong EPS formation on solid media.

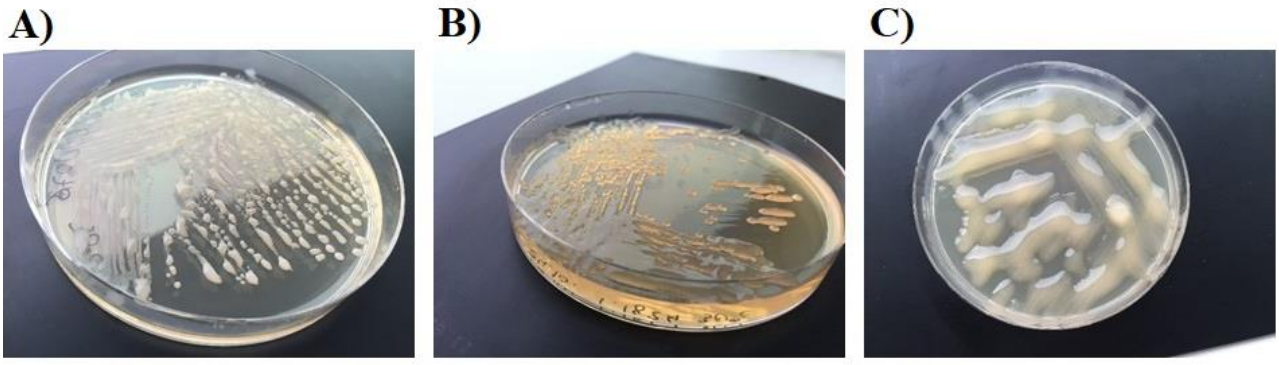

Figure 20. HoPs formation phenotypes of LAB from water kefir on sucMRS. Panel **A)** represents a typical score of 1, with low HoPs formation compared to colony size. Panel **B)** represents a typical score of 2, with almost no colony visible but large amounts of slime and panel **C)** shows a typical score of 3, with drop formation of EPS containing slime visible at the sides of the petri dish and on the solid medium itself.

Table 18. HoPs formation by LAB isolates from WK.

Species	Strain no.	Incubation temperature	
		20 °C	30 °C
Lb. curvatus	TMW 1.624	1	1
Lb. hilgardii	TMW 1.828	1	1
Lb. hordeii	TMW 1.1817	1	1
Lb. hilgardii	TMW 1.1819	0	1
Lb. hordeii	TMW 1.1821	1	1
Lb. hordeii	TMW 1.1822	1	2
Lb. nagelii	TMW 1.1823	0	2
Lb. nagelii	TMW 1.1824	2	2
Lb. satsumensis	TMW 1.1829	1	2
Lb. hordeii	TMW 1.1907	2	2
Lb. hilgardii	TMW 1.2196	3	3
Lc. mesenteroides	TMW 2.1073	2	3
Lc. mesenteroides	TMW 2.1075	1	2
Lc. mesenteroides	TMW 2.1076	1	2
Lc. citreum	TMW 2.1194	3	3
Lc. mesenteroides	TMW 2.1195	1	1

4.4.3. Production of HoPs in beverage-based media

In order to assess the ability of our selected strains to produce HoPs in beverages, we determined the EPS yield in fruit juice-based media. For this purpose, the media were inoculated using standard procedure and EPS yield was determined after 24 and 48 h of fermentation.

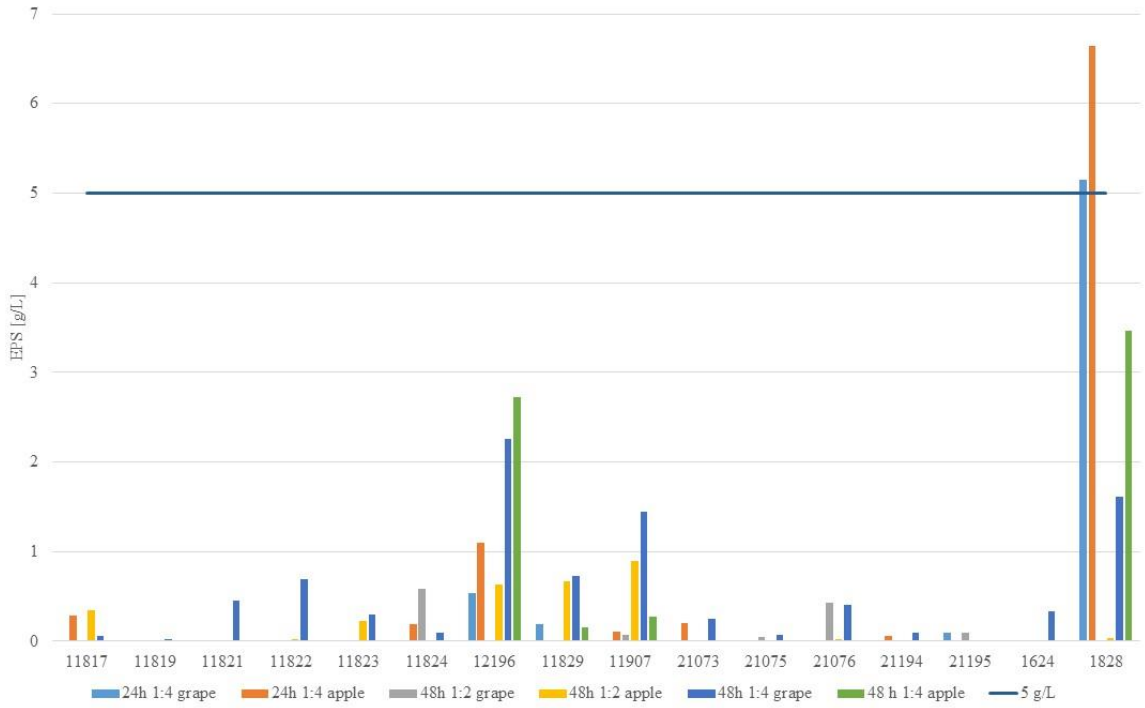

Figure 21. EPS formation of initial strain selection when grown in fruit juice-based media. 2 fruit jucies at different dilutions were used at 30 °C for 24 and 48 hours, each dilution was supplemented with 40 g/L sucrose.

It becomes clear from Figure 21 that certain isolates are more prone to EPS formation than others. All *Lb. hordei* isolates show a certain extent of EPS formation as do all isolates of *Lb. hilgardii*. The goal of 5 g/L is only reached by one isolate, *Lb hilgardii* TMW 1.828 and only under two conditions. Due to the, on average, highest EPS formation in fermentations carried out for 48 h, this fermentation time was chosen as the standard condition for further experiments. This corresponds well to observations from the solid screening reported in the previous chapter, where in most cases no EPS formation was visible after 24 h.

4.4.4. Differentiation of isolates using RAPD PCR

In order to ensure the possibility to subsequently differentiatiate strains, RAPD PCR based on M13 V primers was carried out followed by a UPMGA cluster analysis. The resulting patterns and dendrogram are shown in Figure 22.

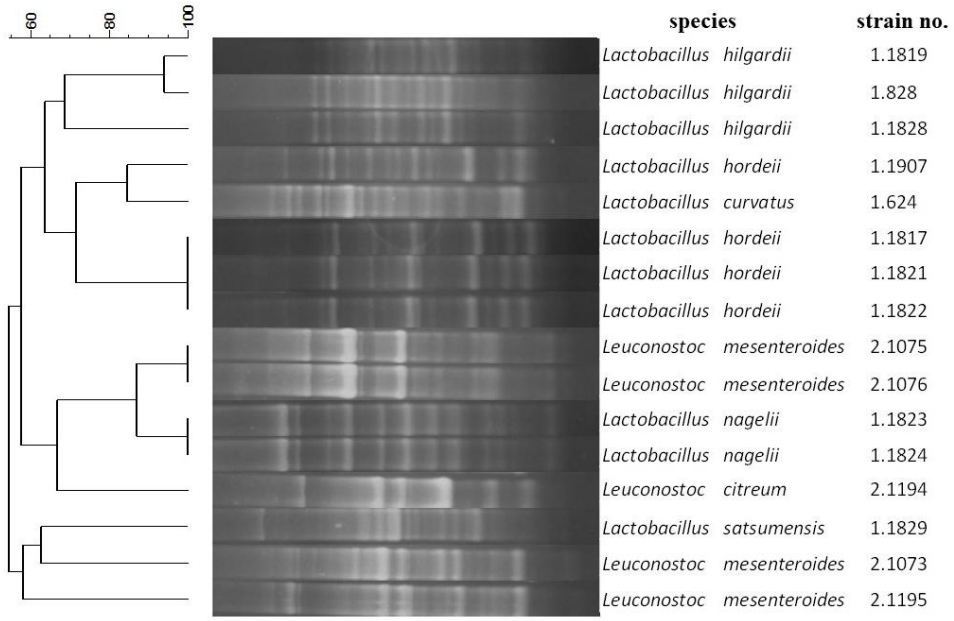

Figure 22. RAPD patterns and UPMGA clustering of strains selected for EPS production.

It becomes clear that some isolates can be more easily distinguished than others. While *Lc. mesenteroides* cluster less closely together, strains of *Lb. hordei* cluster more closely together. It is doubtful whether M13 V RAPD is suitable to distinguish most *Lb. hordei* strains.

4.4.5. Identification of monomer compositions

As described before, exopolysaccharides can be divided into hetero- and homopolysaccharides. In order to evaluate the type of EPS regarding the monomerical makeup of the polysaccharide as glucan or fructan, a complete hydrolysis of isolated EPS was carried out. After hydrolysis, the hydrolysis end-products were determined by HPLC and their concentrations and molecular identity determined. The concentrations were cross-referenced with the concentrations of EPS employed before hydrolysis to ensure stoichiometric hydrolysis. Hydrolysis was found to be complete in all cases. All strains produced a glucan, except *Lc. mesenteroides* TMW 2.1075 and TMW 2.1076, which produced a fructan.

4.4.6. Determination of viscosity using a flow cup

The viscosity is one of the most well known properties of EPS containing solutions. It is one of the reasons EPS are spoilage associated, since the viscous properties are considered detrimental to beverages (Cogan and Jordan 1994), while on the other hand being one of the reasons for the success of the use of e.g., carrageenan as a stabilizing agent (Cao et al. 1990). It is therefore vital to evaluate the viscosity of produced EPS. This was done using a flow cup according to

DIN 53211. In this cup, a defined amount of liquid escapes through a defined opening in the cup. The measured times until the liquid stream at the bottom of the cup breaks or becomes discontinuous is a measure of viscosity, especially for highly viscous liquids like oil or EPS-containing solutions (Fritz 1949). The results of the viscometry experiments are depicted in Figure 23.

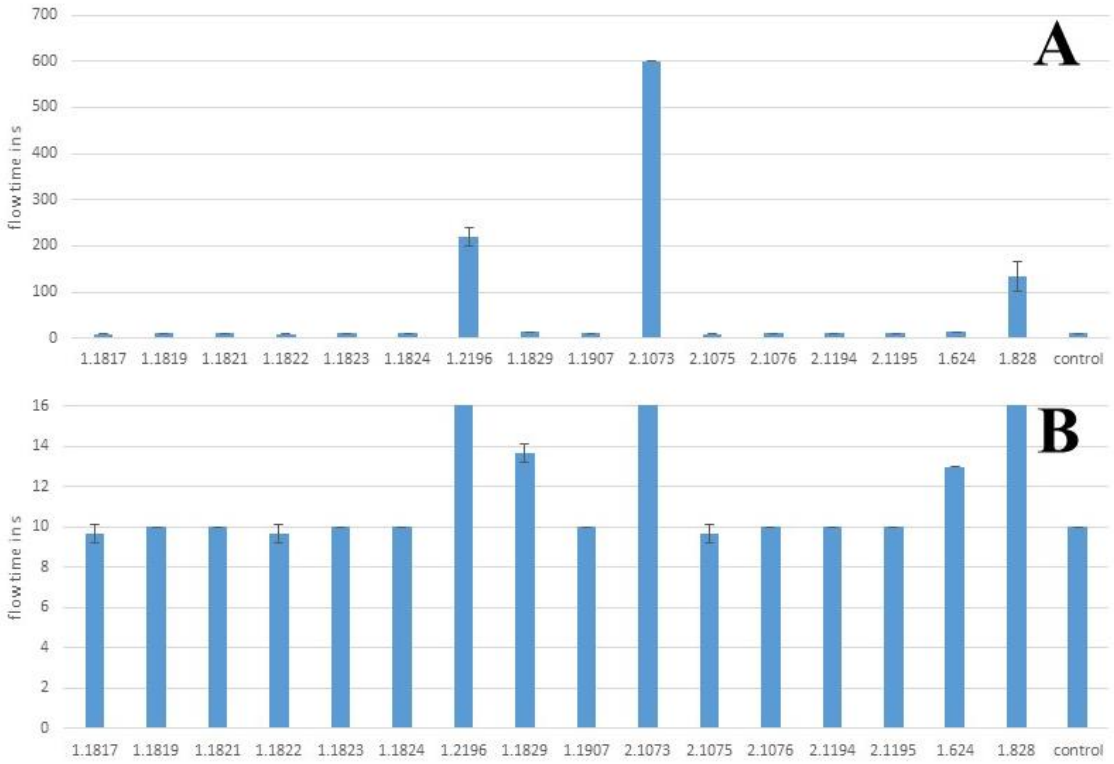

Figure 23. Flow times of 100 mL fermentate as determined by flow cup according to DIN 53211. A shows full range of flow times, **B** shows an expanded view of the flow time range 0-16 s to highlight slower flow times as observed in TMW 1.1829 and 1.624. Error bars show standard deviation of 3 biological replicates, control shows flow time of unfermented medium. In cases where the error bar is not visible, its value is 0.

4.4.7. Overview over the EPS types produced by LAB isolates from water kefir

To gain an initial overview over the EPS types produced by LAB from water kefir, their structure was investigated by AF4-MALS. The EPS investigated in this chapter was derived from the fermentation of MRS pH 6.2 with sucrose as a carbon source. It becomes clear that these organisms produce a great diversity of EPS regarding their molecular size distribution and chromatographic separation pattern, as is evident in Figure 24. While some EPS do not give a good MALS signal like *Lb. hilgardii*, most EPS formed are easily separated and detected

using AF4-MALS. Closer investigation of the EPS revealed that the EPS of *Lactobacillus* isolates are highly similar for each species of *Lactobacillus* when the EPS are produced under the same fermentation conditions, as is evident in Figure 25. In fact, the EPS produced by *Lb. nagelii* and *Lb. hordei* isolates under the same conditions are barely distinguishable within the same species. It also becomes clear that *Lb. hilgardii* EPS is not well suited for analysis using AF4-MALS, due to the high noise stemming from the low overall signal.

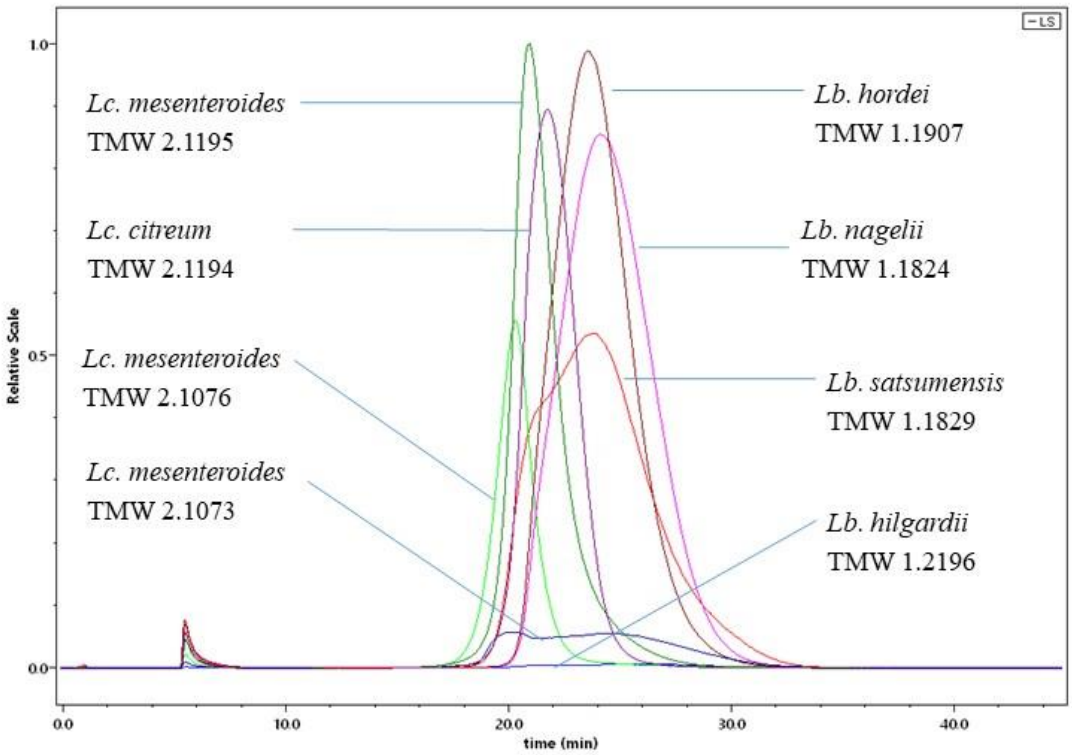

Figure 24. AF4-MALS chromatograms of EPS produced by selected LAB from the initial strain selection. Y axis shows 90° MALS signal.

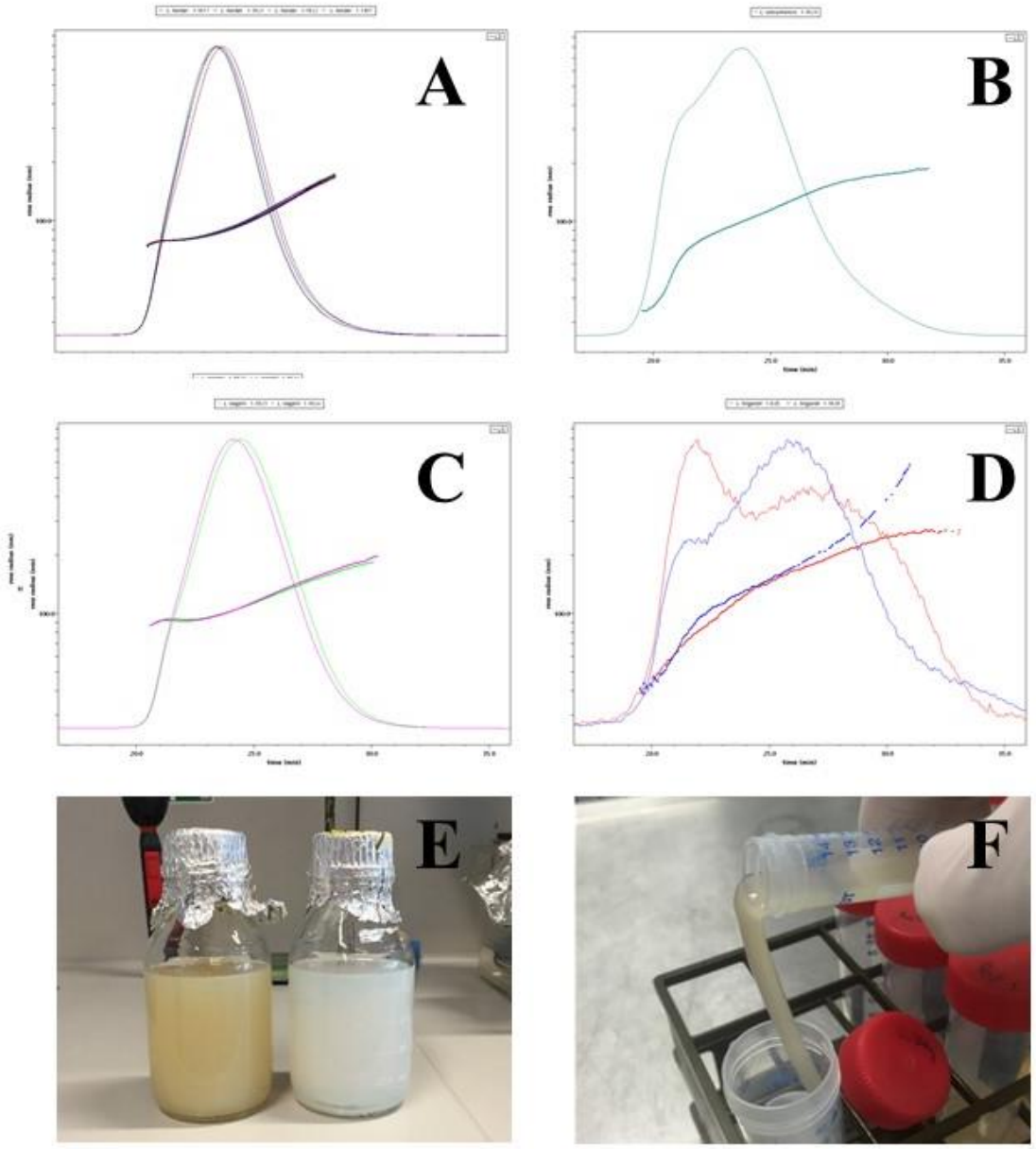

Figure 25. AF4-MALS chromatograms of EPS produced by *Lactobacillus* species from water kefir and a representative photographic image of the fermentation broths. A shows chromatograms of EPS produced by *Lb. hordei* isolates, **B** shows chromatograms of EPS produced by *Lb. satsumensis*, **C** shows chromatograms of EPS produced by *Lb. nagelii* isolates, **D** shows chromatograms of EPS produced by *Lb. hilgardii* isolates. Bottom row shows representative photographic images of EPS produced by the two groups of strains. **E** shows non viscous but highly turbid, cell free fermentate on the left and isolated EPS on the right. **F** shows highly viscous fermentate produced by *Lb. hilgardii*.

4.4.8. Preliminary strain selection

All data from the previous experiments was used to derive a preliminary strain selection. This was done in order to reduce complexity and enable the selection of strains for genomic sequencing. The goal was to select strains from the screening experiments that:

- Grow in full medium.
- Show satisfactory EPS formation on full medium.
- Grow in fruit juice-based media.
- Form glucans not fructans.
- Can be distinguished from other strains.

As indicated above, strains TMW 1.1826 and 1.1827 were excluded due to unsatisfactory growth in full medium. Strain TMW 1.1819 was excluded due to its low EPS production on solid medium.

From the monomer determination, strains TMW 2.1075 and TMW 2.1076 were excluded, due to the reduced stability of fructans at low pH, a property that would be undesireable in beverages. Since some *Lb. hordei* and *Lb. nagelii* strains showed almost no differences in EPS formation as well as RAPD-PCR patterns and are therefore possibly not different strains. For each combination of clearly distinguishable RAPD-PCR patterns and EPS formation, one strain was chosen. Thereby strains *Lb. nagelii* TMW 1.1824 and *Lb. hordei* TMW 1.1817 and TMW 1.1821 were excluded from the selection. *Lactobacillus curvatus* TMW 1.624 was further excluded from the selection since it was only employed as a reference strain.

Based on this exclusion process, 9 strains were preliminarily selected for genomic DNA sequencing, as indicated in Table 19.

Table 19. Preliminary strain selection for EPS production.

Strain		Strain number
Lactobacillus	*hilgardii*	TMW 1.828
Lactobacillus	*hordeii*	TMW 1.1822
Lactobacillus	*nagelii*	TMW 1.1823
Lactobacillus	*hilgardii*	TMW 1.2196
Lactobacillus	*satsumensis*	TMW 1.1829
Lactobacillus	*hordeii*	TMW 1.1907
Leuconostoc	*mesenteroides*	TMW 2.1073
Leuconostoc	*citreum*	TMW 2.1194
Leuconostoc	*mesenteroides*	TMW 2.1195

4.4.9. Genomic DNA sequencing

All strains from the preliminary strain selection were genomically sequenced. Since *Lb. hordei* TMW 1.1822 has already been sequenced in an earlier work (Xu et al. 2019a), only the other 8 strains were sequenced. The sequencing was undertaken to further evaluate metabolism in later experiements and allow for correlation of metabolite kinetics to the genomic data. The key features of the genomic sequences obtained are displayed in Table 20.

Table 20. Characteristics of sequenced LAB genomes derived from this study.

Species	Strain	Contigs	Genome size (Mbp)	GC content (%)	Number of genes	Coding density (%)
Lb. hordei	TMW 1.1907	15	2.37	34.79	2251	86.8
Lb. hilgardii	TMW 1.2196	80	3.2	39.87	3068	84.88
Lb. hilgardii	TMW 1.828	87	3.2	39.86	3084	84.88

4.5. Final strain selection

The data of the flow-time experiment in conjunction with the data for the fruit juice-based media screening were employed to further select strains. In this selection process the goal was to select 4 strains with ideally two groups of similarly EPS-producing strains in each group and stark contrast between the two groups in order to better depict the diversity of EPS produced by water kefir isolates.

- Show high EPS production in full medium as well as in fruit juice-based media.

- Have different EPS production in terms of viscosity and yield.
- Form two groups of 2 strains with similar EPS properties in each group but different EPS properties between the groups.

From the preliminary strain selection, several strains were again removed to select the final 4 strains. Due to the fact that both selected *Lc. mesenteroides* strains produce extremely different EPS when compared to each other (see Figure 24), they were exluded from further experiments. Since for *Lb. nagelii, Lb. satsumensis* and *Lc. citreum* only one strain per species is represented in the strain selection, these species were likewise excluded from the selection.

The final strains that were selected were therefore: *Lb. hordei* TMW 1.1822 and TMW 1.1907 and *Lb. hilgardii* TMW 1.828 and TMW 1.2196. Both *Lb. hordei* strains form a non thickening dextran and *Lb. hilgardii* strains form a thickening dextran.

4.6. Optimization of fermentation conditions in fruit juice-based media

Since the EPS producing isolates showed low yields in pure fruit juices and dilute fruit juices with sucrose addition, an attempt at optimizing the fermentation conditions was conducted to achieve the goal of 5 g/L EPS yield. As prior experiments showed a higher EPS yield at higher initial fermentation pH (data not shown). The effect of pH adjustment and buffering was investigated. For this purpose, the dilute medium, which was most promising in initial experiments (Figure 21), was pH adjusted to pH 7 and supplemented with citrate as a buffering agent. Fermentation was then carried out and EPS yield was determined. The results of this experiment are shown in Figure 26.

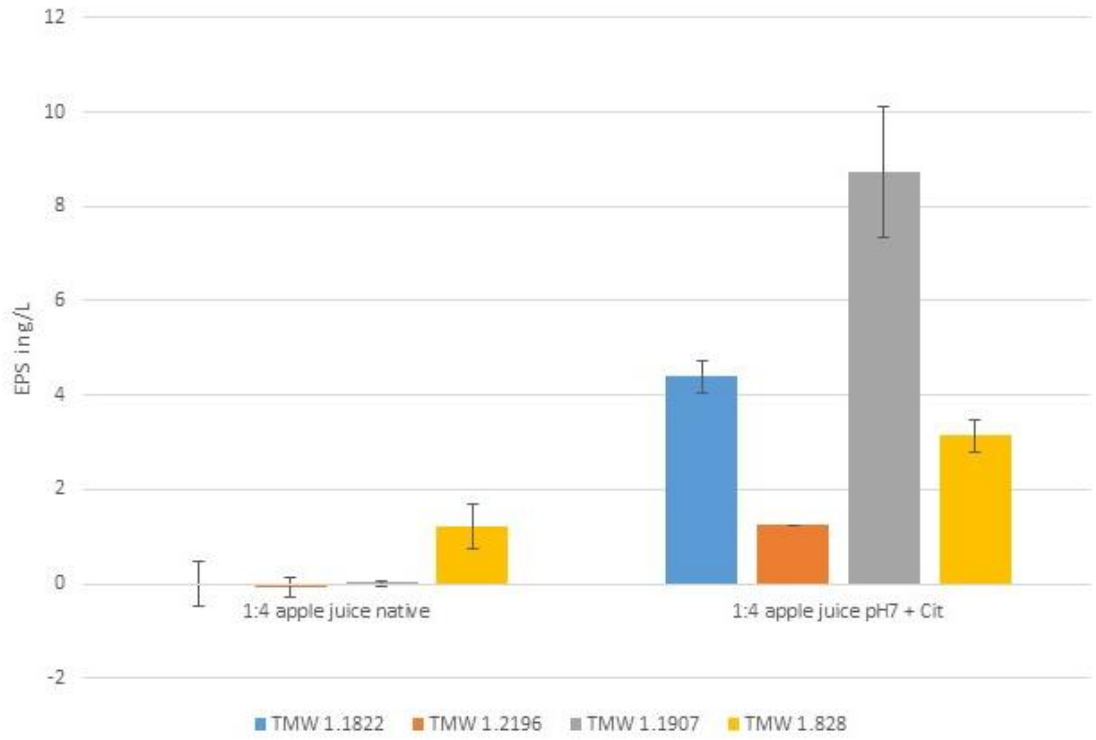

Figure 26. EPS formation by 4 LAB from water kefir in two different fruit juice-based media. Native dilute (25%) apple juice and pH-adjusted dilute apple juice with citrate addition ("+Cit", 5 g/L) after 48 h at 20 °C are shown.

After this promising intial experiment, the experiment was repeated with all strains and for both types of fruit juice. Additionally, a simple pH adjustment without addition of buffering agent was also carried out to investigate whether the buffering agent was necessary, with one version of each juice being only pH adjusted and one version of the medium receiving a supplement of sodium citrate. This was done since the enzymes involved in glucan production are known to have a higher pH optimum than the fruit juices (van Hijum et al. 2006; Waldherr et al. 2010). Because dextransucrases are active extracellularly, the pH of the fermentation broth is paramount for dextran production. Adjusting the medium pH would mean that the pH of the fermentation starts high and ends low, thus necessarily passing through the optimal pH range for dextran production. The citrate ions would then act as a buffering substance that, by slowing the process of lowering the pH or even elevating the final pH, would increase the time the fermentation pH stays in the optimal range for dextran production. The results of this experiment are presented in Figure 27.

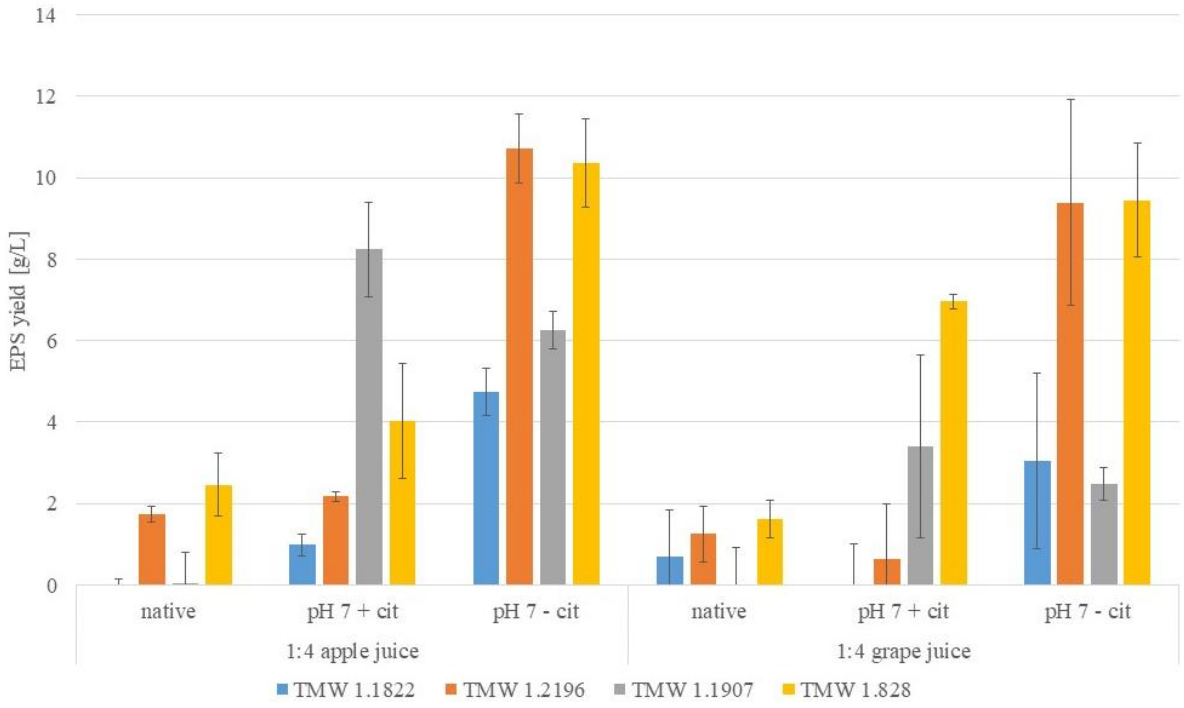

Figure 27. EPS formation by 4 LAB from water kefir. Native pH, pH-adjusted (pH 7 - cit) and pH adjusted with citrate addition (pH 7 + cit) diluted fruit juice-based media were used. Yields shown were determined after 48 h of fermentation at 20 °C.

As is evident from Figure 27, the addition of citrate actually decreases EPS yield under most fermentation conditions. Simple pH adjustment actually leads to sufficient yields concerning the initial goal of 5 g/L. However, *Lb. hordei* TMW 1.1822 does not reach this goal under any condition, while the other isolates reach this goal under many conditions at 20 °C. Due to their suitability for EPS production, these pH-adjusted dilute fruit juices without citrate addition were used for further studies. They will be referred to as An, A7, Gn and G7 (25% apple juice at native pH (pH 3.3), 25% apple juice at pH 7, 25% grape juice at native pH (pH 3.4) and 25% grape juice at pH 7, respectively) in further instances.

4.7. Production of dextrans using *Lb. hordei* TMW 1.1907

While the *Lb. hilgardii* isolates produce high amounts of EPS, their viscous glucans cannot be investigated using AF4-MALS. Additionally, microscopic investigation reveals the presence of cells in the dextran preparations. The glucan once purified, cannot be re-dissolved, hindering the study of these polysaccharides using the methods available (see also Figure 25). Since therefore an investigation of the structure-function relationship of these molecules is not

possible, a stability during storage is not given due to the insoluble nature of the glucan and in fact the product not turbid *Lb. hilgardii* was excluded from further investigations.

Consequently, in-depth analysis of glucan formation focused on the two *Lb. hordei* strains. Due to the low fermentation performance of *Lb. hordei* TMW 1.1822 in fruit juice-based media, as indicated by Figure 27, *Lb. hordei* TMW 1.1822 was excluded from further experiments. Therefore. *Lb. hordei* TMW 1.1907 was selected for the detailed characterization of its glucan yield and glucan properties.

To characterize the formation of turbidity forming dextrans by TMW 1.1907, fermentations were carried out at 10, 20 and 30 °C using native dilute fruit juices as well as pH adjusted fruit juices. The yields from these fermentations after 48 h are shown in Figure 28, cell counts are listed in Table 21.

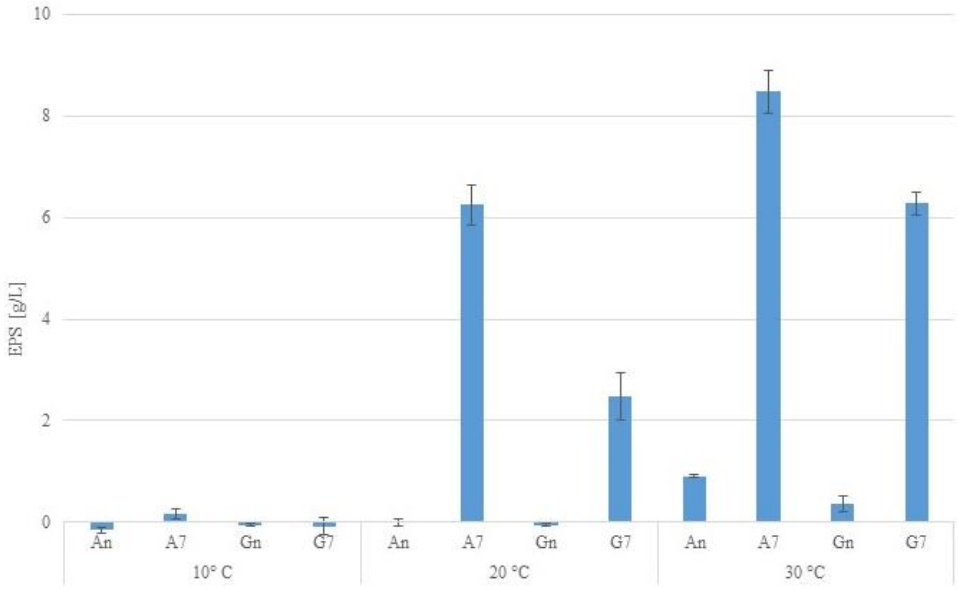

Figure 28. EPS yield in g/L from fermentations of An, A7, Gn and G7 using *Lb. hordei* TMW 1.1907 at 10, 20 and 30 °C. Fermentations were carried out in 15 mL scale for 48 h, error bars show standard deviations from biological triplicates.

Fermentation at 10 °C in all media and non-pH adjusted media at 20 °C resulted in no EPS formation. Consequently, pH adjusted media resulted in the highest yields at all temperatures, while yields were greatest at 30 °C. This once again shows that pH adjustment of the substrates highly increases yield. Despite the low dextran production, *Lb. hordei* was able to grow in native juices, again underlining that glucan formation is pH dependent (Table 21).

Table 21. Key parameters of fermentations of dilute fruit juices carried out using *Lb. hordei* TMW 1.1907 as determined by HPLC analysis and plate counting. A modified version of his table was published in Eckel et al. (2019a).

	Cell count [cfu/mL]	Sucrose [mmol/L]	Glucose [mmol/L]	Fructose [mmol/L]	Malate [mmol/L]	Lactate [mmol/L]
A7 0h	$6.2 \pm 0.5 \times 10^6$	111.2 ± 3.2	35.3 ± 0.6	63.2 ± 1.7	272.1 ± 5.5	n.d.
A7 48h 20 °C	$1.8 \pm 0.8 \times 10^6$	3.3 ± 0.7	18.3 ± 0.5	158.9 ± 9.1	33.6 ± 0.4	32.3 ± 0.2
A7 48h 30 °C	$1.4 \pm 0.3 \times 10^8$	6.3 ± 0.3	18.5 ± 0.9	169.7 ± 10	34 ± 0.4	34.7 ± 9
G7 0h	$6.2 \pm 0.5 \times 10^6$	124.6 ± 0.6	122.9 ± 0.6	133.4 ± 0.7	372.8 ± 11.3	n.d.
G7 48h 20 °C	$1.9 \pm 1.0 \times 10^8$	11.8 ± 0.1	93.6 ± 2.2	221.2 ± 4.3	151.1 ± 4.2	39.1 ± 1.2
G7 48h 30 °C	$2.0 \pm 0.2 \times 10^8$	13.7 ± 0.1	90.9 ± 1.2	206.5 ± 2.6	144.1 ± 3.3	46.6 ± 1.5

pH values of the fermentation broth dropped over the 48 h of fermentation. The final pH values for A7 and G 7 after fermentation at 20 °C were pH 4.4 and 4.7 and for A7 and G7 at 30 °C the pH values after fermentation were pH 3.9 and 4.1. Cell counts of native dilute fruit juices after 48 h were $1.5 \pm 0.5 \times 10^8$ cfu/mL for An at 20 °C, $1.2 \pm 0.6 \times 10^7$ cfu/mL for Gn at 20 °C, $1.7 \pm 0.6 \times 10^8$ cfu/mL for An at 30 °C and $8.2 \pm 0.2 \times 10^7$ cfu/mL for Gn at 30 °C.

HPLC was used to determine the concentration of sugars and acids in the (fermented) fruit juice-based media as shown in Table 21. Ethanol was not produced and while acetate was detected, its levels were below the limit of quantification. Lactate concentrations however increased over the course of fermentation, as would be expected. Sucrose concentrations decreased during fermentations as a result of glucan formation. Sucrose consumption was highest in fermentations using grape juice based media as opposed to apple juice based fermentations. Fructose concentration increased as a result of the glucansucrase reaction. As much as this effect is expected, it is interesting to note that fructose consumption remains relatively low. Malate concentration development was strain dependent.

4.8. Investigation of the structure-function relationship of glucans

AF4-MALS-UV was used to characterize the dextrans produced by *Lb. hordei* TMW 1.1907 *in situ*. To determine the molecular structure of the dextrans in question, polysaccharides were separated using AF4 and detected using MALS. Conformation plots were then constructed using the ASTRA 6.1 software. All obtained chromatograms as well as rms-radius distributions, molecular weight distributions and conformation plots are shown in Figure 29. Additionally, different evaluation models were used to determine rms radii, weight averaged molecular weights, the hydrodynamic coefficients v_G (slopes) that are generated using the conformation plots (Figure 29 D) and their coefficients of determination (R^2) at the peak maxima. Their values are given in Table 22.

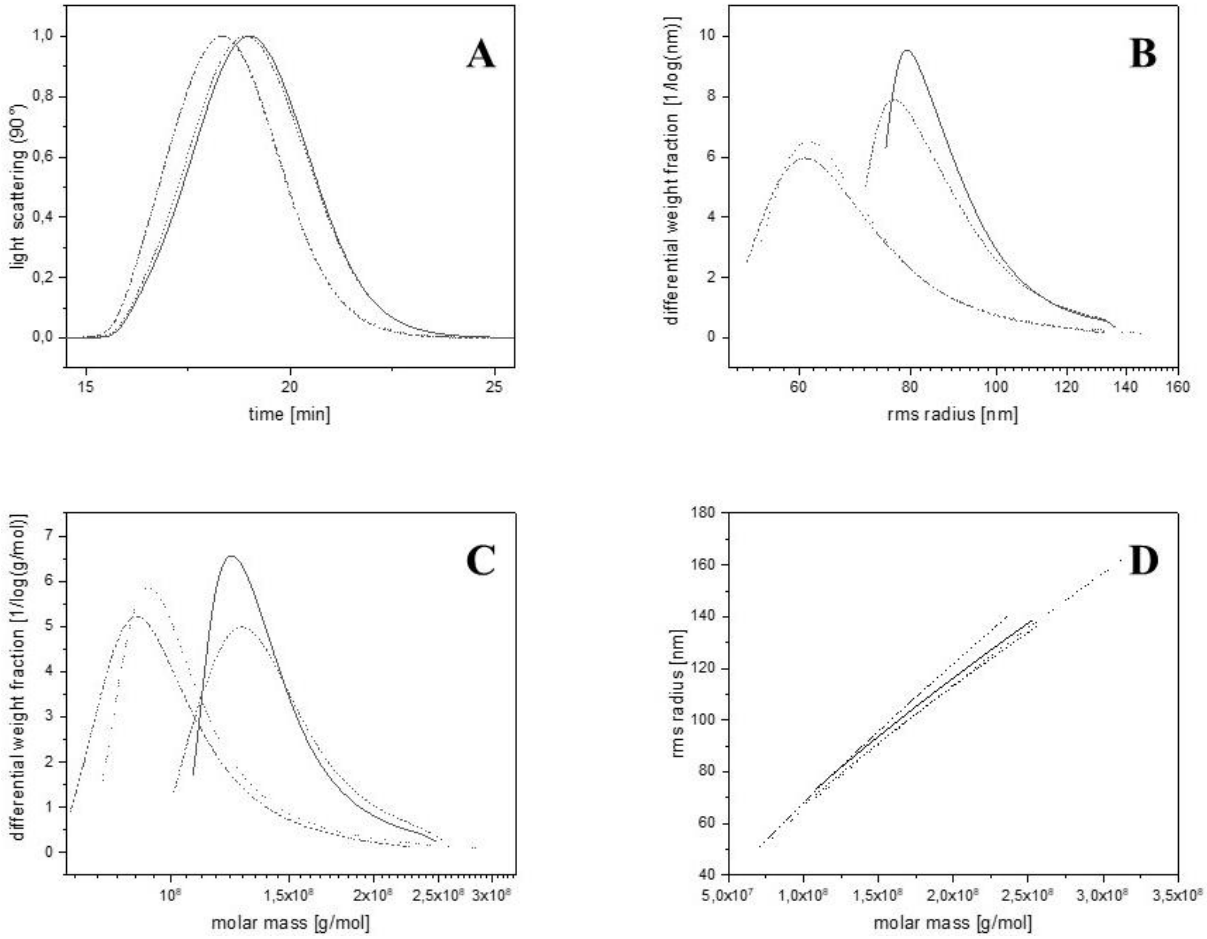

Figure 29. AF4-MALS data of glucan produced by *Lb. hordei* TMW 1.1907. Panels **A** to **C** show AF4-MALS chromatograms: **A** shows 90 ° light scattering signal as a function of time, **B** shows differential weight distributions of rms radii, **C** shows differential weight distributions of molar masses and **D** shows conformation plots (rms radius vs. log molar mass. Solid line shows: A7 20°C, dotted line shows: G7 20 °C, dashed line shows: A7 30 °C, dashed/dotted line shows: G7 30 °C. The Berry model was used for calculation of molar masses and rms radii. Modified from Eckel et al. (2019a).

In summary, dextrans produced at the same temperatures exhibit similar molecular weights and rms radii. This seems largely independent of the used fruit juice-based medium (Figure 29). This is also evident in shifted retention times (20 °C vs. 30 °C; Figure 29 A) as dextrans from fermentations at 20 °C were larger in size. Both the Berry algorithm as well as the random coil algorithm showed similarly high coefficients of determination for all dextrans. The Berry algorithm generally showed the best fit (highest R^2), which suggests the molecular structure to resemble a random coil like form (Table 22).

Table 22. Characteristics of dextrans produced by fermentation using *Lb. hordei* TMW 1.1907. A modified version of his table was published in (Eckel et al. 2019a).

		r (avg, nm)	Mw (mDa)	Retention time (min)	Conformation plot slope	R² (Berry model)	R² (random coil model)	R² (rod model)
20 °C	A7	87.4	141.2882	18.965	0.75	0.9984	0.9956	0.9774
	G7	85.5	141.5691	18.864	0.76	0.9989	0.9975	0.9816
30 °C	A7	66.1	111.8581	18.241	0.79	0.999	0.9985	0.9973
	G7	66.0	103.0909	18.258	0.84	0.9992	0.9987	0.9976

Further investigations using the conformation plots show that the regression lines have slopes with values in between literature values for a typical random coil (ν_G ~ 0.5 – 0.6) and a rod (ν_G ~ 1) molecule (Jakob et al. 2013; Nilsson 2013), as is also evident in Table 22. The structure of the *in situ* produced dextrans might therefore resemble a stretched random coil molecule, with smaller dextrans produced at 30 °C being more elongated (higher ν_G) and larger molecules produced at 20 °C being shorter and more compact. In accordance with this observation, the R^2 is still > 0.99 even though the rod model is used for evaluation of the dextrans, especially for those produced at 30 °C, as indicated by Table 22.

4.9. Stability of the *in situ* produced dextrans towards hydrolysis upon heat-treatment

Heat treatment ("pasteurization") is a typical method to achieve microbial stability in beverage production. Therefore, to evaluate the suitability of the produced dextrans for beverage technological use, we determined the stability of the produced glucans with regard to acidic hydrolysis during heat treatment conditions typical for beverage production (Zhao 2012). For this purpose, dextrans were dissolved in McIlvaine buffer (pH 3 or pH 7), subjected to a typical heat treatment and the structure of heat-treated dextrans was compared to those before heat treatment (controls). Dextrans dissolved at pH 3 showed a lower molecular weight and rms

radius compared to non heat treated dextrans in contrast to those dissolved at pH 7, which showed no difference in rms radius and molecular weight before and after heat treatment (Figure 30). Nonetheless, the heat treated dextran fraction with reduced molecular weight still showed sufficient turbidity-forming properties for both the glucan produced at 30 °C as well as 20 °C, as shown in Figure 30.

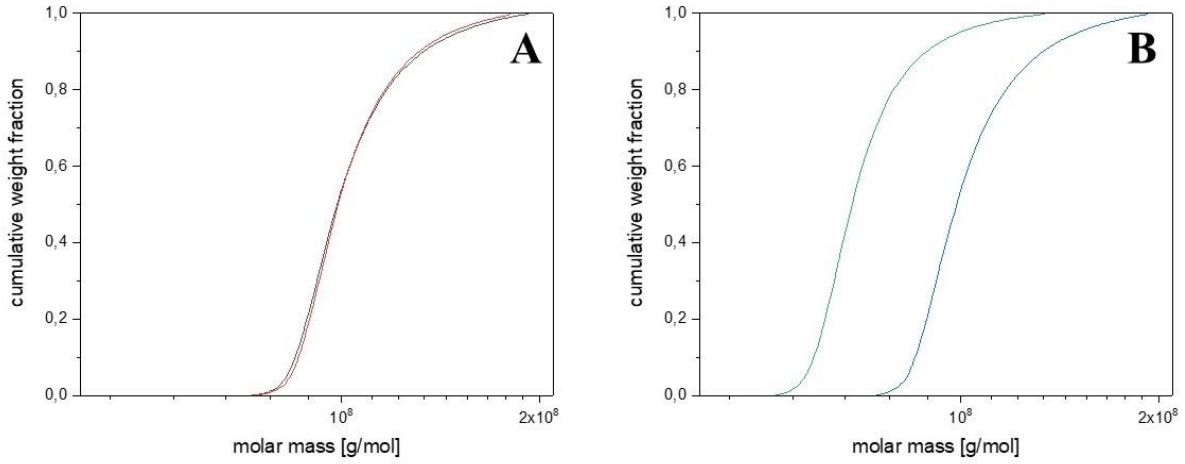

Figure 30. Molar mass distributions as cumulative weight fraction vs. molar mass for A7 at 30 °C dextran before and after heat treatment at pH 7 (A) and pH 3 (B). Purified dextrans were dissolved in phosphate citrate buffers of the respective pH at the concentrations present in the fermentate. Blue and red shows before heat treatment, black and green shows after heat treatment. Modified from Eckel et al. (2019a).

4.10. Stability of the dextran-based cloud systems towards hydrolysis during long-term storage

Acid hydrolysis of bacterial polysaccharides is typically enhanced by high temperatures (Wolff et al. 1953). However, while high temperatures speed up the reaction, acid hydrolysis of polysaccharides theoretically occurs at low rates even at low temperatures. We therefore assessed the stability of the produced dextrans towards hydrolysis at room temperature over time in a long-term storage experiment. For this purpose, the fermentation broth as well as the isolated and redissolved dextran from these fermentations, redissolved in McIlvaine buffer (pH 3) were heat treated for microbial stability and stored over the course of 3 months. To measure turbidity, the optical densitiy at 400 nm (OD_{400}) was recorded as a function of time. The turbidity of dextran-containing fruit juice-based fermentates (Figure 31 A) decreased during the first 3 weeks of storage, when a relatively steady turbidity level was reached. This level of turbidity roughly corresponded to the level of turbidity observed in the redissolved isolated

dextrans. After week 3, the observed levels of turbidity were nearly constant over the course of the experiment. Additionally, no sedimentation of particles was observed visually upon storage.

The visual appearance of the *in situ* produced glucans after 3 months of storage is shown in Figure 31 B in contrast to the unfermented juice shown in Figure 31 C.

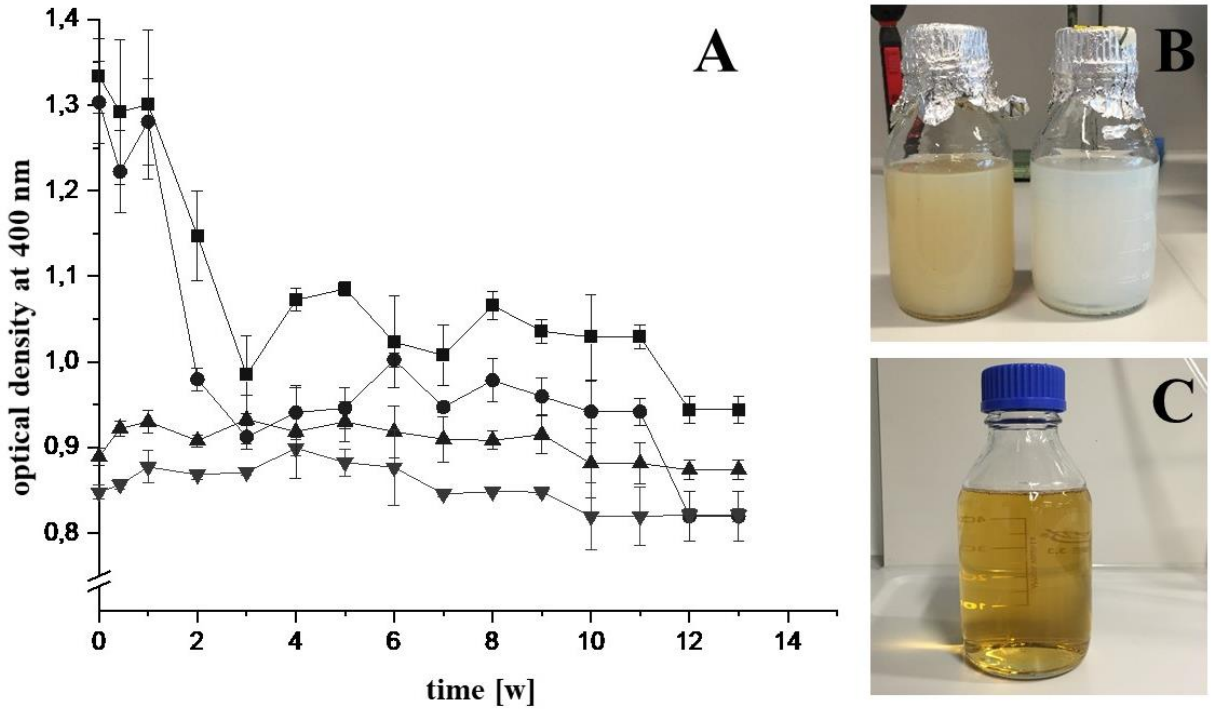

Figure 31. Level of turbidity exhibited by dextran-containing solutions produced using *Lb. hordei* TMW 1.1907 over a storage duration of 13 weeks. A: Optical density as a function of storage time in weeks [w], circles show dilute apple juice pH 7 fermented at 20 °C (A7, cell-free); squares show dilute apple juice pH 7 fermented at 30 °C (A7, cell-free); downward facing triangles show isolated dextran from apple juice (A7, 20 °C) resuspended in McIlvain buffer pH 3; upward facing triangle show isolated dextran from apple juice (A7, 30 °C) resuspended in McIlvain buffer pH 3. **B**: dextran-containing, fermented apple juice (left; A7, 30 °C, 48 h, cell-free) and the corresponding dextran resuspended in McIlvain buffer pH 3 to the same concentration (right); recorded after 3 months of storage. **C**: unfermented dilute apple juice pH 7. Modified from Eckel et al. (2019a).

5. Discussion

The main results and theses of this work, with regard to the initial working hypotheses and postulates, can be summarized as follows:

A re-evaluation of the water kefir microbiota indeed revealed novel isolates of the genus *Bifidobacterium*. These bifidobacteria can be isolated using a selective medium and subsequently cultured. These isolates are closely related to other bifidobacteria from foods. While their functional applicability remains uncertain and no phenotypic and genomic HoPs formation by these isolates was found, these strains could be characterized in detail. This includes their phenotypic placement as well as physiologic insights. Additionally, strong adaptations to the water kefir habitat on a genomic and physiologic level could be shown, revealing an intriguing lifestyle in strong contrast to known probiotic bifidobacteria.

Regarding the production of HoPs for beverage technological applications, *Lactobacillus* isolates from water kefir show great potential. While several strains could be found that produce glucans, especially *Lb. hordei* and *Lb. hilgardii* show great potential for glucan production in full medium and fruit juice-based media. By optimizing these fruit-juice based media, the yield of EPS could be increased to over 5 g/L under several conditions, meeting the initial yield goal and technological demands. While *Lb. hilgardii* produces a strongly viscosifying, non-turbid glucan, *Lb. hordei* produces a non viscosifying, opaque glucan. The latter glucan could be shown to produce a strong turbidity in solution and to be be highly stable towards heat treatment and prolongued storage, as encountered in beverage technological environments. This underlines their suitability for beverage technological use. A structure-function relationship for these molecules could be established and the structure of the fermentation product can be controlled by varying the fermentation conditions. Media based on two different fruit jucies were characterized and the resulting fermentation broths were investigated with regard to the metabolites formed and the suitability for use in beverages. In chapters 5.1-5.3, these findings are discussed in detail.

5.1. Description of *Bifidobacterium tibiigranuli* spec. nov.

The isolation of hitherto unknown bifidobacterial isolates, as described in chapter 4.1 and 4.2, highlights water kefir as a rich source of microorganisms with a high biodiversity. Additionally, it underlines the fact that the water kefir fermentation consortium is likely not fully characterized yet. As outlined above, diverse bioinformatics and physiologic investigations were carried out to delineate a novel species and discern our novel isolates from all previously known isolates of the genus *Bifidobacterium*. Genetic investigations including 16S rRNA identity, ANIb and isDDH values concerning the relatedness of our novel isolates to known species of the genus *Bifidobacterium* are below the thresholds used for species delineation (Kim et al. 2014; Meier-Kolthoff et al. 2014). Bioinformatic calculations show *B. subtile* DSM 20096^T as the closest phylogenetic relative with 98.35%. 87.91% according to ANIb and isDDH, and 35.80% for 16S rRNA identity. These values support the placement of the isolates as a new species within the genus *Bifidobacterium*. The close relation to *B. subtile* is highlighted by the similar peptidoglycan and cellular fatty acid profiles. These findings are additionally underlined by the topologies of the phylogenetic trees generated from 16S rRNA gene and concatenated marker gene sequences. The general topology of these trees is confirmed when considering different algorithms, highlighting their robustness and the independent standing of our isolates in the clade, as our isolates cluster closely with *B. subtile*, *B. aquikefiri*, *B. crudilactis* and *B. psychraerophilum*. Interestingly, these species have all been described to occur in the water kefir consortium or even to only have been isolated from such sources (Gulitz et al. 2013; Laureys et al. 2016; Laureys and De Vuyst 2014). They have also recently been classified as a new phylogenetic group, the "psychraerophilum group" based on phylogenomic calculations (Lugli et al. 2018). This is in accordance with the tree topologies obtained in this study. In addition, strains TMW 2.2057^T and TMW 2.1764 can be phenotypically distinguished from each other and all other described taxa of the genus *Bifidobacterium* when physiological properties, genetic fingerprinting, 16S rRNA gene sequences, concatenated marker gene sequences and genomic comparison data are considered. Considering these finding, the proposal of a new species of the genus *Bifidobacterium* according to the recommended minimal standards for the description of new species is warranted (Mattarelli et al. 2014). A novel species has therefore since been validly published (Eckel et al. 2019b). For the novel species, we proposed the name *Bifidobacterium tibiigranuli* (ti.bi.i.gra'nu.li. N.L). The name is derived from the N.L. *tibium*=Tibi the traditional name for water kefir; and L. neut. n. *granulum* a granule; resulting in N.L. gen. n. *tibiigranuli* ("of a Tibi granule"). The isolation and description of a novel species of the genus *Bifidobacterium* from water kefir shows the great potential of

this beverage for the isolation of hitherto unknown bifidobacteria. Unlike the bifidobacteria currently used as probiotics they are suited for the fermentation of sucrose rich beverages and have a broad growth range when pH and temperature are considered. They are less oxygen-sensitive and grow on readily available substrates like water kefir medium. This makes them ideal candidates for industrial production, since they can be propagated *in situ* in foods and drinks and are more tolerant to environmental stress factors like pH and atmospheric oxygen. However, their role in the fermentation consortium is still unclear.

The presence of fructanases has been described for bifidobacteria from genomic (Liu et al. 2015) and physiologic data (Ávila-Fernández et al. 2016). Additionally, bifidobacteria have been described to possess dextranolytic activity (Kim et al. 2015), while no dextransucrase or fructansucrase activity has been described for bifidobacteria to our knowledge. However, none of these four enzymes could be found in the genomes of the two isolated bifidobacteria, making their direct involvement in the granule formation doubtful.

It is also noteworthy that *B. psychraerophilum*. *B. aquikefiri* and *B. tibiigranuli* have all been described to not form acid from inulin (Eckel et al. 2019b; Laureys et al. 2016; Simpson et al. 2004). However, FOS-containing isolation media have been frequently used for their isolation. In fact, the mTY medium used by Gulitz et al. (2013) and Laureys et al. (2016) sources 50% of its main carbon source from inulin. In our study we therefore used an isolation medium using D-raffinose as the carbon source, because acid formation has been described from this sugar rendering it as suitable carbon source for their isolation (Eckel et al. 2019b; Laureys et al. 2016; Simpson et al. 2004). However, while using a more suitable sugar, the organisms isolated using our procedure proved to posess a higher optimal growth temperature than the one employed for their isolation and we only isolated one species of *Bifidobacterium*. These facts highlighted the nececessity for further investigations regarding the development of isolation media and procedures of these organisms from water kefir.

5.2. Investigation of the physiological properties, role in the consortium as well as application potential of bifidobacteria exclusively found in water kefir

The genomic investigations of bifidobacteria from water kefir as described in chapter 4.3, yielded several noteworthy facts. Generally, compared to the genomes of the probiotic group, the genomes of the water kefir group are bigger. Large genome sizes are a typical trait of free-living, non host-adapted bacteria due to the inherent metabolic flexibility, which is in

accordance with their habitat, where temperature and substrate availability as well as other environmental factors vary greatly (Toft and Andersson 2010). Among the genes shared by the water kefir isolates but not by the intestinal probiotics are several genes underlining the specific adaptations to the water kefir habitat that these organisms share. The observed adaptations include genes that are linked to the high osmolarity, low amino acids availability, acid stress and plant specific sugars like sucrose and fructose.

The exclusive presence of genes in the genomes of water kefir isolates was especially prominent in genes involved in amino acid metabolism, which highlights the importance of amino acids synthesis for this organism group. In fact, genomic data predict their prototrophy for amino acids. The mutations in the aspartate kinase reported by Verce et al. (2019) were not evident in this study *in silico*, which is in accordance with the ability of the strains in the water kefir group for full amino acid biosynthesis *in vitro*. Still, strain specific differences were observed concerning the growth in the absence of single amino acids. For example, *B. aquikefiri* CCUG 67145^{T} showed lower maximum OD in the absence of methionine.

pH values of below 3.5, that are commonly found in water kefir fermentations (Laureys and De Vuyst 2014) are in strong contrast to the higher pH values that are found in the intestinal tract of mammals. This fact suggests a strong acid tolerance of bacteria that are involved in water kefir fermentations. It is therefore surprising that the ADI pathway, which is typically discussed as an important mechanism of pH tolerance in many LAB (Rimaux et al. 2011; Tonon and Lonvaud-Funel 2002), was not encoded in the genomes of the water kefir group. Instead, a predominance of genes of the asparagine and aspartate metabolism was identified. These genes form a pathway, which is depicted in Figure 16. This pathway represents an alternative pathway to the ADI pathway and is a possible adaptation to the high acid-stress encountered in water kefir. By converting glutamine and asparagine to glutamate, ammonia is formed intracellularly. Intracellular ammonia formation is generally linked to an increase of the intracellular pH and therefore acid tolerance (Cotter and Hill 2003). This pathway is especially noteworthy, since water kefir derived yeast strains have been shown to liberate glutamine in the presence of LAB from water kefir for cross-feeding-reactions (Xu et al. 2019b). This suggests that bifidobacteria possibly also take advantage of these cross-feeding reactions with yeast, thereby alleviating pH stress. Several additional amino acid syntheses-related genes were derived from comparative genomics. These genes can also contribute to enhanced acid tolerance.

Osmotic stress is an additional important environmental challenge for the organisms of the water kefir microbiota. Typical countermeasures to this stress are for example accumulation of

compatible solutes like trehalose. Trehalose and trehalose-6-phosphate synthesis has been shown to be part of the osmotolerance of *E. coli* (Csonka and Hanson 1991; Giaever et al. 1988; Larsen et al. 1987). It is therefore not surprising, that trehalose-6-phosphate synthase and a possible trehalose phoshphate hydrolase are present in the genomes of the water kefir group. They likely present a way to deal with the osmotic stress encountered in water kefir. The incompleteness of the ots operon however makes an interpretation of the physiological role of these genes speculative. The two genes might simply serve a catabolic utilization of trehalose.

Due to the fact that the tibi grains are handled under atmospheric conditions during household fermentation, all members of the consortium are regularly exposed to oxidative stress. The duration and amount of oxygen present during the handling steps would likely kill most strictly anerobic bifidobacteria. The presence of oxidative stress tolerance genes would therefore be needed for bifidobacteria involved in water kefir fermentation and transfer to the next fermentation. This is in fact predicted from a genomic level in these organisms, as they possess several oxidative stress tolerance genes. Two of these genes are CydC and D. They have been shown to be part of gluthathione transport (Pittman et al. 2005) and cysteine transport (Holyoake et al. 2016) outside of the cell in gram negative bacteria. CydCD thus increases radical and oxidative stress resistance (Goldman et al. 1996). In LAB on the other hand, the role of CydCD is less clear. There, these genes are also likely linked to cellular resistance to oxidative stress, possibly through their involvement in gluthathione uptake (Pophaly et al. 2012). Gluthathione peroxidase has previously been shown to contribute to oxidative stress tolerance (Cabiscol Català et al. 2000; Moore and Sparling 1996). Rubrerythrin is described to posess a superoxide dismutase like function (LeGall et al. 1988; Lehmann et al. 1996), while superoxide dismutase, also exclusively found in the water kefir organisms, is a classic example of an oxygen detoxification enzyme (Scandalios 1993). SbxB is a pyruvate oxidase that has been shown to increase oxygen tolerance of *Streptococcus pneumoniae*, similarly to bifidobacteria a non-respiratory bacterium, but is in the case of *Streptococcus pneumoniae* an aerotolerant bacterium (Pericone et al. 2003). YaaA has been described as part of oxidative stress resistance in *E. coli* (Liu et al. 2011). Additionally, cysthathionine γ-lyase is part of an oxidative stress tolerance related mechanism in *Lactobacillus reuteri*, which works through a gluthathione independent pathway that involves cysteine (Lo et al. 2009). It might therefore contribute to acid tolerance in these organisms as well. The presence of this group of genes reveals several adaptations involved in oxygen tolerance. This is in accordance with recent findings, that describe these species as being aerotolerant in contrast to the classic obligately anaerobic bifidobacteria (Eckel et al. 2019b; Laureys et al. 2016; Scardovi 1986a).

Water kefir initially mainly contains sucrose. However, this sucrose is soon metabolized and previous data suggests the main carbohydrate in the first hours of water kefir fermentations is actually fructose (Laureys and De Vuyst 2014; Stadie 2013). This fructose is produced via extracellular dextransucrase (mainly by lactobacilli and *Leuconostoc*) and invertase (mainly by yeast) reactions, making fructose the main carbohydrate of early stage water kefir fermentations (Laureys and De Vuyst 2014; Stadie 2013; Xu et al. 2019a). The glucose moiety ends up in glucans or is consumed by the bacteria and yeasts, respectively. The presence of two possible mechanisms involved in fructose uptake in the genomes of the water kefir bifidobacteria, based on genomic predictions, is therefore not surprising. The ABC transporter present in the genomes only encodes 1 permease unit, which is in contrast to the typical two. However, it is homologous to an ABC transporter described for *B. longum* NCC2705. While early research by Parche et al. (2006) implied it as a ribose transporter, it was shown by Wei et al. (2012) that this transporter actually has a higher affinity for fructose. The fact that the ABC transporter has only one permease domain sheds doubt on its functionality. Its presence in all three strains of the water kefir group however is remarkable. The fact that two putative fructose uptake pathways are encoded apparently underlines the adaptation of these organisms to fructose rich growth media. It is interesting to note that neither *B. tibiigranuli* nor *B. aquikefiri* have a complete mannitol uptake pathway, which is in contrast to reports indicating they produce acid from mannitol (Eckel et al. 2019b; Laureys et al. 2016). Mannitol utilization is not unusual in the context of water kefir, since LAB from water kefir have also been shown to be able to metabolize mannitol (Xu et al. 2019a). This mannitol is likely produced from fructose by acetic acid bacteria that are also part of the water kefir environment (Gulitz et al. 2011). Therefore, the genomic background of mannitol utilization in these strains remains unclear, namely with respect to annotated uptake systems.

5.3. Production of novel beverage additives

5.3.1. Strain selection

The strain selection process, as outlined in chapters 4.4 and 4.5, focused on several goals. 4 strains were to be selected from LAB and bifidobacterial strains originating from water kefir. These strains should produce glucans in MRS + suc as well as fruit juice-based media. These strains should achieve a yield of 5 g/L glucan in fruit-juice based media and be distinguishable from each other. The produced glucan should be soluble, ideally non-viscous and exhibit stable cloud forming properties. While the *Bifidobacterium* isolates, which were characterized in early chapters proved as non/low HoPs-producing and therefore not suitable for the purpose of HoPs

production in fruit juice-based media, several promising candidate strains comprising LAB from water kefir could be pre-selected. The initial strain selection comprised 20 strains. Based on the criteria growth in full medium, EPS production in fruit juice-based media, viscosity of produced EPS and bacterial species, a final selection of 4 strains was chosen, as outlined in chapter 4.4.

Among the finally selected 4 water kefir isolates *Lb. hordei* and *Lb. hilgardii* are part of fermentation consortium of water kefirs as described in several independent studies (Gulitz et al. 2013; Gulitz et al. 2011; Laureys 2017; Laureys and De Vuyst 2014; Pidoux 1989). While *Lb. hilgardii* has been long known to be a wine spoilage organism (Douglas and Cruess 1936), it has more recently been discussed as a starter organism in wine fermentation for its sensory characteristics (du Toit et al. 2011). However, the use of *Lb. hordei* as a pure culture for beverage fermentation is thus far not explored. Generally, to our knowledge, fermentation of fruit juice-based beverages with dextran producing water kefir LAB has not been investigated before. Still, these bacteria offer a great potential for the fermentation of (dilute) fruit juices, due to the similar high sugar and low nitrogen content in the medium, as especially our isolates are derived from the fermentation of a medium that exhibits these traits.

Initial experiments confirmed this theory, as all 4 isolates showed glucan production in fruit juice-based media. This glucan production could be further increased by artificially elevating the medium pH to pH 7, likely by increasing the time the dextransucrase is exposed to its pH optimum. Using this technique, sufficient yields over 5 g/L can be achieved under different conditions in different media.

Viscosity determinations revealed that glucan produced by *Lb. hilgardii* is highly viscous, a trait undesirable in beverages and associated with spoilage (Fraunhofer et al. 2018; Werning et al. 2006). Additionally, initial experiments revealed that the glucan produced by *Lb. hilgardii* is non-soluble after purification. It can therefore not be analyzed, nor could it be used in beverages after purification due to the insolubility of the polysaccharide, which is in stark contrast to the dextrans produced by *Lb. hordei* that are non thickening, soluble and intrinsically turbid. In fact, when crude enzyme preparations of the dextransucrase of *Lb. hilgardii* were used for the production of glucan, the resulting dextran solution was still viscous, but clear in its native state. This is in accordance with microscopic findings that show associated cells in the purified glucan preparations. These cells are associated with or entrapped by the polysaccharide molecules, a common mechanism among bacterial exopolysaccharides. They cannot, after production of the polysaccharide, be removed by centrifugation since they are

attached to the polysaccharide matrix. When embedded in the polysaccharide matrix, they scatter visible light and thus contribute to the turbid appearance of the fermentation broth rather than the polysaccharide itself. Because of the fact that the glucan are highly viscous and are not turbid upon closer inspection, *Lb. hilgardii* was excluded from further experiments. Following work including a more in-depth characterization of glucan formation was therefore focused on *Lb. hordei*. However, *Lb. hordei* TMW 1.1822 showed lower dextran yields than *Lb. hordei* TMW 1.1907. The fact that both strains show different glucan yields in the same medium underlines the complex metabolic adaptations described for different water kefir organisms (Bechtner et al. 2019; Xu et al. 2019a; Xu et al. 2018). *Lb. hordei* TMW 1.1907 was therefore subsequently chosen for the in-depth characterization of the glucan-forming properties.

5.3.2. Production of clouding dextrans in fruit juice-based fermentation media

Lb. hordei TMW 1.1907 was isolated from water kefir in previous works and has been reported to produce dextrans from sucrose (Gulitz et al. 2011; Xu et al. 2018). The dextran yields in the tested apple and grape juice based media were lower than those reported in MRS supplemented with 8% (w/v) sucrose (Stadie 2013), which is likely due to lower substrate concentrations and overall nutrient content. In any case, growth also occurred in non-pH adjusted dilute fruit juices as described in the results section, while no (20 °C) or very low levels (30 °C) of dextran were produced in these dilute juices (Figure 28).

This suggests the dextransucrase is not properly operating at 20 °C or is not being released when low medium pH and low temperature occur together. Moreover, the pH of the fermentation medium appears to be crucial for dextran biosynthesis by *Lb. hordei*, as artificial elevation of pH of fruit juices to pH 7 resulted in much higher dextran yields at all temperatures for all strains. Dextransucrases, especially the one of *Lb. hilgardii* TMW 1.828, typically possess a pH optimum in a moderately acidic environment from pH ~ 4.0 - 6.0 (van Hijum et al. 2006; Waldherr et al. 2010). Additionally, it has been shown for other LAB that dextransucrases are released more efficiently at higher pH (Otts and Day 1988). This shows that higher dextran yields obtained in the pH-adjusted media can be based on the fact that the higher pH causes a higher level of dextransucrase secretion as well as a higher dextransucrase activity. In our experiments, dextran yields were not only influenced by the initial medium pH but also by the fermentation temperature. This suggests that both cell growth and environmental pH affect dextran biosynthesis. The fact that no dextran production took place in TMW 1.1907 at 10 °C is likely a consequence of no growth occuring at this temperature, which is contrary to effects observed in other LAB. In *Lb. sakei* for example, dextran production was highest at 10

°C as opposed to 30 °C and takes place through cell wall anchored dextransucrases. This again shows the diverse adaptations of LAB from different foods to their habitats and the resulting different EPS production patterns (Prechtl et al. 2018a; Prechtl et al. 2018b).

In the context of foods, it is important to consider the metabolites present after fermentation. In the case of our fermentations, fermentation with *Lb. hordei* TMW 1.1907 led to decreasing sucrose and glucose concentrations while fructose concentrations increased. This shows that under our fermentation conditions *Lb. hordei* TMW 1.1907 preferentially metabolized glucose upon growth in fruit juices. The increasing fructose concentrations can be attributed to the release of fructose during e.g. the dextransucrase reaction. This is also corroborated by the fact that fructose concentrations at the end of fermentation (48 h) corresponded well to the sum of initial fructose and the fructose that would be liberated from the glucansucrase reaction, which indicates that only little fructose is consumed during the fermentation. This is especially noteworty as fructose has been shown to be the main carbohydrate present in early water kefir fermentations and might therefore be assumed to be readiy used by bacteria of this consortium (Laureys and De Vuyst 2014; Stadie 2013; Xu et al. 2019a).

When *Lb. hordei* TMW 1.1907 is used for fermentation of fruit juice-based media, malate levels decrease of the course of fermentation. This is in accordance with the presence of a malate transporter (CRI84_05185), malate permease (CRI84_09915) and malic enzyme (CRI84_09410) in the genome of *Lb. hordei* TMW 1.1907 (Accession No: PDDD00000000). The first two enzymes mediate malate uptake, while malic enzyme catalyzes the reaction from malate to pyruvate. The measurements of dereasing malate concentrations together with the genomic data suggest that *Lb. hordei* can catabolize malate from fruit jucies, a common metabolic trait of *Lactobacillus* in the fermentation of fruit juice (du Toit et al. 2011). The low levels of acetate likely result from the phosphoketolase pathway in the genome of *Lb. hordei*, which has been described for *Lb. hordei* before (Rouse et al. 2008; Xu et al. 2019a; Zheng et al. 2015). The concentration of lactic acid found in our fermentations is comparable to that described for water kefir fermentations but higher than the concentration described for other lactic beverages (Laureys and De Vuyst 2017; Malbaša et al. 2008).

Yields of dextran yields in A7 medium were highest at those temperatures, which enabled growth. This is surprising, since at inoculation levels of OD 0.1, glucan formation might be expected even when no growth occurs. The fact that the utilized apple juice contained lower intrinsic levels of sucrose than the grape juice (see Table 21) means that sucrose consumption by *Lb hordei* was higher in grape juice based fermentations than apple juice based

fermentations, in which dextran production was highest. The higher sucrose consumption without corresponding glucan formation could be explained by a putative invertase (CRI84_06255) that is present in the genome of *Lb. hordei* TMW 1.1907 and is, in addition to the dextransucrase, capable of sucrose utilization. This putative invertase has a KxYKxGKxW signal peptide that suggests cellular export and an extracellular localization of this protein, which might suggest that extracellular sucrose utilization may not only occur by the dextransucrase (Bensing et al. 2014; Bensing et al. 2007). In any case, dextransucrases are also capable of sucrose hydrolysis without dextran formation releasing glucose and fructose (Leemhuis et al. 2013). Therefore, the apparent sucrose hydrolysis might be simply a result of dextransucrase hydrolysis reaction, which occurs more under the respective fermentation conditions.

When considering dextran yields, apple juice seems to be a more suitable fermentation substrate for *Lb. hordei* TMW 1.1907, since yields in apple juice are always higher (Figure 28). In the light of industrial use however, an apple flavour might be undesirable and grape juice might be chosen for its sensory properties, in which case production in G7 medium could be preferable. The growth of LAB in juices has long been studied especially in the context of wine, but also with the goal of producing novel probiotic drinks (Mousavi et al. 2011; Wibowo et al. 1985). While this research mainly focused on organoleptic properties, the formation of exopolysaccharides was investigated mainly in the context of spoilage due to the thickening properties of some of these EPS (Martínez-Viedma et al. 2008; Werning et al. 2006). In contrast to that research, no thickening of aqueous solutions of the purified dextrans or the dextrans produced *in situ* by *Lb. hordei* was observed in this study. These results thus show that *Lb. hordei* is highly suited for the fermentation of neutralized dilute apple and grape juice and for *in situ* production of dextrans that exhibit novel cloud-forming techno-functional properties.

5.3.3. Characterization of the macromolecular properties and the stability of *in situ* produced dextrans

Acidic hydrolysis of dextrans at high temperatures is a typical effect observed in aqueous solutions containing dextrans (Senti et al. 1955). Heat treatment however is a typical method to achieve microbial stability for acidic beverages. It is therefore critical to assess the stability of the produced dextrans towards acidic hydrolysis, as a high level of hydrolysis would mean a compromised functionality of the molecules in question. Our tests revealed that the properties of the *in situ* produced dextrans concerning light scattering and turbidity formation were not strongly affected by pasteurization, as it is applied in beverage filling plants. This highlights

the suitability of these molecules for their use in beverage related applications and their filling procedures. Concerning their long-term stability, the observed initial drop in optical density (Figure 31) might be due to a time-dependent breakdown of aggregates that are present in the fermented juice. The turbidity level in the solution prepared with purified dextran (Figure 31 B) was stable throughout the storage period and correlated to the level of turbidity in the untreated fermentate after the initial loss of turbidity. This clearly shows that the dextrans found in the fermentate are mainly responsible observed turbidity in the cell free fermented fruit juices.

The data obtained from AF4-MALS measurements showed that the type of fruit juice has a smaller impact on the molecular size of the polysaccharide molecules than the temperature during fermentation. Dextrans produced at 20 °C showed a higher molecular weight than dextrans produced at 30 °C, an effect which has similarly been described for the formation of dextran molecules that had a comparatively smaller size when produced at higher temperatures (Prechtl et al. 2018a). Additionally underlining these findings of Prechtl et al. (2018a), a higher molecular weight of dextran produced by *Lb. hordei* resulted in more intense light scattering in this work.

6. Summary

Water kefir is a fermented beverage produced using back-slopping of a microbial consortium, which is immobilized in granula. The constituents of the water kefir microbiome and their functional properties are not yet fully understood and their use in industrial beverage fermentations thus underexplored. Namely, not yet cultured members of the granulum microbiome and the naturally stable, cloudy appearance of the water kefir liquid phase has not been addressed for any explanation of their role or their use as cloud-forming agent in turbid beverages.

In the first part of this study, the potential of water kefir for the isolation and identification of bacteria for novel types of food fermentations was explored. By isolating and characterizing new strains of bifidobacteria from water kefir the presence of hitherto unknown and uncultured species in water kefir was shown. Bifidobacteria are commonly associated with health benefits. It is therefore of great interest to the food industry, but also for food microbiological research to characterize novel species. During this work, *Bifidobacterium tibiigranuli* has been validly described as a novel species. It has been placed in the Psychraerophilum group of bifidobacteria, which it shares with other isolates derived from food fermentations, and which underlines its adaptation to this niche. Using comparative genomics and phenotypic characterizations, their amino acid and sugar utilization patterns were predicted. They suggest a strong adaptation to the water kefir habitat, with full amino acid synthesis pathways, oxidative stress tolerance genes and a high number of genes for sugar utilization. These genomic findings correlate well with their phenotype.

In the second part of this thesis the basics for a novel promising application of LAB from water kefir exploiting their ability to produce cloud-forming dextrans was established. For this purpose, the EPS-formation properties of LAB from water kefir were characterized. Starting with a selection of 18 strains, four *Lactobacillus* strains that appeared especially suited for glucan production in the context of beverage fermentation were identified. Out of the 4 isolates, *Lb. hordei* TMW 1.1907 was identified as the most promising strain for the production of cloud-forming dextrans. *Lb. hordei* TMW 1.1907 produces dextrans in food grade media that form a very stable turbidity while being non-viscosifying. The yield and molecular size of produced dextrans can be varied by varying fermentation conditions. A relationship was established between the molecular size distributions of these molecules and their viscosity / cloud-forming properties. These dextrans could be produced in fruit juice-based media at yields of over 5 g/L. The produced dextrans are stable towards acid hydrolysis and long term storage at native as

well as acidic pH. These molecules can be used to substitute current emulsion-based clouding systems with the benefit of being natural ingredients that do not rely on the addition of additives to retard emulsion decay. Additionally, the manufacture of these glucan containing fermentates is readily achievable and can be up-scaled at laboratory scale.

7. Zusammenfassung

Wasserkefir ist ein fermentiertes Getränk, welches auf traditionelle Weise durch Fermentation mit einem mikrobiellen Konsortium hergestellt wird, welches auf der Wasserkefir-Granule immobilisiert ist. Die Bestandteile des Konsortiums und seine funtionellen Eigenschaften sind noch nicht vollständig erfasst und die Verwendung der involvierten Mikroorganismen in industriellen Fermentationen noch untererforscht. Dies gilt insbesondere für die noch nicht kultivierten Teile des Konsortiums, deren Rolle in der Fermentation unklar ist, und die stabile, natürliche Trübung des Wasserkefirs, deren getränketechnologische Nutzung bis jetzt keine Beachtung fand.

Im ersten Teil dieser Arbeit wurde das Potential von Wasserkefir für die Isolation von Mikroorganismen für neuartige Lebensmittelfermentationen erforscht. Durch die Charakterisierung und Beschreibung neuartiger *Bifidobacterium* Stämme konnte eine neue, bisher unkultivierte Spezies etabliert werden. Aufgrund der Assoziation von Bifidobakterien mit positiven Effekten auf die Gesundheit sind diese Organismen von großem Interesse für die Lebensmittelindustrie und für die lebensmittelmikrobiologische Forschung. Die Beschreibung von *Bifidobacterium tibiigranuli* wurde im Rahmen dieser Arbeit veröffentlicht und die Spezies wird der Psychraerophilum-Gruppe zugeordnet, der auch andere Isolate aus Lebensmittelfermentationen angehören. Dies unterstreicht die Anpassung der Spezies an Lebensmittelfermentationen. Zusätzlich wurden die Aminosäure-prototropie und der genomische Hintergrund ihrer Kohlenhydratnutzung gezeigt. Außerdem zeigen die bioinformatischen Daten aus der vorliegenden Arbeit eine hohe Zahl an Genen der oxidativen Stressresistenz und Zuckerausnutzung. Diese genomischen Daten korrelieren mit den phänotypischen Daten wie z.B. der Aerotoleranz der Spezies.

Im zweiten Teil dieser Arbeit wurde die Möglichkeit zur Nutzung von Milchsäurebakterien aus Wasserkefir für neuartige Lebensmittelfermentationen zur Herstellung einer natürlichen, stabilen Trübung erforscht. Dazu wurden exopolysaccharidbildende Milchsäurebakterien aus Wasserkefir hinsichtlich ihrer polysaccharidbildenden Eigenschaften untersucht. Ausgehend von einer Stammauswahl von 18 Isolaten konnten 4 Isolate identifiziert werden, welche in Gertänkesubstraten hohe Glucanausbeuten zeigen. Aus diesen 4 Stämmen wurde *Lb. hordei* TMW 1.1907 als besonders geeignet identifiziert. *Lb. hordei* TMW 1.1907 produziert in fruchtsaft-basierten Medien trübungsbildende Dextrane, die nicht viskosifizierend wirken. Durch Variation der Fermentationsbedingungen kann das produzierte Dextran gezielt in seiner Größe beeinflusst werden. Für das derartig hergestellte Glucan konnte eine Struktur-

Funktionsbeziehung zwischen der Molekülgröße und der Funktionalität als Trübungsbildner aufgezeigt werden. Die produzierten Dextrane sind stark trübungsbildend und lager- sowie hitzestabil. Dadurch stellen sie eine natürliche Alternative zu aktuell verwendeten Trübungsbildnern und Getränkeemulsionen dar.

8. List of publications and conference contributions derived from this work

Peer reviewed papers

2020	Eckel VPL and Vogel RF, Genomic and physiologic insights into the lifestyle of *Bifidobacterium* species from water kefir. *Arch Micobiol 2020*
2019	Eckel VPL, Vogel RF and Ehrmann M, Description of *B. tibiigranuli* sp. nov. isolated from homemade water kefir. *Int J Syst Evol Microbiol* 2019; 003936: 1-8
	Eckel VPL, Vogel RF and Jakob F, *In situ* production and characterization of cloud forming dextrans in fruit-juices. *Int J Food Microbiol* 2019; 306: 108261
	Münkel F, Bechtner J, Eckel V, Fischer A, Herbi F, Jakob F, and Wefers D. Detailed structural characterization of glucans produced from *Leuconostoc citreum* TMW 2.1194. *J Agric Food Chem.* 2019, 67, 24:6856-6866

Conference contributions

2019	Eckel VPL, Vogel RF and Jakob F, *In situ* production and characterization of cloud forming dextrans on fruit-juice based media. Poster presentation, *8th Congress of European Microbiologists*, Glasgow, Scotland
	Jakob F, Bechtner J, Eckel VPL, Schmid J, and Vogel RF, Nutzbarmachung molekularer Prozesse in Wasserkefir und Kombucha für Lebensmittelentwicklungen. Oral presentation, *VLB-Symposium Alkoholfreie fermentierte Getränke - Sauer macht lustig*, Berlin, Germany
	Huchtmann J, Rossmann S, Kindsvater V, Eckel VPL, Jakob F and Schneider J, Microbial exopolysaccharides as an alternative for declarable stabilizers and opacifiers. Posterbeitrag, *EBC Congress 2019*, Antwerp, Belgium
2018	Jakob F, Prechtl RM, Fraunhofer ME, Brandt JU, Ua-Arak T, Bechtner JU, Eckel VPL, Schmid J, and Vogel RF, Exopolysaccharide production by food-grade lactic and acetic acid bacteria. Oral presentation, *CDZ-Symposium*, Universität Hohenheim, Stuttgart, Germany
	Huchtmann J, Rossmann S, Kindsvater V, Eckel VPL, Jakob F and Schneider J, Mikrobielle Exopolysaccharide als Alternative für deklarationspflichtige Stabilisatoren und Trübungsbildner, *Kongress Lebensmitteltechnologie 2018*, Bremerhaven, Germany
2017	Eckel VPL, Bechtner J, Jakob F and Vogel RF, Molecular diversity of glucan production in *Lactobacillus* strains from water kefir. Poster presentation, *13th International Symposium on Lactic Acid Bacteria*, Egmond aan Zee, Netherlands

9. References

Adams MR, Hall CJ (1988) Growth inhibition of food-borne pathogens by lactic and acetic acids and their mixtures. Int J Food Sci Tech 23:287-292. doi:10.1111/j.1365-2621.1988.tb00581.x

Arslan S (2015) A review: chemical, microbiological and nutritional characteristics of kefir. CyTA - Journal of Food 13:340-345. doi:10.1080/19476337.2014.981588

Aryana KJ, Olson DW (2017) A 100-Year Review: Yogurt and other cultured dairy products. J Dairy Sci 100:9987-10013. doi:https://doi.org/10.3168/jds.2017-12981

Ault RG (1965) Spoilage bacteria in brewing - a review. Journal of the Institute of Brewing 71:376-391. doi:10.1002/j.2050-0416.1965.tb06362.x

Ávila-Fernández Á, Cuevas-Juárez E, Rodríguez-Alegría ME, Olvera C, López-Munguía A (2016) Functional characterization of a novel β-fructofuranosidase from *Bifidobacterium longum* subsp. *infantis* ATCC 15697 on structurally diverse fructans. J Appl Microbiol 121:263-276. doi:10.1111/jam.13154

Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K et al. (2008) The RAST Server: rapid annotations using subsystems technology. BMC Genomics 9:75-75. doi:10.1186/1471-2164-9-75

Badel S, Bernardi T, Michaud P (2011) New perspectives for lactobacilli exopolysaccharides. Biotechnol Adv 29:54-66

Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM et al. (2012) SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455-477. doi:10.1089/cmb.2012.0021

Bechtner J, Xu D, Behr J, Vogel RF (2019) Comparative proteomic analysis of *Lactobacillus nagelii* and *Lactobacillus hordei* in the presence of *Saccharomyces cerevisiae* isolated from water kefir. Front Microbiol 10:325

Behr J, Geissler AJ, Schmid J, Zehe A, Vogel RF (2016) The identification of novel Diagnostic Marker Genes for the detection of beer spoiling *Pediococcus damnosus* strains using the BlAst Diagnostic Gene findEr. PLoS One 11:e0152747. doi:10.1371/journal.pone.0152747

Bensing BA, Seepersaud R, Yen YT, Sullam PM (2014) Selective transport by SecA2: An expanding family of customized motor proteins. Biochim Biophys Acta 1843:1674-1686. doi:https://doi.org/10.1016/j.bbamcr.2013.10.019

Bensing BA, Siboo IR, Sullam PM (2007) Glycine residues in the hydrophobic core of the GspB signal sequence route export toward the accessory Sec pathway. J Bacteriol 189:3846-3854. doi:10.1128/jb.00027-07

Biavati B, Mattarelli P (2006) The family *Bifidobacteriaceae*. In: The prokaryotes. Springer, pp 322-382

Booten K, De SJ, Frippiat A (1998) Fructan- and/or polydextrose-containing dairy powders, process for preparing same and their use

Bottacini F, Motherway MOC, Kuczynski J, O'Connell KJ, Serafini F, Duranti S, Milani C et al. (2014) Comparative genomics of the *Bifidobacterium breve* taxon. BMC Genomics 15:170

Bovey F (1959) Enzymatic polymerization. I. Molecular weight and branching during the formation of dextran. J Polym Sci 35:167-182

Buchholz K, Monsan P (2001) Dextransucrases. In: R. WJ (ed) Handbook of food enzymology. Marcel Dekker, New York,

Buckenhüskes HJ (1993) Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. FEMS Microbiol Rev 12:253-271

Bunesova V, Musilova S, Geigerova M, Pechar R, Rada V (2015) Comparison of mupirocin-based media for selective enumeration of bifidobacteria in probiotic supplements. J Microbiol Methods 109:106-109. doi:https://doi.org/10.1016/j.mimet.2014.12.016

Burger J, Kirchner M, Bramanti B, Haak W, Thomas MG (2007) Absence of the lactase-persistence-associated allele in early Neolithic Europeans. Proceedings of the National Academy of Sciences 104:3736-3741. doi:10.1073/pnas.0607187104

Cabiscol Català E, Tamarit Sumalla J, Ros Salvador J (2000) Oxidative stress in bacteria and protein damage by reactive oxygen species. Int Microbiol 3:3-8

Campbell-Platt G (1994) Fermented foods — a world perspective. Food Res Int 27:253-257. doi:https://doi.org/10.1016/0963-9969(94)90093-0

Cao Y, Dickinson E, Wedlock DJ (1990) Creaming and flocculation in emulsions containing polysaccharide. Food Hydrocoll 4:185-195. doi:https://doi.org/10.1016/S0268-005X(09)80151-3

Cerning J (1990) Exocellular polysaccharides produced by lactic acid bacteria. FEMS Microbiol Lett 87:113-130. doi:https://doi.org/10.1016/0378-1097(90)90701-Q

Cogan TM, Jordan KN (1994) Metabolism of *Leuconostoc* bacteria. J Dairy Sci 77:2704-2717. doi:https://doi.org/10.3168/jds.S0022-0302(94)77213-1

Cotter PD, Hill C (2003) Surviving the acid test: responses of gram-positive bacteria to low pH. Microbiol Mol Biol Rev 67:429-453. doi:10.1128/mmbr.67.3.429-453.2003

Cronin M, Zomer A, Fitzgerald G, van Sinderen D (2012) Identification of iron-regulated genes of *Bifidobacterium breve* UCC2003 as a basis for controlled gene expression. Bioengineered 3:159-169. doi:10.4161/bbug.18985

Csonka LN, Hanson AD (1991) Prokaryotic osmoregulation: genetics and physiology. Annu Rev Microbiol 45:569-606

De Man J, Rogosa d, Sharpe ME (1960) A medium for the cultivation of lactobacilli. J Appl Bacteriol 23:130-135

De Vuyst L, Degeest B (1999) Heteropolysaccharides from lactic acid bacteria. FEMS Microbiol Rev 23:153-177

De Vuyst L, Vandamme EJ (1994) Antimicrobial potential of lactic acid bacteria. In: Bacteriocins of lactic acid bacteria. Springer, pp 91-142

Decock P, Cappelle S (2005) Bread technology and sourdough technology. vol 16. doi:10.1016/j.tifs.2004.04.012

Deguchi Y, Morishita T, Mutai M (1985) Comparative studies on synthesis of water-soluble vitamins among human species of Bifidobacteria Agric Biol Chem 49:13-19. doi:10.1080/00021369.1985.10866683

Delcenserie V, Gavini F, Beerens H, Tresse O, Franssen C, Daube G (2007) Description of a new species, *Bifidobacterium crudilactis* sp. nov., isolated from raw milk and raw milk cheeses. Syst Appl Microbiol 30:381-389. doi:https://doi.org/10.1016/j.syapm.2007.01.004

Dhanashree, Rajashekharan S, Krishnaswamy B, Kammara R (2017) Bifid shape is intrinsic to *Bifidobacterium adolescentis*. Front Microbiol 8. doi:10.3389/fmicb.2017.00478

Dickinson E (1994) Colloidal aspects of beverages. Food Chem 51:343-347. doi:https://doi.org/10.1016/0308-8146(94)90184-8

Doleyres Y, Schaub L, Lacroix C (2005) Comparison of the functionality of exopolysaccharides produced *in situ* or added as bioingredients on yogurt properties. J Dairy Sci 88:4146-4156. doi:https://doi.org/10.3168/jds.S0022-0302(05)73100-3

Douglas HC, Cruess WV (1936) A *Lactobacillus* from california wine: *Lactobacillus hilgardii*. J Food Sci 1:113-119. doi:10.1111/j.1365-2621.1936.tb17774.x

du Toit M, Engelbrecht L, Lerm E, Krieger-Weber S (2011) *Lactobacillus*: the next generation of malolactic fermentation starter cultures—an overview. Food and Bioprocess Technology 4:876-906. doi:10.1007/s11947-010-0448-8

Durso L, Hutkins R (2003) Starter cultures. In: Caballero B (ed) Encyclopedia of food sciences and nutrition (Second Edition). Academic Press, Oxford, pp 5583-5593. doi:https://doi.org/10.1016/B0-12-227055-X/01146-9

Eckel V, Vogel R (2020) Genomic and physiological insights into the lifestyle of Bifidobacterium species from water kefir. Arch Microbiol

Eckel VPL, Vogel RF, Jakob F (2019a) In situ production and characterization of cloud forming dextrans in fruit-juices. Int J Food Microbiol 306:108261. doi:https://doi.org/10.1016/j.ijfoodmicro.2019.108261

Eckel VPL, Ziegler L-M, Vogel RF, Ehrmann M (2019b) *Bifidobacterium tibiigranuli* sp. nov. isolated from homemade water kefir. Int J Syst Evol Microbiol. doi:https://doi.org/10.1099/ijsem.0.003936

Ehrmann MA, Müller MRA, Vogel RF (2003) Molecular analysis of sourdough reveals *Lactobacillus mindensis* sp. nov. Int J Syst Evol Microbiol 53:7-13. doi:doi:10.1099/ijs.0.02202-0

Fels L, Jakob F, Vogel RF, Wefers D (2018) Structural characterization of the exopolysaccharides from water kefir. Carbohydr Polym 189:296-303. doi:https://doi.org/10.1016/j.carbpol.2018.02.037

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783-791

Ferrario C, Duranti S, Milani C, Mancabelli L, Lugli GA, Turroni F, Mangifesta M et al. (2015) Exploring amino acid auxotrophy in *Bifidobacterium bifidum* PRL2010. Front Microbiol 6. doi:10.3389/fmicb.2015.01331

Fleming H, McFeeters R (1981) Use of microbial cultures: vegetable products. Food Technol 35:84-87

Franzetti L, Galli A, Pagani M, Noni Id (1998) Microbiological and chemical investigations on sugar kefir drink. Annali di Microbiologia ed Enzimologia (Italy)

Fraunhofer ME, Geissler AJ, Wefers D, Bunzel M, Jakob F, Vogel RF (2018) Characterization of β-glucan formation by *Lactobacillus brevis* TMW 1.2112 isolated from slimy spoiled beer. Int J Biol Macromol 107:874-881. doi:https://doi.org/10.1016/j.ijbiomac.2017.09.063

Fritz W (1949) Bemerkungen zum Auslaufbecher DIN 53211. Chem Ing Tech 21:103-105. doi:doi:10.1002/cite.330210505

Gaby W, Hadley C (1957) Practical laboratory test for the identification of *Pseudomonas aeruginosa*. J Bacteriol 74:356

Gardy JL, Laird MR, Chen F, Rey S, Walsh CJ, Ester M, Brinkman FSL (2004) PSORTb v.2.0: Expanded prediction of bacterial protein subcellular localization and insights gained from comparative proteome analysis. Bioinformatics 21:617-623. doi:10.1093/bioinformatics/bti057

Giaever H, Styrvold OB, Kaasen I, Strøm A (1988) Biochemical and genetic characterization of osmoregulatory trehalose synthesis in *Escherichia coli*. J Bacteriol 170:2841-2849

Goldman BS, Gabbert KK, Kranz RG (1996) The temperature-sensitive growth and survival phenotypes of *Escherichia coli* cydDC and cydAB strains are due to deficiencies in cytochrome bd and are corrected by exogenous catalase and reducing agents. J Bacteriol 178:6348-6351. doi:10.1128/jb.178.21.6348-6351.1996

Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, Tiedje JM (2007) DNA–DNA hybridization values and their relationship to whole-genome sequence similarities. Int J Syst Evol Microbiol 57:81-91. doi:doi:10.1099/ijs.0.64483-0

Gram HC (1884) Ueber die isolirte Färbung der Schizomyceten: in Schnitt-und Trockenpräparaten. Fortschr Med 2:185-189

Granato D, Branco GF, Nazzaro F, Cruz AG, Faria JAF (2010) Functional foods and nondairy probiotic food development: Trends, concepts, and products. Comprehensive Reviews in Food Science and Food Safety 9:292-302. doi:10.1111/j.1541-4337.2010.00110.x

Gulitz A, Stadie J, Ehrmann MA, Ludwig W, Vogel RF (2013) Comparative phylobiomic analysis of the bacterial community of water kefir by 16S rRNA gene amplicon sequencing and ARDRA analysis. J Appl Microbiol 114:1082-1091. doi:doi:10.1111/jam.12124

Gulitz A, Stadie J, Wenning M, Ehrmann MA, Vogel RF (2011) The microbial diversity of water kefir. Int J Food Microbiol 151:284-288

Gulitz AJ (2013) Analysis of the diversity of water kefir microbiota by culture-dependent and -independent approaches. Technische Universitaet Muenchen, Munich, Germany

Guyonnet D, Chassany O, Ducrotte P, Picard C, Mouret M, Mercier C-H, Matuchansky C (2007) Effect of a fermented milk containing *Bifidobacterium animalis* DN-173 010 on the health-related quality of life and symptoms in irritable bowel syndrome in adults in primary care: a multicentre, randomized, double-blind, controlled trial. Aliment Pharmacol Ther 26:475-486. doi:doi:10.1111/j.1365-2036.2007.03362.x

Haft DH, DiCuccio M, Badretdin A, Brover V, Chetvernin V, O'Neill K, Li W et al. (2018) RefSeq: an update on prokaryotic genome annotation and curation. Nucleic Acids Res 46:D851-d860. doi:10.1093/nar/gkx1068

Hamada S, Slade HD (1980) Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol Rev 44:331

Hammes WP, Gänzle M (1998) Sourdough breads and related products. In: Microbiology of fermented foods. Springer, pp 199-216

Han YW (1990) Microbial levan. In: Advances in applied microbiology, vol 35. Elsevier, pp 171-194

Hansen EB (2002) Commercial bacterial starter cultures for fermented foods of the future. Int J Food Microbiol 78:119-131. doi:https://doi.org/10.1016/S0168-1605(02)00238-6

Hansen EC (1881) Undersøgelser over Alkoholgjoersvampenes Fysiologi og Morfologi. 1. Om *Saccharomyces apiculatus* og dens Kredsløb i den frie Natur. Meddr Carlsberg Lab 1:293-327

Holyoake LV, Hunt S, Sanguinetti G, Cook GM, Howard MJ, Rowe ML, Poole RK et al. (2016) CydDC-mediated reductant export in *Escherichia coli* controls the transcriptional wiring of

energy metabolism and combats nitrosative stress. Biochem J 473:693-701. doi:10.1042/BJ20150536

Hornsey IS (2003) A history of beer and brewing. vol 34. Royal Society of Chemistry,

Hugenholtz P (1998) The genera of lactic acid bacteria. Blackie Academic & Professional, London, UK.

Huptas C, Scherer S, Wenning M (2016) Optimized Illumina PCR-free library preparation for bacterial whole genome sequencing and analysis of factors influencing de novo assembly. BMC Res Notes 9:269. doi:10.1186/s13104-016-2072-9

Itan Y, Powell A, Beaumont MA, Burger J, Thomas MG (2009) The origins of lactase persistence in europe. PLoS Comp Biol 5:e1000491. doi:10.1371/journal.pcbi.1000491

Jakob F (2014) Novel fructans from acetic acid bacteria. Technische Universität München,

Jakob F, Meißner D, Vogel RF (2012a) Comparison of novel GH 68 levansucrases of levan-overproducing *Gluconobacter* species. Acetic Acid Bacteria 1:e2-e2

Jakob F, Pfaff A, Novoa-Carballal R, Rübsam H, Becker T, Vogel RF (2013) Structural analysis of fructans produced by acetic acid bacteria reveals a relation to hydrocolloid function. Carbohydr Polym 92:1234-1242. doi:https://doi.org/10.1016/j.carbpol.2012.10.054

Jakob F, Steger S, Vogel RF (2012b) Influence of novel fructans produced by selected acetic acid bacteria on the volume and texture of wheat breads. Eur Food Res Technol 234:493-499

Jian W, Zhu L, Dong X (2001) New approach to phylogenetic analysis of the genus *Bifidobacterium* based on partial HSP60 gene sequences. Int J Syst Evol Microbiol 51:1633-1638. doi:doi:10.1099/00207713-51-5-1633

Johnson AJ (2016) Artisanal food microbiology. Nature Microbiology 1:16039. doi:10.1038/nmicrobiol.2016.39

Jungersen M, Wind A, Johansen E, Christensen JE, Stuer-Lauridsen B, Eskesen D (2014) The science behind the probiotic strain *Bifidobacterium animalis* subsp. *lactis* BB-12®. Microorganisms 2:92-110

Kaditzky S, Vogel RF (2008) Optimization of exopolysaccharide yields in sourdoughs fermented by lactobacilli. Eur Food Res Technol 228:291. doi:10.1007/s00217-008-0934-7

Kandler O (1983) Carbohydrate metabolism in lactic acid bacteria. Antonie Van Leeuwenhoek 49:209-224

Kebler LF (1921) California bees. J Am Pharm Assoc Am Pharm Assoc 10:939-943

Kern CC, Vogel RF, Behr J (2014) Differentiation of *Lactobacillus brevis* strains using Matrix-Assisted-Laser-Desorption-Ionization-Time-of-Flight Mass Spectrometry with respect to their beer spoilage potential. Food Microbiol 40:18-24. doi:https://doi.org/10.1016/j.fm.2013.11.015

Kim BJ, Kim H-Y, Yun Y-J, Kim B-J, Kook Y-H (2010) Differentiation of *Bifidobacterium* species using partial RNA polymerase β-subunit (rpoB) gene sequences. Int J Syst Evol Microbiol 60:2697-2704. doi:doi:10.1099/ijs.0.020339-0

Kim JK, Shin S-Y, Moon JS, Li L, Cho SK, Kim T-J, Han NS (2015) Isolation of dextran-hydrolyzing intestinal bacteria and characterization of their dextranolytic activities. Biopolymers 103:321-327. doi:10.1002/bip.22615

Kim M, Oh H-S, Park S-C, Chun J (2014) Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. Int J Syst Evol Microbiol 64:346-351. doi:doi:10.1099/ijs.0.059774-0

Klein G, Pack A, Bonaparte C, Reuter G (1998) Taxonomy and physiology of probiotic lactic acid bacteria. Int J Food Microbiol 41:103-125. doi:https://doi.org/10.1016/S0168-1605(98)00049-X

Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870-1874

Kuykendall LD, Roy MA, Neil JJ, Devine TE (1988) Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. Int J Syst Evol Microbiol 38:358-361. doi:https://doi.org/10.1099/00207713-38-4-358

Lane D (1991) 16S/23S rRNA sequencing. Nucleic acid techniques in bacterial systematics:115-175

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F et al. (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947-2948. doi:10.1093/bioinformatics/btm404

Larsen PI, Sydnes LK, Landfald B, Strøm AR (1987) Osmoregulation in *Escherichia coli* by accumulation of organic osmolytes: betaines, glutamic acid, and trehalose. Arch Microbiol 147:1-7. doi:10.1007/BF00492896

Laureys D (2017) Microbial species diversity, community dynamics, substrate consumption, and metabolite production during water kefir fermentation. Ghent University,

Laureys D, Aerts M, Vandamme P, De Vuyst L (2018) Oxygen and diverse nutrients influence the water kefir fermentation process. Food Microbiol 73:351-361. doi:https://doi.org/10.1016/j.fm.2018.02.007

Laureys D, Cnockaert M, De Vuyst L, Vandamme P (2016) *Bifidobacterium aquikefiri* sp. nov., isolated from water kefir. Int J Syst Evol Microbiol 66:1281-1286. doi:doi:10.1099/ijsem.0.000877

Laureys D, De Vuyst L (2014) Microbial species diversity, community dynamics, and metabolite kinetics of water kefir fermentation. Appl Environ Microbiol 80:2564-2572. doi:10.1128/aem.03978-13

Laureys D, De Vuyst L (2017) The water kefir grain inoculum determines the characteristics of the resulting water kefir fermentation process. J Appl Microbiol 122:719-732. doi:doi:10.1111/jam.13370

Laureys D, Van Jean A, Dumont J, De Vuyst L (2017) Investigation of the instability and low water kefir grain growth during an industrial water kefir fermentation process. Appl Microbiol Biotechnol 101:2811-2819

Leahy SC, Higgins DG, Fitzgerald GF, van Sinderen D (2005) Getting better with bifidobacteria. J Appl Microbiol 98:1303-1315. doi:10.1111/j.1365-2672.2005.02600.x

LeBlanc JG, Milani C, de Giori GS, Sesma F, van Sinderen D, Ventura M (2013) Bacteria as vitamin suppliers to their host: a gut microbiota perspective. Curr Opin Biotechnol 24:160-168. doi:https://doi.org/10.1016/j.copbio.2012.08.005

Lee J-H, Karamychev VN, Kozyavkin SA, Mills D, Pavlov AR, Pavlova NV, Polouchine NN et al. (2008) Comparative genomic analysis of the gut bacterium *Bifidobacterium longum* reveals loci susceptible to deletion during pure culture growth. BMC Genomics 9:247-247. doi:10.1186/1471-2164-9-247

Lee J-H, O'Sullivan DJ (2010) Genomic insights into Bifidobacteria. Microbiol Mol Biol Rev 74:378-416. doi:10.1128/mmbr.00004-10

Lee LW, Cheong MW, Curran P, Yu B, Liu SQ (2015) Coffee fermentation and flavor – An intricate and delicate relationship. Food Chem 185:182-191. doi:https://doi.org/10.1016/j.foodchem.2015.03.124

Leemhuis H, Pijning T, Dobruchowska JM, van Leeuwen SS, Kralj S, Dijkstra BW, Dijkhuizen L (2013) Glucansucrases: Three-dimensional structures, reactions, mechanism, α-glucan analysis and their implications in biotechnology and food applications. J Biotechnol 163:250-272. doi:https://doi.org/10.1016/j.jbiotec.2012.06.037

LeGall J, Prickril BC, Moura I, Xavier AV, Moura JJG, Huynh Boi H (1988) Isolation and characterization of rubrerythrin, a non-heme iron protein from *Desulfovibrio vulgaris* that contains rubredoxin centers and a hemerythrin-like binuclear iron cluster. Biochemistry 27:1636-1642. doi:10.1021/bi00405a037

Legras J-L, Merdinoglu D, Cornuet J-M, Karst F (2007) Bread, beer and wine: Saccharomyces cerevisiae diversity reflects human history. Mol Ecol 16:2091-2102. doi:10.1111/j.1365-294X.2007.03266.x

Lehmann Y, Meile L, Teuber M (1996) Rubrerythrin from *Clostridium perfringens*: cloning of the gene, purification of the protein, and characterization of its superoxide dismutase function. J Bacteriol 178:7152-7158. doi:10.1128/jb.178.24.7152-7158.1996

Leroy F, Aymerich T, Champomier-Vergès M-C, Cocolin L, De Vuyst L, Flores M, Leroi F et al. (2018) Fermented meats (and the symptomatic case of the Flemish food pyramid): Are we heading towards the vilification of a valuable food group? Int J Food Microbiol 274:67-70. doi:https://doi.org/10.1016/j.ijfoodmicro.2018.02.006

Linke C, Drusch S (2016) Turbidity in oil-in-water-emulsions — Key factors and visual perception. Food Res Int 89:202-210. doi:https://doi.org/10.1016/j.foodres.2016.07.019

Liu L, Wang J, Rosenberg D, Zhao H, Lengyel G, Nadel D (2018) Fermented beverage and food storage in 13,000 y-old stone mortars at Raqefet Cave, Israel: Investigating Natufian ritual feasting. Journal of Archaeological Science: Reports 21:783-793. doi:https://doi.org/10.1016/j.jasrep.2018.08.008

Liu S, Ren F, Zhao L, Jiang L, Hao Y, Jin J, Zhang M et al. (2015) Starch and starch hydrolysates are favorable carbon sources for Bifidobacteria in the human gut. BMC Microbiol 15:54. doi:10.1186/s12866-015-0362-3

Liu Y, Bauer SC, Imlay JA (2011) The YaaA protein of the *Escherichia coli* OxyR regulon lessens hydrogen peroxide toxicity by diminishing the amount of intracellular unincorporated iron. J Bacteriol 193:2186-2196

Ljungh A, Wadstrom T (2006) Lactic acid bacteria as probiotics. Curr Issues Intestinal Microbiol 7:73-90

Lo R, Turner MS, Barry DG, Sreekumar R, Walsh TP, Giffard PM (2009) Cystathionine γ-lyase is a component of cystine-mediated oxidative defense in *Lactobacillus reuteri* BR11. J Bacteriol 191:1827-1837. doi:10.1128/jb.01553-08

Lonvaud-Funel A (1999) Lactic acid bacteria in the quality improvement and depreciation of wine. In: Konings WN, Kuipers OP, In 't Veld JHJH (eds) Lactic Acid Bacteria: Genetics, Metabolism and Applications: Proceedings of the Sixth Symposium on lactic acid bacteria: genetics, metabolism and applications, 19–23 September 1999, Veldhoven, The Netherlands. Springer Netherlands, Dordrecht, pp 317-331. doi:10.1007/978-94-017-2027-4_16

Lücke F-K (1998) Fermented sausages. In: Microbiology of fermented foods. Springer, pp 441-483

Lugli GA, Milani C, Duranti S, Mancabelli L, Mangifesta M, Turroni F, Viappiani A et al. (2018) Tracking the taxonomy of the genus *Bifidobacterium* based on a phylogenomic approach. Appl Environ Microbiol 84:e02249-02217

Lutz L (1899a) Recherches biologiques sur la constitution du tibi. Bulletin trimestrel de la societé mycologique de france

Lutz L (1899b) Recherches biologiques sur la constitution du tibi. Bul Trim Soc Myc Fr 15:68

MacFaddin J (1972) Biochemical Tests for Identification of Medical Bacteria Baltimore. MD: Williams and Wilkins Company

Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, Pavlov A et al. (2006a) Comparative genomics of the lactic acid bacteria. Proceedings of the National Academy of Sciences 103:15611-15616

Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, Pavlov A et al. (2006b) Comparative genomics of the lactic acid bacteria. Proceedings of the National Academy of Sciences 103:15611-15616. doi:10.1073/pnas.0607117103

Malbaša R, Lončar E, Djurić M, Došenović I (2008) Effect of sucrose concentration on the products of Kombucha fermentation on molasses. Food Chem 108:926-932. doi:https://doi.org/10.1016/j.foodchem.2007.11.069

Marsh AJ, Hill C, Ross RP, Cotter PD (2014) Fermented beverages with health-promoting potential: Past and future perspectives. Trends Food Sci Technol 38:113-124. doi:https://doi.org/10.1016/j.tifs.2014.05.002

Marsh AJ, O'Sullivan O, Hill C, Ross RP, Cotter PD (2013) Sequence-based analysis of the microbial composition of water kefir from multiple sources. FEMS Microbiol Lett 348:79-85. doi:10.1111/1574-6968.12248

Martí-Arbona R, Xu C, Steele S, Weeks A, Kuty GF, Seibert CM, Raushel FM (2006) Annotating enzymes of unknown function: N-formimino-l-glutamate deiminase is a member of the amidohydrolase superfamily. Biochemistry 45:1997-2005. doi:10.1021/bi0525425

Martínez-Viedma P, Abriouel H, Omar NB, Valdivia E, López RL, Gálvez A (2008) Inactivation of exopolysaccharide and 3-hydroxypropionaldehyde-producing lactic acid bacteria in apple juice and apple cider by enterocin AS-48. Food Chem Toxicol 46:1143-1151. doi:https://doi.org/10.1016/j.fct.2007.11.010

Mattarelli P, Biavati B, Holzapfel WH, Wood BJ (2017) The Bifidobacteria and related organisms: biology, taxonomy, applications. Academic Press,

Mattarelli P, Holzapfel W, Franz CMAP, Endo A, Felis GE, Hammes W, Pot B et al. (2014) Recommended minimal standards for description of new taxa of the genera *Bifidobacterium*, *Lactobacillus* and related genera. Int J Syst Evol Microbiol 64:1434-1451. doi:doi:10.1099/ijs.0.060046-0

Mäyrä-Mäukinen A, Mnninen M, Gyllenberg H (1983) The adherence of lactic acid bacteria to the columnar epithelial cells of pigs and calves. J Appl Bacteriol 55:241-245. doi:10.1111/j.1365-2672.1983.tb01321.x

McClements DJ (2005) Food emulsions : principles, practices, and techniques. CRC series in contemporary food science, 2nd edn. CRC Press, Boca Raton

McCleskey CS, Faville LW, Barnett RO (1947) Characteristics of *Leuconostoc mesenteroides* from cane juice. J Bacteriol 54:697-708

McGovern PE (2013) Ancient wine: the search for the origins of viniculture. Princeton University Press,

McIlvaine T (1921) A buffer solution for colorimetric comparison. J Biol Chem 49:183-186

Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M (2013) Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bioinformatics 14:60. doi:10.1186/1471-2105-14-60

Meier-Kolthoff JP, Klenk H-P, Göker M (2014) Taxonomic use of DNA G+C content and DNA–DNA hybridization in the genomic age. Int J Syst Evol Microbiol 64:352-356. doi:doi:10.1099/ijs.0.056994-0

Meile L, Ludwig W, Rueger U, Gut C, Kaufmann P, Dasen G, Wenger S et al. (1997) *Bifidobacterium lactis* sp. nov., a Moderately Oxygen Tolerant Species Isolated from Fermented Milk. Syst Appl Microbiol 20:57-64. doi:https://doi.org/10.1016/S0723-2020(97)80048-3

Michelini S, Modesto M, Pisi AM, Filippini G, Sandri C, Spiezio C, Biavati B et al. (2016) *Bifidobacterium eulemuris* sp. nov., isolated from faeces of black lemurs (*Eulemur macaco*). Int J Syst Evol Microbiol 66:1567-1576. doi:doi:10.1099/ijsem.0.000924

Milani C, Lugli GA, Duranti S, Turroni F, Bottacini F, Mangifesta M, Sanchez B et al. (2014) Genomic Encyclopedia of Type Strains of the Genus *Bifidobacterium*. Appl Environ Microbiol 80:6290-6302. doi:10.1128/aem.02308-14

Miller LT (1982) Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxy acids. Journal of Clinical Microbiology 16:584-586

Miranda RO, Carvalho AFd, Nero LA (2014) Development of a selective culture medium for bifidobacteria, Raffinose-Propionate Lithium Mupirocin (RP-MUP) and assessment of its usage with Petrifilm™ Aerobic Count plates. Food Microbiol 39:96-102. doi:https://doi.org/10.1016/j.fm.2013.11.010

Mirhosseini H, Tan CP, Hamid NSA, Yusof S (2008) Effect of Arabic gum, xanthan gum and orange oil contents on ζ-potential, conductivity, stability, size index and pH of orange beverage emulsion. Colloids Surf Physicochem Eng Aspects 315:47-56. doi:https://doi.org/10.1016/j.colsurfa.2007.07.007

Mitsuoka T (1990) Bifidobacteria and their role in human health. J Ind Microbiol 6:263-267. doi:10.1007/bf01575871

Modesto M, Michelini S, Stefanini I, Sandri C, Spiezio C, Pisi A, Filippini G et al. (2015) *Bifidobacterium lemurum* sp. nov., from faeces of the ring-tailed lemur (*Lemur catta*). Int J Syst Evol Microbiol 65:1726-1734. doi:doi:10.1099/ijs.0.000162

Monsan P, Bozonnet S, Albenne C, Joucla G, Willemot R-M, Remaud-Siméon M (2001) Homopolysaccharides from lactic acid bacteria. Int Dairy J 11:675-685. doi:https://doi.org/10.1016/S0958-6946(01)00113-3

Moore T, Sparling PF (1996) Interruption of the gpxA gene increases the sensitivity of *Neisseria meningitidis* to paraquat. J Bacteriol 178:4301-4305

Moroni AV, Dal Bello F, Arendt EK (2009) Sourdough in gluten-free bread-making: An ancient technology to solve a novel issue? Food Microbiol 26:676-684. doi:https://doi.org/10.1016/j.fm.2009.07.001

Mousavi ZE, Mousavi SM, Razavi SH, Emam-Djomeh Z, Kiani H (2011) Fermentation of pomegranate juice by probiotic lactic acid bacteria. World J Microbiol Biotechnol 27:123-128. doi:10.1007/s11274-010-0436-1

Naessens M, Cerdobbel A, Soetaert W, Vandamme EJ (2005) *Leuconostoc* dextransucrase and dextran: production, properties and applications. J Chem Technol Biotechnol 80:845-860. doi:doi:10.1002/jctb.1322

Nilsson L (2013) Separation and characterization of food macromolecules using field-flow fractionation: A review. Food Hydrocoll 30:1-11. doi:https://doi.org/10.1016/j.foodhyd.2012.04.007

Noda H, Akasaka N, Ohsugi M (1994) Biotin Production by Bifidobacteria. vol 40. doi:10.3177/jnsv.40.181

Orban JI, Patterson JA (2000) Modification of the phosphoketolase assay for rapid identification of bifidobacteria. J Microbiol Methods 40:221-224. doi:https://doi.org/10.1016/S0167-7012(00)00133-0

Orla-Jensen S (1919) The lactic acid bacteria. Host & Son, Copenhagen, Denmark.

Otts D, Day DF (1988) Dextransucrase secretion in *Leuconostoc mesenteroides* depends on the presence of a transmembrane proton gradient. J Bacteriol 170:5006-5011

Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA et al. (2014) The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res 42:D206-D214. doi:10.1093/nar/gkt1226

Panesar PS, Marwaha SS, Kennedy JF (2006) Zymomonas mobilis: an alternative ethanol producer. J Chem Technol Biotechnol 81:623-635. doi:10.1002/jctb.1448

Parche S, Beleut M, Rezzonico E, Jacobs D, Arigoni F, Titgemeyer F, Jankovic I (2006) Lactose-over-glucose preference in *Bifidobacterium longum* NCC2705: glcP, encoding a glucose transporter, is subject to lactose repression. J Bacteriol 188:1260-1265. doi:10.1128/JB.188.4.1260-1265.2006

Pasteur L (1876) Etudes sur la bière, ses maladies avec une théorie nouvelle de la fermentation. Gauthier-Villars,

Paul Ross R, Morgan S, Hill C (2002) Preservation and fermentation: past, present and future. Int J Food Microbiol 79:3-16. doi:https://doi.org/10.1016/S0168-1605(02)00174-5

Pederson CS, Albury MN (1969) Bulletin number 824: The sauerkraut fermentation.

Perdigon G, Alvarez S, Macias MEND, Roux ME, Holgado APdR (1990) The Oral Administration of Lactic Acid Bacteria Increase the Mucosal Intestinal Immunity in Response to Enteropathogens. J Food Prot 53:404-410. doi:10.4315/0362-028x-53.5.404

Pericone CD, Park S, Imlay JA, Weiser JN (2003) Factors contributing to hydrogen peroxide resistance in *Streptococcus pneumoniae* include pyruvate oxidase (SpxB) and avoidance of the toxic effects of the Fenton reaction. J Bacteriol 185:6815-6825

Petry S, Furlan S, Crepeau MJ, Cerning J, Desmazeaud M (2000) Factors affecting exocellular polysaccharide production by *Lactobacillus delbrueckii* subsp. *bulgaricus* grown in a chemically defined medium. Appl Environ Microbiol 66:3427-3431

Pidoux M (1989) The microbial flora of sugary kefir grain (the gingerbeer plant): biosynthesis of the grain from *Lactobacillus hilgardii* producing a polysaccharide gel. World J Microbiol Biotechnol 5:223-238

Pidoux M, De Ruiter G, Brooker B, Colquhoun I, Morris V (1990) Microscopic and chemical studies of a gelling polysaccharide from *Lactobacillus hilgardii*. Carbohydr Polym 13:351-362

Pittman MS, Robinson HC, Poole RK (2005) A bacterial glutathione transporter (*Escherichia coli* CydDC) exports reductant to the periplasm. J Biol Chem 280:32254-32261. doi:10.1074/jbc.M503075200

Poelmans E, Swinnen JF (2011) A brief economic history of beer. The economics of beer:3-28

Pokusaeva K, Fitzgerald GF, van Sinderen D (2011) Carbohydrate metabolism in Bifidobacteria. Genes Nutr 6:285-306. doi:10.1007/s12263-010-0206-6

Pophaly SD, Singh R, Pophaly SD, Kaushik JK, Tomar SK (2012) Current status and emerging role of glutathione in food grade lactic acid bacteria. Microbial cell factories 11:114-114. doi:10.1186/1475-2859-11-114

Prechtl RM, Wefers D, Jakob F, Vogel RF (2018a) Cold and salt stress modulate amount, molecular and macromolecular structure of a *Lactobacillus sakei* dextran. Food Hydrocoll 82:73-81. doi:https://doi.org/10.1016/j.foodhyd.2018.04.003

Prechtl RM, Wefers D, Jakob F, Vogel RF (2018b) Structural characterization of the surface-associated heteropolysaccharide of *Lactobacillus plantarum* TMW 1.1478 and genetic analysis of its putative biosynthesis cluster. Carbohydr Polym 202:236-245. doi:https://doi.org/10.1016/j.carbpol.2018.08.115

Reddy NR, Pierson MD (1994) Reduction in antinutritional and toxic components in plant foods by fermentation Food Res Int 27:281-290. doi:https://doi.org/10.1016/0963-9969(94)90096-5

Reid SJ, Abratt VR (2005) Sucrose utilisation in bacteria: genetic organisation and regulation. Appl Microbiol Biotechnol 67:312-321

Richter M, Rosselló-Móra R, Oliver Glöckner F, Peplies J (2016) JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. Bioinformatics 32:929-931. doi:10.1093/bioinformatics/btv681

Rimada PS, Abraham AG (2003) Comparative study of different methodologies to determine the exopolysaccharide produced by kefir grains in milk and whey. Lait 83:79-87

Rimaux T, Vrancken G, Pothakos V, Maes D, De Vuyst L, Leroy F (2011) The kinetics of the arginine deiminase pathway in the meat starter culture *Lactobacillus sakei* CTC 494 are pH-dependent. Food Microbiol 28:597-604. doi:https://doi.org/10.1016/j.fm.2010.11.016

Rouse S, Canchaya C, van Sinderen D (2008) *Lactobacillus hordei* sp. nov., a bacteriocinogenic strain isolated from malted barley. Int J Syst Evol Microbiol 58:2013-2017. doi:doi:10.1099/ijs.0.65584-0

Roy D (2001) Media for the isolation and enumeration of bifidobacteria in dairy products. Int J Food Microbiol 69:167-182. doi:https://doi.org/10.1016/S0168-1605(01)00496-2

Ruas-Madiedo P, Hugenholtz J, Zoon P (2002a) An overview of the functionality of exopolysaccharides produced by lactic acid bacteria. Int Dairy J 12:163-171

Ruas-Madiedo P, Hugenholtz J, Zoon P (2002b) An overview of the functionality of exopolysaccharides produced by lactic acid bacteria. Int Dairy J 12:163-171. doi:https://doi.org/10.1016/S0958-6946(01)00160-1

Rühmkorf C, Bork C, Mischnick P, Rübsam H, Becker T, Vogel RF (2013) Identification of *Lactobacillus curvatus* TMW 1.624 dextransucrase and comparative characterization with *Lactobacillus reuteri* TMW 1.106 and *Lactobacillus animalis* TMW 1.971 dextransucrases. Food Microbiol 34:52-61. doi:https://doi.org/10.1016/j.fm.2012.11.002

Rühmkorf C, Jungkunz S, Wagner M, Vogel RF (2012a) Optimization of homoexopolysaccharide formation by lactobacilli in gluten-free sourdoughs. Food Microbiol 32:286-294. doi:https://doi.org/10.1016/j.fm.2012.07.002

Rühmkorf C, Rübsam H, Becker T, Bork C, Voiges K, Mischnick P, Brandt MJ et al. (2012b) Effect of structurally different microbial homoexopolysaccharides on the quality of gluten-free bread. Eur Food Res Technol 235:139-146. doi:10.1007/s00217-012-1746-3

Ryan P, Ross R, Fitzgerald G, Caplice N, Stanton C (2015) Sugar-coated: exopolysaccharide producing lactic acid bacteria for food and human health applications. Food Funct 6:679-693

Rzhetsky A, Nei M (1993) Theoretical foundation of the minimum-evolution method of phylogenetic inference. Mol Biol Evol 10:1073-1095. doi:10.1093/oxfordjournals.molbev.a040056

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425. doi:10.1093/oxfordjournals.molbev.a040454

Salque M, Bogucki PI, Pyzel J, Sobkowiak-Tabaka I, Grygiel R, Szmyt M, Evershed RP (2012) Earliest evidence for cheese making in the sixth millennium bc in northern Europe. Nature 493:522. doi:10.1038/nature11698

https://www.nature.com/articles/nature11698#supplementary-information

Salvetti E, Harris HMB, Felis GE, O'Toole PW (2018) Comparative genomics of the genus *Lactobacillus* reveals robust phylogroups that provide the basis for reclassification. Appl Environ Microbiol 84:e00993-00918. doi:10.1128/aem.00993-18

Scandalios JG (1993) Oxygen stress and superoxide dismutases. Plant Physiol 101:7

Scardovi V (1986a) Genus *Bifidobacterium*. Bergey's manual of systematic bacteriology:1418-1434

Scardovi V (1986b) Genus *Bifidobacterium*. In: Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore, pp 1418-1434

Schell MA, Karmirantzou M, Snel B, Vilanova D, Berger B, Pessi G, Zwahlen MC et al. (2002) The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. Proc Natl Acad Sci U S A 99:14422-14427. doi:10.1073/pnas.212527599

Schifferdecker AJ, Dashko S, Ishchuk OP, Piškur J (2014) The wine and beer yeast *Dekkera bruxellensis*. Yeast 31:323-332. doi:10.1002/yea.3023

Schleifer KH (1987) Recent changes in the taxonomy of lactic acid bacteria. FEMS Microbiol Rev 3:201-203. doi:10.1111/j.1574-6968.1987.tb02460.x

Schleifer KH, Kandler O (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev 36:407-477

Schumann P (2011) Peptidoglycan Structure. Methods in Microbiology 38:101-129. doi:10.1016/B978-0-12-387730-7.00005-X

Senti FR, Hellman NN, Ludwig NH, Babcock GE, Tobin R, Glass CA, Lamberts BL (1955) Viscosity, sedimentation, and light-scattering properties of fraction of an acid-hydrolyzed dextran. J Polym Sci 17:527-546. doi:doi:10.1002/pol.1955.120178605

Seymour FR, Knapp RD, Bishop SH (1976) Determination of the structure of dextran by 13C-nuclear magnetic resonance spectroscopy. Carbohydr Res 51:179-194. doi:https://doi.org/10.1016/S0008-6215(00)83325-X

Simpson PJ, Ross RP, Fitzgerald GF, Stanton C (2004) *Bifidobacterium psychraerophilum* sp. nov. and *Aeriscardovia aeriphila* gen. nov., sp. nov., isolated from a porcine caecum. Int J Syst Evol Microbiol 54:401-406. doi:doi:10.1099/ijs.0.02667-0

Smibert RM, Krieg NR (1981) Manual of Methods for General Bacteriology. American Society for Microbiology:409-443

Stadie J (2013) Metabolic activity and symbiotic interaction of bacteria and yeasts in water kefir. Dissertation, Technische Universität München, München, Germany

Stadie J, Gulitz A, Ehrmann MA, Vogel RF (2013) Metabolic activity and symbiotic interactions of lactic acid bacteria and yeasts isolated from water kefir. Food Microbiol 35:92-98. doi:https://doi.org/10.1016/j.fm.2013.03.009

Steinkraus KH, Hui Y, Meunier-Goddik L, Hansen A, Josephsen J, Nip W, Stanfeld P et al. (2004) Origin and history of food fermentations. FOOD SCIENCE AND TECHNOLOGY-NEW YORK-MARCEL DEKKER-:1-8

Stijn W, Sander W, Meehan CJ, Lebeer S (2019) A genome-based species taxonomy of the *Lactobacillus* genus complex. MSystems 4

Stiles ME, Holzapfel WH (1997) Lactic acid bacteria of foods and their current taxonomy. Int J Food Microbiol 36:1-29. doi:https://doi.org/10.1016/S0168-1605(96)01233-0

Stokes GG (1845) On the theories of the internal friction of fluids in motion. Trans Cam Phil Soc 8:287 - 305

Sutherland I (1979) Microbial exopolysaccharides. Trends Biochem Sci 4:55-59

Taherian AR, Fustier P, Ramaswamy HS (2007) Effects of added weighing agent and xanthan gum on stability and rheological properties of beverage cloudemulsions formulated using modified starch. J Food Process Eng 30:204-224. doi:doi:10.1111/j.1745-4530.2007.00109.x

Talwalkar A, Kailasapathy K (2004) Comparison of selective and differential media for the accurate enumeration of strains of *Lactobacillus acidophilus*, *Bifidobacterium* spp. and *Lactobacillus casei* complex from commercial yoghurts. Int Dairy J 14:143-149. doi:https://doi.org/10.1016/S0958-6946(03)00172-9

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10:512-526

Tan C-T (1998) Beverage flavor emulsion—A form of emulsion liquid membrane microencapsulation. In: Contis ET, Ho CT, Mussinan CJ, Parliment TH, Shahidi F, Spanier AM (eds) Developments in Food Science, vol 40. Elsevier, pp 29-42. doi:https://doi.org/10.1016/S0167-4501(98)80030-7

Tatusova T, DiCuccio M, Badretdin A, Chetvernin V, Nawrocki EP, Zaslavsky L, Lomsadze A et al. (2016) NCBI prokaryotic genome annotation pipeline. Nucleic Acids Res 44:6614-6624. doi:10.1093/nar/gkw569

Thiele C, Gänzle MG, Vogel RF (2002) Contribution of sourdough Lactobacilli, yeast, and cereal enzymes to the generation of amino acids in dough relevant for bread flavor. Cereal Chem 79:45-51. doi:10.1094/cchem.2002.79.1.45

Tissier H (1899) La reaction chromophile d'Escherich et le Bacterium coli. C R Soc Biol 51:943-945

Tissier H (1900) Recherches sur la flore intestinale des nourrissons (état normal et pathologique). Méd.--Paris,

Toft C, Andersson SGE (2010) Evolutionary microbial genomics: insights into bacterial host adaptation. Nat Rev Genet 11:465-475. doi:10.1038/nrg2798

Tonon T, Lonvaud-Funel A (2002) Arginine metabolism by wine Lactobacilli isolated from wine. Food Microbiol 19:451-461. doi:https://doi.org/10.1006/fmic.2002.0502

Trindade MI, Abratt VR, Reid SJ (2003) Induction of sucrose utilization genes from *Bifidobacterium lactis* by sucrose and raffinose. Appl Environ Microbiol 69:24-32. doi:10.1128/aem.69.1.24-32.2003

Ua-Arak T, Jakob F, Vogel RF (2017a) Fermentation pH modulates the size distributions and functional properties of *Gluconobacter albidus* TMW 2.1191 levan. Front Microbiol 8. doi:10.3389/fmicb.2017.00807

Ua-Arak T, Jakob F, Vogel RF (2017b) Influence of levan-producing acetic acid bacteria on buckwheat-sourdough breads. Food Microbiol 65:95-104. doi:https://doi.org/10.1016/j.fm.2017.02.002

Usbeck JC, Kern CC, Vogel RF, Behr J (2013) Optimization of experimental and modelling parameters for the differentiation of beverage spoiling yeasts by Matrix-Assisted-Laser-Desorption/Ionization–Time-of-Flight Mass Spectrometry (MALDI–TOF MS) in response to varying growth conditions. Food Microbiol 36:379-387. doi:https://doi.org/10.1016/j.fm.2013.07.004

Van Geel-Schutten G, Faber E, Smit E, Bonting K, Smith M, Ten Brink B, Kamerling J et al. (1999) Biochemical and structural characterization of the glucan and fructan

exopolysaccharides synthesized by the *Lactobacillus reuteri* wild-type strain and by mutant strains. Appl Environ Microbiol 65:3008-3014

van Hijum SAFT, Kralj S, Ozimek LK, Dijkhuizen L, van Geel-Schutten IGH (2006) Structure-function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. Microbiol Mol Biol Rev 70:157-176. doi:10.1128/mmbr.70.1.157-176.2006

Ventura M, van Sinderen D, Fitzgerald GF, Zink R (2004) Insights into the taxonomy, genetics and physiology of bifidobacteria. Antonie Van Leeuwenhoek 86:205-223. doi:10.1023/B:ANTO.0000047930.11029.ec

Verce M, De Vuyst L, Weckx S (2019) Shotgun metagenomics of a water kefir fermentation ecosystem reveals a novel *Oenococcus* species. Front Microbiol 10:479

Versalovic J, Schneider M, De Bruijn F, Lupski JR (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol Cell Biol 5:25-40

Vijaya Kumar B, Vijayendra SVN, Reddy OVS (2015) Trends in dairy and non-dairy probiotic products - a review. J Food Sci Technol 52:6112-6124. doi:10.1007/s13197-015-1795-2

Villarreal-Soto SA, Beaufort S, Bouajila J, Souchard J-P, Taillandier P (2018) Understanding Kombucha tea fermentation: A review. J Food Sci 83:580-588. doi:10.1111/1750-3841.14068

Vincent S, Brandt M, Cavadini C, Hammes WP, Neeser J-R, Waldbuesser S (2005) Levan-producing *Lactobacillus* strain and method of preparing human or pet food products using the same. Google Patents,

Vogel RF, Knorr R, Müller MR, Steudel U, Gänzle MG, Ehrmann MA (1999) Non-dairy lactic fermentations: the cereal world. Antonie Van Leeuwenhoek 76:403-411

Vriesekoop F, Krahl M, Hucker B, Menz G (2012) 125th Anniversary Review: Bacteria in brewing: The good, the bad and the ugly. Journal of the Institute of Brewing 118:335-345. doi:10.1002/jib.49

Vuyst L, Degeest B (1999) Heteropolysaccharides from lactic acid bacteria. FEMS Microbiol Rev 23:153-177. doi:doi:10.1111/j.1574-6976.1999.tb00395.x

Waldherr FW, Doll VM, Meissner D, Vogel RF (2010) Identification and characterization of a glucan-producing enzyme from *Lactobacillus hilgardii* TMW 1.828 involved in granule formation of water kefir. Food Microbiol 27:672-678. doi:10.1016/j.fm.2010.03.013

Walker GM, Stewart GG (2016) *Saccharomyces cerevisiae* in the production of fermented beverages. Beverages 2:30

Ward HM (1892) V. The ginger-beer plant, and the organisms composing it: a contribution to the study of fermentation-yeasts and bacteria. Philos Trans R Soc London, Ser B:125-197

Watanabe K, Makino H, Sasamoto M, Kudo Y, Fujimoto J, Demberel S (2009) *Bifidobacterium mongoliense* sp. nov., from airag, a traditional fermented mare's milk product from Mongolia. Int J Syst Evol Microbiol 59:1535-1540. doi:doi:10.1099/ijs.0.006247-0

Wei X, Guo Y, Shao C, Sun Z, Zhurina D, Liu D, Liu W et al. (2012) Fructose uptake in *Bifidobacterium longum* NCC2705 is mediated by an ATP-binding cassette transporter. J Biol Chem 287:357-367. doi:10.1074/jbc.M111.266213

Werning ML, Ibarburu I, Duenas MT, Irastorza ANA, Navas J, López P (2006) *Pediococcus parvulus* gtf gene encoding the GTF glycosyltransferase and its application for specific PCR detection of β-d-glucan–producing bacteria in foods and beverages. J Food Prot 69:161-169. doi:10.4315/0362-028X-69.1.161

Wibowo D, Eschenbruch R, Davis CR, Fleet GH, Lee TH (1985) Occurrence and growth of lactic acid bacteria in wine: A review. Am J Enol Viticult 36:302

Wolff IA, Watson PR, Sloan JW, Rist CE (1953) Controlled thermal depolymerization of dextran. Ind Eng Chem 45:755-759. doi:10.1021/ie50520a030

Wood BJ (2012) Microbiology of fermented foods. Springer Science & Business Media,

Xu D, Bechtner J, Behr J, Eisenbach L, Geißler AJ, Vogel RF (2019a) Lifestyle of *Lactobacillus hordei* isolated from water kefir based on genomic, proteomic and physiological characterization. Int J Food Microbiol 290:141-149. doi:https://doi.org/10.1016/j.ijfoodmicro.2018.10.004

Xu D, Behr J, Geißler AJ, Bechtner J, Ludwig C, Vogel RF (2019b) Label-free quantitative proteomic analysis reveals the lifestyle of *Lactobacillus hordei* in the presence of *Sacchromyces cerevisiae*. Int J Food Microbiol 294:18-26. doi:https://doi.org/10.1016/j.ijfoodmicro.2019.01.010

Xu D, Fels L, Wefers D, Behr J, Jakob F, Vogel RF (2018) *Lactobacillus hordei* dextrans induce *Saccharomyces cerevisiae* aggregation and network formation on hydrophilic surfaces. Int J Biol Macromol 115:236-242. doi:https://doi.org/10.1016/j.ijbiomac.2018.04.068

Yang Y, Shevchenko A, Knaust A, Abuduresule I, Li W, Hu X, Wang C et al. (2014) Proteomics evidence for kefir dairy in Early Bronze Age China. Journal of Archaeological Science 45:178-186. doi:https://doi.org/10.1016/j.jas.2014.02.005

Yu NY, Wagner JR, Laird MR, Melli G, Rey S, Lo R, Dao P et al. (2010) PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. Bioinformatics 26:1608-1615. doi:10.1093/bioinformatics/btq249

Yuryev VP, Tomasik P, Bertoft E (2007) Starch : achievements in understanding of structure and functionality. Nova Science Publishers, New York

Zannini E, Waters DM, Coffey A, Arendt EK (2016) Production, properties, and industrial food application of lactic acid bacteria-derived exopolysaccharides. Appl Microbiol Biotechnol 100:1121-1135

Zeidan AA, Poulsen VK, Janzen T, Buldo P, Derkx PM, Øregaard G, Neves AR (2017) Polysaccharide production by lactic acid bacteria: from genes to industrial applications. FEMS Microbiol Rev 41:S168-S200

Zhao Y (2012) Specialty foods : processing technology, quality, and safety. CRC Press, Boca Raton

Zheng J, Ruan L, Sun M, Gänzle M (2015) A genomic view of Lactobacilli and Pediococci demonstrates that phylogeny matches ecology and physiology. Appl Environ Microbiol 81:7233-7243. doi:10.1128/aem.02116-15

10. Appendix

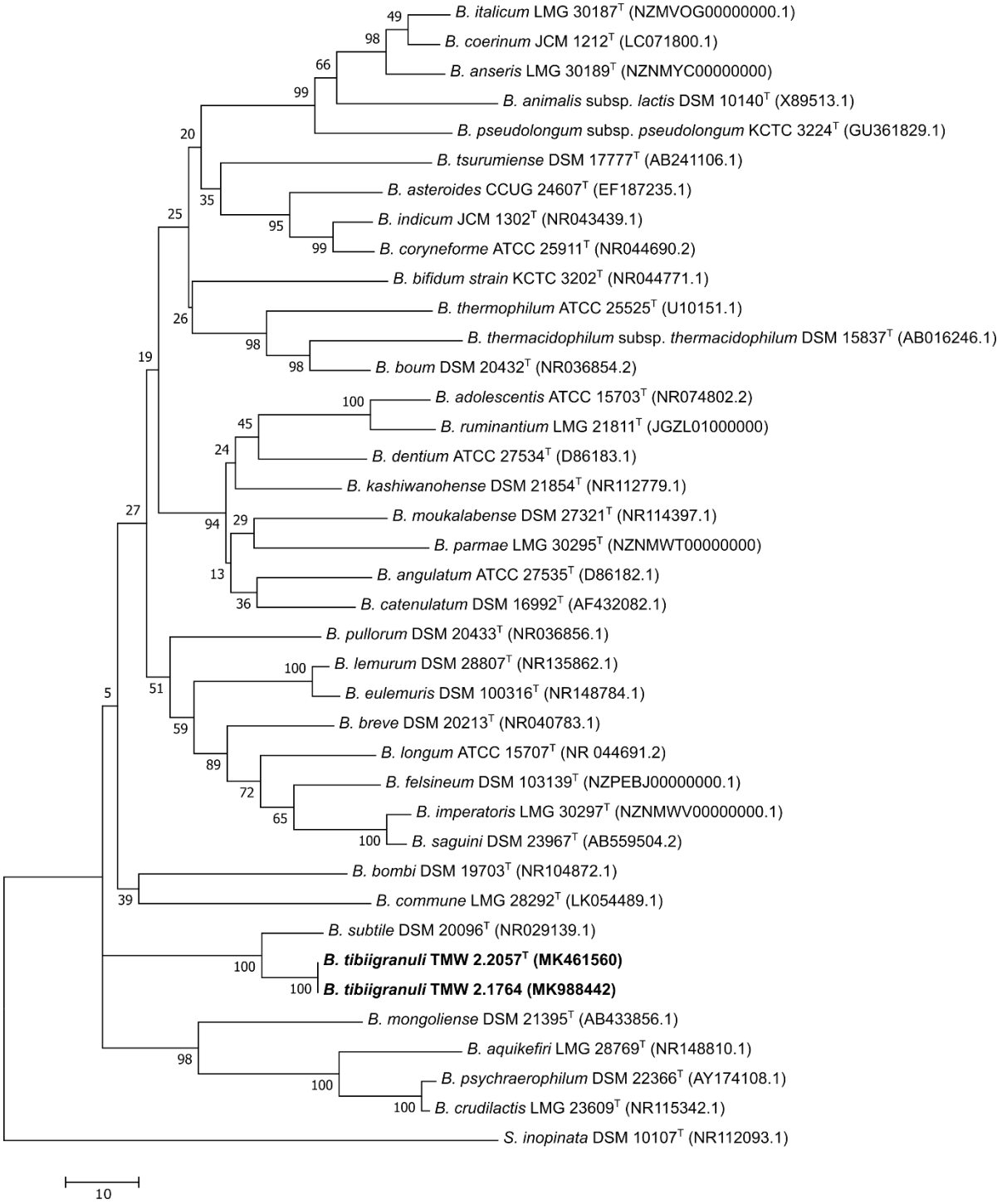

Figure A1. 16S rRNA gene sequence-based tree showing the relationship of novel strains to others of the genus *Bifidobacterium* using the minimum-evolution method.The percentage of replicate trees for the bootstrap test (1000 replicates) are shown at branch nodes. The *Scardovia inopinata* 16S rRNA gene sequence was used as an outgroup. Scale bar shows base differences per sequence. (Eckel et al. 2019b)

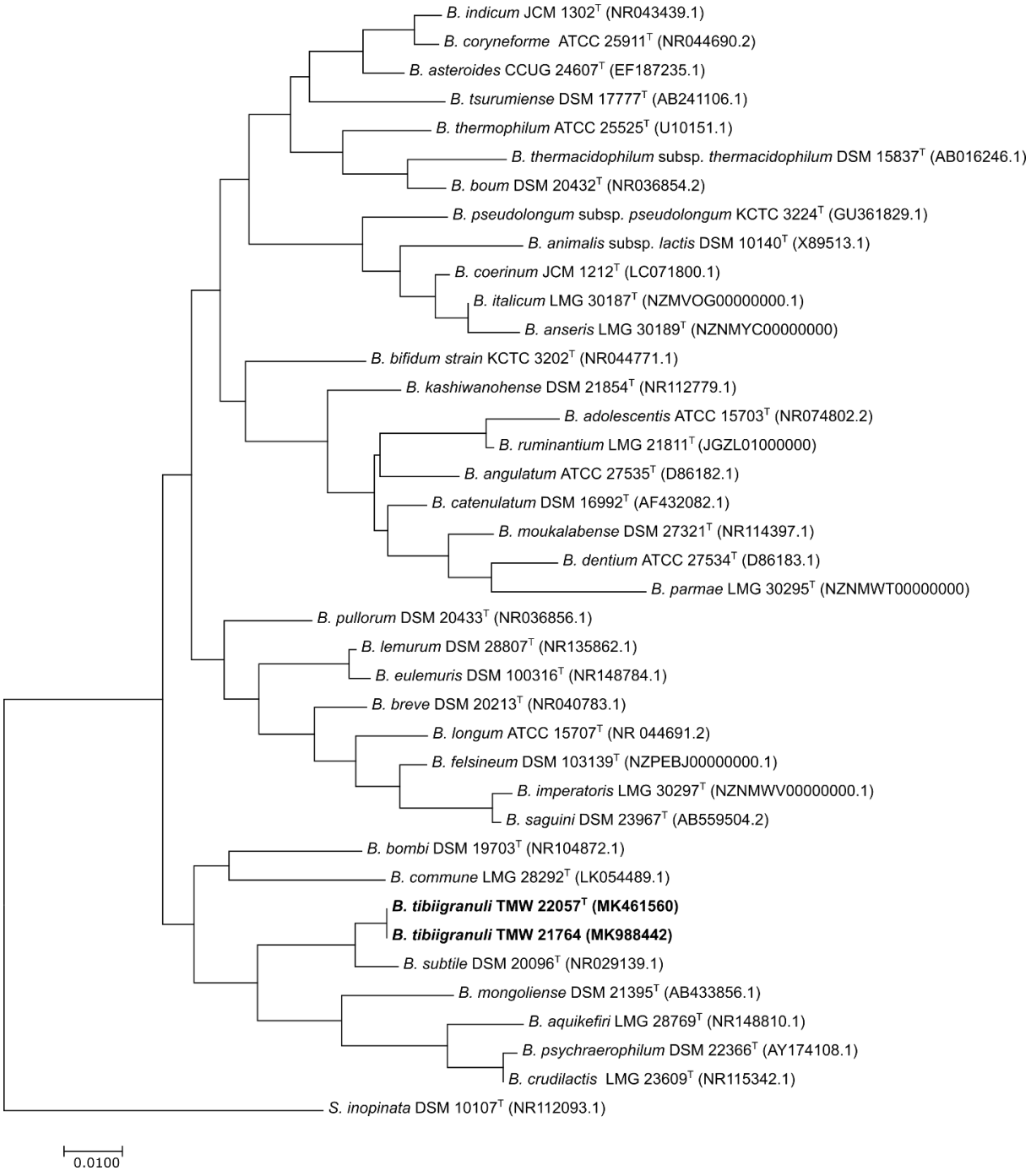

Figure A2. 16S rRNA gene sequence-based tree showing the relationship of novel strains to others of the genus *Bifidobacterium* using the maximum likelihood method. The *Scardovia inopinata* 16S rRNA gene sequence was used as an outgroup. Scale bar shows base differences per sequence. (Eckel et. al 2019b)

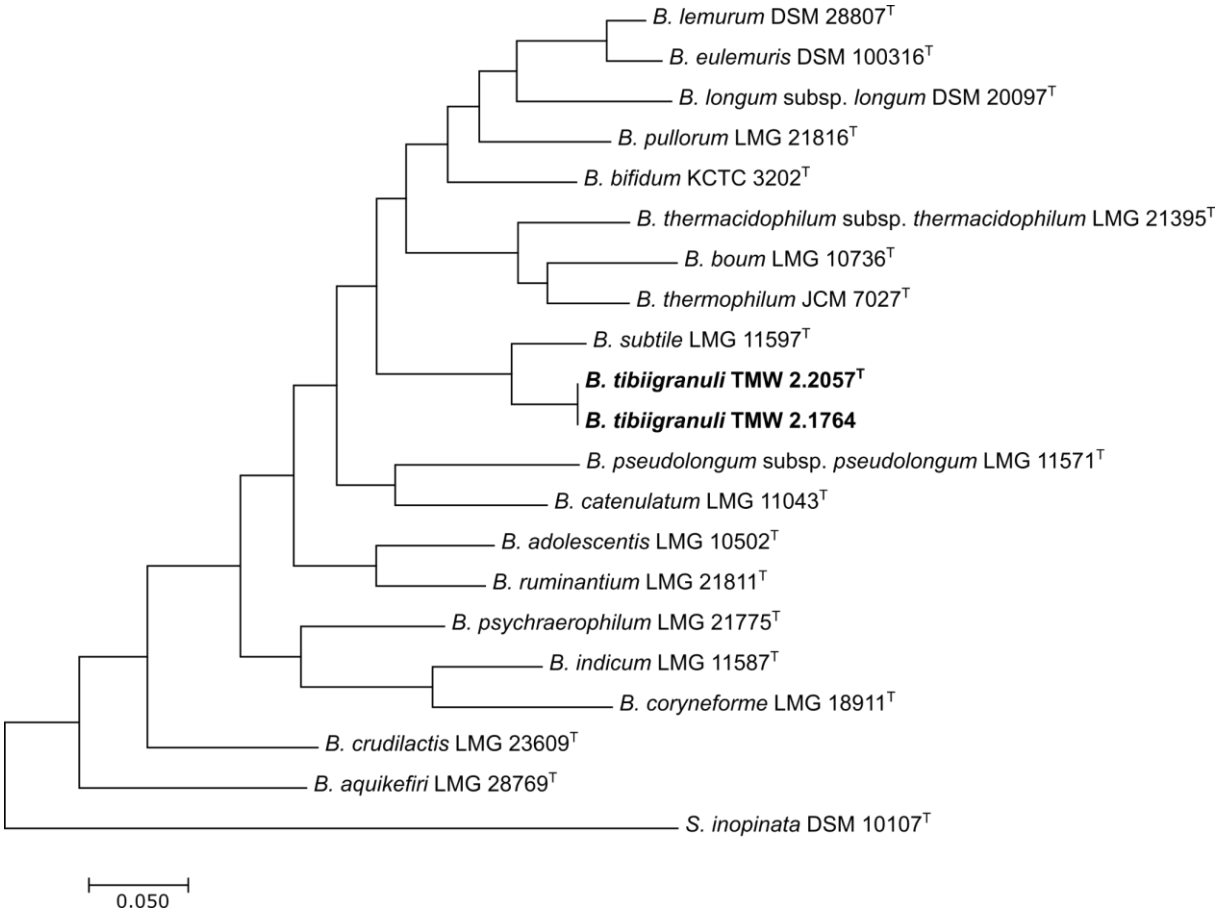

Figure A3. Phylogenetic tree generated using the concatenated partial sequences of hsp60, rpoB, clpC, dnaG and dnaB showing the relationship of strains TMW 2.2057^{T} and TMW 2.1764 to closely related strains of the genus *Bifidobacterium*. Phylogenetic tree was constructed using the Maximum Likelyhood method. The concatenated marker gene sequence of *Scardovia inopinata* was used as an outgroup. Scale bar shows substitutions per site. (Eckel et. al 2019b)

Table A1. Full list of physiologic characteristics of isolates TMW 2.2057^{T} and TMW 2.1764 when compared to other closely related bifidobacterial species (Eckel et. al 2019b).

Characteristic	1	2	3	4*	5	6	7*	8	9*
Growth									
Temperature (°C)	15-40	15-40	15-40	25-40	25-40	20-40	25-40	30-40	4-37
Optimal temperature (°C)	30	30	30-37	37	35-37	35	37	37	28
pH	4.0-8.5	4.0-8.5	4.0-8.5	5.5-7.0	4.5-7.5	4.5-8.5	5.0-7.5	4.5-7.5	4.0-8.0
Optimal pH	6.5-7.0	6.5-7.0	6.5-7.0	6.0	6.0-6.5	5.5-6.0	6.0	6.5	n. d.
Enzyme activity									
β-Galactosidase	-	-	-	+	+	+	+	+	+
β-Glucosidase	+	+	w	+	v	+	+	+	+
α-Arabinosidase	-	-	-	+	-	+	+	+	n. d.
β-N-Acetyl-*β*-Glucosaminidase	-	-	-	w	w	-	w	w	-
α-Fucosidase	-	-	-	w	-	-	-	-	-
Alkaline phosphatase	+	+	v	-	-	-	-	-	-
Leucyl glycine arylamidase	+	+	+	+	+	+	-	+	n. d.
Alanine arylamidase	+	+	+	+	-	+	+	+	n. d.
Glutamyl glutamic acid arylamidase	-	-	-	v	-	-	-	-	n. d.
Production of acid from*									
L-Arabinose	-	-	-	+	-	+	-	+	+
D-Xylose	-	-	-	+	-	+	+	+	-
Methyl-*β*D-Xylopyranoside	-	-	-	+	-	-	-	-	-
D-Mannose	-	-	v	+	+	-	+	-	+
L-Sorbose	+	+	v	-	-	-	-	+	-
Dulcitol	-	-	-	-	-	-	+	-	-
Inositol	-	v	-	-	-	-	-	-	-
D-Mannitol	+	+	-	+	+	-	-	-	w
D-Sorbitol	+	+	+	-	+	-	-	-	-
Methyl-*α*D-Mannopyranoside	-	-	v	-	-	-	-	-	-
Methyl-*α*D-Glucopyranoside	+	+	+	+	-	+	-	+	+
N-Acetylglucosamine	-	-	-	+	+	-	-	-	w
Amygdalin	-	-	-	+	-	+	+	-	w
Arbutin	+	+	-	+	-	+	v	-	-
Esculin/ferric citrate	+	+	+	+	+	+	+	+	-
Salicin	-	-	+	+	v	+	+	-	-
D-Cellobiose	-	-	-	+	-	+	-	-	-
D-Maltose	+	+	+	+	+	-	-	+	+
D-Lactose	-	-	-	-	+	-	+	+	-
D-Melibiose	+	+	+	+	+	-	+	+	+
D-Trehalose	+	+	-	+	-	-	-	+	-
D-Melezitose	+	+	+	v	-	-	-	+	-
D-Raffinose	+	+	+	+	+	-	+	+	+
Amidon	-	-	+	-	-	-	-	+	-
Glycogen	-	-	+	-	-	-	-	-	-
Gentiobiose	-	-	v	+	v	+	+	-	+
D-Lyxose	v	-	-	-	v	-	-	-	-
potassium gluconate	+	+	v	-	-	-	+	-	+
potassium-2-Ketogluconate	-	-	-	-	-	-	+	-	-
potassium-5-Ketogluconate	+	+	v	+	+	+	+	+	-

Strains: 1, TMW 2.2057^T; 2, TMW 2.1764, 3 B. subtile DSM 20096^T, 4 B. eulemuris DSM 100216^T, 5 B. breve DSM 20213^T, 6 B. indicum DSM 20214^T, 7 B. lemurum DSM 28807^T, 8 B. longum subsp. longum DSM 20219^T, 9 B. aquikefiri LMG 28769^T. The physiological traits were determined as present (+), weak (w), absent (-) and variable (v).

†assessed at 30 °C.

‡assessed at 37 °C.

*Data taken from (Modesto et al. 2015), (Michelini et al. 2016) and (Laureys et al. 2016).

Table A2. List of putative dextransucrase sequences used for genomic investigations.

Species	Accession number	Annotation
Lactobacillus hordei	WP_141055456.1	hypothetical protein
Lactobacillus nagelii	AUJ33173.1	hypothetical protein BSQ50_03510
Lactobacillus fermentum	AAU08008.1	glucansucrase [Lactobacillus fermentum]
Lactobacillus parabuchneri	AAU08006.1	glucansucrase [Lactobacillus parabuchneri]
Lactobacillus reuteri	AAU08001.1	glucansucrase [Lactobacillus reuteri]
Lactobacillus sakei	AAU08011.1	glucansucrase
Leuconostoc citreum	ACY92456.2	dextransucrase
Leuconostoc citreum	WP_004908547.1	glycosyltransferase
Leuconostoc mesenteroides	AAS79426.1	dextransucrase
Leuconostoc mesenteroides	AAG61158.1	dextransucrase DsrD
Leuconostoc citreum	WP_040177214.1	glycosyl hydrolase
Leuconostoc mesenteroides	AAB95453.1	glycosyltransferase
Leuconostoc mesenteroides	AAB40875.1	dextransucrase
Leuconostoc mesenteroides	AAD10952.1	dextransucrase
Streptococcus mutans	WP_002352262.1	glycosyltransferase-S
Streptococcus salivarius	AAC41413.1	glycosyltransferase
Streptococcus salivarius	WP_045768851.1	glycosyltransferase
Weisella cibaria	ACK38203.1	glucansucrase
Lactobacillus reuteri	AAU08004.1	glucansucrase
Streptococcus downei	WP_115325031.1	glycosyl transferase
Streptococcus mutans	WP_002352268.1	glycosyltransferase-I
Streptococcus mutans	WP_002352269.1	glycosyltransferase-SI
Streptococcus salivarius	WP_084871409.1	glycosyltransferase
Lactobacillus reuteri	AAY86923.1	Ir1943
Leuconostoc mesenteroides	CAB65910.2	alternansucrase
Leuconostoc mesenteroides	BAA90527.1	dextransucrase
Streptococcus salivarius	WP_084871217.1	glucosyltransferase
Lactobacillus reuteri	AAU08015.1	glucansucrase
Lactobacillus reuteri	WP_081372298.1	hypothetical protein
Lactobacillus reuteri	ASA47881.1	putative GTFB
Lactobacillus hilgardii	CBJ19544.1	glycosyltransferase
Bifidobacterium gallicum	WP_006295413.1	CHAP-domain containing protein

Table A3. List of putative dextranase sequences used for genomic investigations.

Species	Accession number	Annotation
Streptococcus mutans	D49430.1	Dextranase
Paenibacillus sabinae	AHV95999.1	Dextranase
Eubacterium sp.	CCY70005.1	Dextranase
Clostridium sp.	WP009172537.1	Dextranase
Bifidobacterium psychraerophilum	KFI82023.1	Dextranase
Bifidobacterium pseudolongum	WP118238591.1	Dextranase
Pseudarthrobacter oxydans	AAX09503.1	Dextranase
Arthrobacter sp.	JQ0878	Dextranase
Symbiodinium microadriaticum	OLP88843.1	Dextranase
Talaromyces minioluteus	AAB47720.1	Dextranase
Streptomyces sp.	RLV64308.1	Dextranase
Chaetomium globosum	AXN77607.1	Dextranase
Adhaeribacter sp.	RDC65695.1	Dextranase
Lactobacillus phage EV3	CBZ13171.1	Dextranase
Thermoanaerobacter sp.	ADN55721.1	Dextranase
Lipomyces sarkeyi	AAS90631.1	Dextranase
Sphingobacterium sp.	BAE92747.1	Dextranase
Vagococcus entomophilus	WP126824598.1	Dextranase
Actinomyces viscosus	WP126414582.1	Dextranase
Neisseria sp.	WP123805824.1	Dextranase
Enterococcus gallinarum	ROY85585.1	Dextranase
Saccharothrix texasensis	ROP35059.1	Dextranase
Petrotoga olearia	RMA76540.1	Dextranase
Mycetocola reblochoni	RLP71175.1	Dextranase
Fusarium oxysporum	RKK89715.1	Dextranase
Bifidobacteriaceae	WP040591521.1	Dextranase
Marinilabilia salmonicolor	RCW34626.1	Dextranase
Micromonospora saelicesensis	RAO62881.1	Dextranase
Gelidibacter algens	RAJ27695.1	Dextranase
Dysgonomonas alginatilytica	PXV63029.1	Dextranase
Purpureocillium lilacinum	PWI76590.1	Dextranase
Bifidobacterium sp.	RSX49244.1	Dextranase
Bifidobacterium asteroides	WP110424912.1	Dextranase

Table A4. List of putative fructanase sequences used for genomic investigations.

Species	Accession number	Annotation
Prevotella loescheii	BAI39501.1	Fructanase
Streptococcus mutans	AAN57863.1	Fructanase
Arthrobacter sp.	KIA74147.1	Inulinase
Bifidobacterium adolescentis	AJE06289.1	Inulinase
Bifidobacterium longum	AAN23970.1	Inulinase
Bifidobacterium animalis subsp. lactis	EDT88945.1	Inulinase
Paenibacillus sp.	ODP26905.1	*β*-Fructofuranosidase
Vibrio scophthalmi	ANU38700.1	*β*-Fructofuranosidase
Bifidobacterium longum subsp. infantis	ALY05367.1	*β*-Fructofuranosidase
Microbulbifer sp.	BAL70276.1	*β*-Fructofuranosidase
Bacillus licheniformis	AGR40655.1	exo-Inulinase
Pseudomonas mucidolens	AAF44125.1	exo-Inulinase
Geobacillus stearothermophilus	BAC45010.1	exo-Inulinase
Burkholderia ubonensis	AJX13591.1	exo-Inulinase
Pseudarthrobacter siccitolerans	CCQ47137.1	exo-Inulinase
Halobacillus sp.	WPO82794489.1	endo-Inulinase
Pontibacillus halophilus	KGX92856.1	endo-Inulinase
Fimbiimonas ginsengisoli	AIE84515.1	endo-Inulinase
Catenovulum agarivorans	EWH11901.1	endo-Inulinase
Bacteroides fragilis	CAH08872.1	Levanase
Clavibacter michiganensis	WPO15489156.1	Levanase
Klebsiella michiganensis	AEX04434.1	Levanase
Fibrella aestuarina	CCH00523.1	Levanase
Saccharopolyspora erythraea	CAM01582.1	Levanase
Gluconacetobacter diazotrophicus	ACI51309.1	Levanase
Beijerinokiav inolica subsp. indica	ACB95642	Levanase
Burkholderia ambifaria	ACB66634.1	Levanase
Frankia sp.	ABW16365.1	Levanase
Pseudoalteromonas	ABG42323.1	Levanase
Capnocytophaga ochracea	ACU91786.1	Levanase
Rhodothermus marinus	ACY47581.1	Levanase
Lactobacillus paracasei	AGP67222.1	Levanase
Lactobacillus plantarum	BBA81815.1	*β*-Fructofuranusidase

Table A5. BADGE output „Water Kefir vs. Probiotic“ (Eckel and Vogel 2020).

DMG_ID	percent_ occurrence	dc_blast_ hit	max_blastn_ hit_length	ORF_ID	ORF_length	annotation	contig	start	stop
DMG_1	100	no	31	BAQU_0017	1320	GntR family transcriptional regulator	MWXA01000001_1	21410	22729
				DDE84_01930	1323	PLP-dependent aminotransferase family protein	Bifidobacterium_spec_TMW21764_seq2	77440	76118
				DDF78_02555	1323	PLP-dependent aminotransferase family protein	lcl_Bifidobacterium_spec_TMW22057_seq2	233255	231933
DMG_2	100	no	29	BAQU_0018	1098	D-alanine--D-alanine ligase	MWXA01000001_1	22800	23897
				DDE84_01925	1170	D-alanine--D-alanine ligase	Bifidobacterium_spec_TMW21764_seq2	75986	74817
				DDF78_02550	1170	D-alanine--D-alanine ligase	lcl_Bifidobacterium_spec_TMW22057_seq2	231801	230632
DMG_3	100	no	22	BAQU_0087	615	DNA-3-methyladenine glycosylase	MWXA01000001_1	93812	94426
				DDE84_02370	636	DNA-3-methyladenine glycosylase	Bifidobacterium_spec_TMW21764_seq2	190127	190762
				DDF78_08590	636	DNA-3-methyladenine glycosylase	lcl_Bifidobacterium_spec_TMW22057_seq9	43509	44144
DMG_4	100	no	38	BAQU_0091	246	hypothetical protein	MWXA01000001_1	96142	96387
				BAQU_1977	246	hypothetical protein	MWXA01000014_1	4078	4323
				DDE84_10210	264	hypothetical protein	Bifidobacterium_spec_TMW21764_seq11	73385	73648
				DDF78_10090	264	hypothetical protein	lcl_Bifidobacterium_spec_TMW22057_seq11	73051	73314
DMG_5	100	no	39	BAQU_0092	333	toxin-antitoxin system protein	MWXA01000001_1	96404	96736
				BAQU_1978	333	toxin-antitoxin system protein	MWXA01000014_1	4340	4672
				DDE84_04355	189	hypothetical protein	Bifidobacterium_spec_TMW21764_seq4	10166	10354
				DDF78_04145	189	hypothetical protein	lcl_Bifidobacterium_spec_TMW22057_seq4	10166	10354
DMG_6	100	no	32	BAQU_0128	228	hypothetical protein	MWXA01000001_1	132966	132739
				DDE84_02630	231	hypothetical protein	Bifidobacterium_spec_TMW21764_seq2	228945	228715
				DDF78_08850	231	hypothetical protein	lcl_Bifidobacterium_spec_TMW22057_seq9	82327	82097
DMG_7	100	no	37	BAQU_0132	1320	Phage integrase family	MWXA01000001_1	135544	134225
				DDE84_02680	1293	site-specific integrase	Bifidobacterium_spec_TMW21764_seq2	237054	235762

				DDF78_08900	1293	hypothetical protein	lcl_Bifidobacterium_spec_TMW22057_seq9	90436	89144
DMG_8	100	yes	206	BAQU_0143	2832	haloacid dehalogenase	MWXA01000001_1	148339	151170
				DDE84_09935	2970	HAD family hydrolase	Bifidobacterium_spec_TMW21764_seq10	135218	132249
				DDF78_02180	2970	haloacid dehalogenase	lcl_Bifidobacterium_spec_TMW22057_seq2	135218	132249
DMG_9	100	no	53	BAQU_0152	2394	trehalose 6-phosphate synthase_phosphatase	MWXA01000001_1	163187	165580
				DDE84_01660	2634	trehalose-phosphatase	Bifidobacterium_spec_TMW21764_seq2	5519	2886
				DDF78_02285	2634	trehalose-phosphatase	lcl_Bifidobacterium_spec_TMW22057_seq2	161334	158701
DMG_10	100	no	31	BAQU_0164	582	PTS cellobiose transporter subunit IIC	MWXA01000002_1	3613	3032
				DDE84_02050	627	PTS cellobiose transporter subunit IIC	Bifidobacterium_spec_TMW21764_seq2	110860	111486
				DDF78_02675	627	PTS cellobiose transporter subunit IIC	lcl_Bifidobacterium_spec_TMW22057_seq2	266675	267301
DMG_11	100	no	34	BAQU_0170	195	XRE family transcriptional regulator	MWXA01000002_1	11026	10832
				DDE84_08580	195	XRE family transcriptional regulator	Bifidobacterium_spec_TMW21764_seq8	160175	160369
				DDF78_07565	195	transcriptional regulator	lcl_Bifidobacterium_spec_TMW22057_seq7	160175	160369
DMG_12	100	no	48	BAQU_0194	690	magnesium transporter MgtC	MWXA01000002_1	40520	39831
				DDE84_07750	750	MgtC_SapB family protein	Bifidobacterium_spec_TMW21764_seq7	172192	172941
				DDF78_09385	750	MgtC_SapB family protein	lcl_Bifidobacterium_spec_TMW22057_seq10	35317	34568
DMG_13	100	no	29	BAQU_0221	1014	sugar-binding protein	MWXA01000002_1	73107	74120
				DDE84_06525	969	sugar-binding protein	Bifidobacterium_spec_TMW21764_seq6	64461	65429
				DDF78_06315	969	sugar-binding protein	lcl_Bifidobacterium_spec_TMW22057_seq6	64461	65429
DMG_14	100	no	38	BAQU_0230	1152	dihydrofolate reductase	MWXA01000002_1	86566	85415
				DDE84_02360	1140	diphosphomevalonate decarboxylase	Bifidobacterium_spec_TMW21764_seq2	186060	187199
				DDF78_08580	1140	diphosphomevalonate decarboxylase	lcl_Bifidobacterium_spec_TMW22057_seq9	39442	40581
DMG_15	100	no	32	BAQU_0236	993	nitrate reductase	MWXA01000002_1	93096	94088
				DDE84_01725	1584	NCS1 family nucleobase_cation symporter-1	Bifidobacterium_spec_TMW21764_seq2	23195	21612
				DDF78_02350	1584	NCS1 family nucleobase_cation symporter-1	lcl_Bifidobacterium_spec_TMW22057_seq2	179010	177427
DMG_16	100	no	42	BAQU_0237	747	Asp_Glu racemase	MWXA01000002_1	94116	94862
				DDE84_01720	735	Asp_Glu racemase	Bifidobacterium_spec_TMW21764_seq2	21422	20688
				DDF78_02345	735	Asp_Glu racemase	lcl_Bifidobacterium_spec_TMW22057_seq2	177237	176503

DMG_17	100	no	40	BAQU_0238	1278	serine--pyruvate transaminase	MWXA01000002_1	94963	96240
				DDE84_01715	1224	alanine--glyoxylate aminotransferase family protein	Bifidobacterium_spec_TMW21764_seq2	20566	19343
				DDF78_02340	1224	aminotransferase	lcl_Bifidobacterium_spec_TMW22057_seq2	176381	175158
DMG_18	100	no	34	BAQU_0239	1353	Zn-dependent hydrolase	MWXA01000002_1	96237	97589
				DDE84_01710	1314	Zn-dependent hydrolase	Bifidobacterium_spec_TMW21764_seq2	19253	17940
				DDF78_02335	1314	Zn-dependent hydrolase	lcl_Bifidobacterium_spec_TMW22057_seq2	175068	173755
DMG_19	100	no	45	BAQU_0240	1341	allantoinase	MWXA01000002_1	97628	98968
				DDE84_01705	1410	allantoinase AllB	Bifidobacterium_spec_TMW21764_seq2	17806	16397
				DDF78_02330	1410	allantoinase AllB	lcl_Bifidobacterium_spec_TMW22057_seq2	173621	172212
DMG_20	100	no	44	BAQU_0241	1374	aspartyl_glutamyl-tRNA_asn_Gln_ amidotransferase subunit A	MWXA01000002_1	98997	100370
				DDE84_01695	1587	DUF3225 domain-containing protein	Bifidobacterium_spec_TMW21764_seq2	15242	13656
				DDF78_02320	1587	glutamyl-tRNA amidotransferase	lcl_Bifidobacterium_spec_TMW22057_seq2	171057	169471
DMG_21	100	no	36	BAQU_0271	792	GntR family transcriptional regulator	MWXA01000002_1	137808	137017
				DDE84_07745	852	GntR family transcriptional regulator	Bifidobacterium_spec_TMW21764_seq7	171277	172128
				DDF78_09390	852	GntR family transcriptional regulator	lcl_Bifidobacterium_spec_TMW22057_seq10	36232	35381
DMG_22	100	no	31	BAQU_0272	1365	4-aminobutyrate aminotransferase	MWXA01000002_1	139391	138027
				DDE84_07740	1365	aspartate aminotransferase family protein	Bifidobacterium_spec_TMW21764_seq7	169709	171073
				DDF78_09395	1365	aspartate aminotransferase family protein	lcl_Bifidobacterium_spec_TMW22057_seq10	37800	36436
DMG_23	100	no	35	BAQU_0273	1509	amino acid ABC transporter permease	MWXA01000002_1	141058	139550
				DDE84_07735	1500	amino acid permease	Bifidobacterium_spec_TMW21764_seq7	168042	169541
				DDF78_09400	1500	amino acid ABC transporter permease	lcl_Bifidobacterium_spec_TMW22057_seq10	39467	37968
DMG_24	100	no	28	BAQU_0274	1068	phosphotransferase	MWXA01000002_1	142235	141168
				DDE84_07730	1068	phosphotransferase	Bifidobacterium_spec_TMW21764_seq7	166799	167866
				DDF78_09405	1068	phosphotransferase	lcl_Bifidobacterium_spec_TMW22057_seq10	40710	39643
DMG_25	100	no	75	BAQU_0288	852	ABC transporter ATP-binding protein	MWXA01000002_1	156558	157409
				DDE84_02970	732	ABC transporter ATP-binding protein	Bifidobacterium_spec_TMW21764_seq2	313359	312628

				DDF78_09190	732	ABC transporter ATP-binding protein	lcl_Bifidobacterium_spec_TMW22057_seq9	166741	166010
DMG_26	100	no	46	BAQU_0289	912	ABC transporter permease	MWXA01000002_1	157421	158332
				DDE84_02965	765	ABC transporter permease	Bifidobacterium_spec_TMW21764_seq2	312631	311867
				DDF78_09185	765	ABC transporter permease	lcl_Bifidobacterium_spec_TMW22057_seq9	166013	165249
DMG_27	100	no	43	BAQU_0290	1023	ABC transporter substrate-binding protein	MWXA01000002_1	158411	159433
				DDE84_02960	1044	ABC transporter substrate-binding protein	Bifidobacterium_spec_TMW21764_seq2	311732	310689
				DDF78_09180	1044	ABC transporter substrate-binding protein	lcl_Bifidobacterium_spec_TMW22057_seq9	165114	164071
DMG_28	100	no	79	BAQU_0292	1371	amidase	MWXA01000002_1	159658	161028
				DDE84_02950	1371	amidase	Bifidobacterium_spec_TMW21764_seq2	310459	309089
				DDF78_09170	1371	amidase	lcl_Bifidobacterium_spec_TMW22057_seq9	163841	162471
DMG_29	100	no	34	BAQU_0293	786	transcriptional regulator	MWXA01000002_1	161855	161070
				DDE84_02945	789	IclR family transcriptional regulator	Bifidobacterium_spec_TMW21764_seq2	308220	309008
				DDF78_09165	789	hypothetical protein	lcl_Bifidobacterium_spec_TMW22057_seq9	161602	162390
DMG_30	100	no	44	BAQU_0294	1449	major facilitator transporter	MWXA01000002_1	161991	163439
				DDE84_02940	1428	MFS transporter	Bifidobacterium_spec_TMW21764_seq2	308131	306704
				DDF78_09160	1428	hypothetical protein	lcl_Bifidobacterium_spec_TMW22057_seq9	161513	160086
DMG_31	100	no	44	BAQU_0295	1521	PTS ascorbate transporter subunit IIC	MWXA01000002_1	165069	163549
				DDE84_07955	1530	PTS ascorbate transporter subunit IIC	Bifidobacterium_spec_TMW21764_seq8	11418	12947
				DDF78_06935	1530	PTS ascorbate transporter subunit IIC	lcl_Bifidobacterium_spec_TMW22057_seq7	11418	12947
DMG_32	100	no	28	BAQU_0296	273	PTS ascorbate transporter subunit IIB	MWXA01000002_1	165394	165122
				DDE84_07950	273	PTS sugar transporter subunit IIB	Bifidobacterium_spec_TMW21764_seq8	11096	11368
				DDF78_06930	273	PTS ascorbate transporter subunit IIB	lcl_Bifidobacterium_spec_TMW22057_seq7	11096	11368
DMG_33	100	no	25	BAQU_0297	450	PTS ascorbate transporter subunit IIA	MWXA01000002_1	165909	165460
				DDE84_07945	450	PTS sugar transporter subunit IIA	Bifidobacterium_spec_TMW21764_seq8	10540	10989
				DDF78_06925	450	PTS sugar transporter subunit IIA	lcl_Bifidobacterium_spec_TMW22057_seq7	10540	10989
DMG_34	100	no	36	BAQU_0302	1569	glutamine ABC transporter permease	MWXA01000002_1	170676	172244
				DDE84_04805	1494	ABC transporter permease subunit	Bifidobacterium_spec_TMW21764_seq4	105313	103820
				DDF78_04595	1494	glutamine ABC transporter permease	lcl_Bifidobacterium_spec_TMW22057_seq4	105313	103820

DMG_35	100	no	32	BAQU_0303	1008	L-asparaginase 1	MWXA01000002_1	173492	172485
				DDE84_01480	1008	asparaginase	Bifidobacterium_spec_TMW21764_seq1	328696	327689
				DDF78_00170	1008	asparaginase	lcl_Bifidobacterium_spec_TMW22057_seq1	29370	30377
DMG_36	100	yes	86	BAQU_0304	1554	L-asparagine permease	MWXA01000002_1	175104	173551
				DDE84_01485	1515	amino acid permease	Bifidobacterium_spec_TMW21764_seq1	330210	328696
				DDF78_00165	1515	L-asparagine permease	lcl_Bifidobacterium_spec_TMW22057_seq1	27856	29370
DMG_37	100	no	30	BAQU_0352	480	phage tail protein	MWXA01000003_1	9503	9982
				DDE84_10515	462	phage tail protein	Bifidobacterium_spec_TMW21764_seq12	25181	24720
				DDF78_10620	462	phage tail protein	lcl_Bifidobacterium_spec_TMW22057_seq12	72267	72728
DMG_38	100	no	56	BAQU_0357	969	ABC transporter	MWXA01000003_1	16444	17412
				DDE84_06100	882	ABC transporter ATP-binding protein	Bifidobacterium_spec_TMW21764_seq5	188912	189793
				DDF78_05245	882	ABC transporter	lcl_Bifidobacterium_spec_TMW22057_seq5	34902	34021
DMG_39	100	no	35	BAQU_0373	540	FMN reductase	MWXA01000003_1	38533	39072
				DDE84_05250	546	NAD_P_H-dependent oxidoreductase	Bifidobacterium_spec_TMW21764_seq4	213901	214446
				DDF78_05040	546	NADPH-dependent FMN reductase	lcl_Bifidobacterium_spec_TMW22057_seq4	213901	214446
DMG_40	100	yes	97	BAQU_0376	1440	manganese transporter	MWXA01000003_1	42742	41303
				DDE84_09880	1389	divalent metal cation transporter	Bifidobacterium_spec_TMW21764_seq10	115846	114458
				DDF78_02125	1389	divalent metal cation transporter	lcl_Bifidobacterium_spec_TMW22057_seq2	115846	114458
DMG_41	100	no	24	BAQU_0377	807	DtxR family transcriptional regulator	MWXA01000003_1	42995	43801
				DDE84_05730	765	metal-dependent transcriptional regulator	Bifidobacterium_spec_TMW21764_seq5	101697	102461
				DDF78_05615	765	metal-dependent transcriptional regulator	lcl_Bifidobacterium_spec_TMW22057_seq5	122117	121353
DMG_42	100	no	44	BAQU_0382	1311	major facilitator superfamily protein	MWXA01000003_1	49652	48342
				DDE84_06380	1305	MFS transporter	Bifidobacterium_spec_TMW21764_seq6	30012	28708
				DDF78_06170	1305	MFS transporter	lcl_Bifidobacterium_spec_TMW22057_seq6	30012	28708
DMG_43	100	no	31	BAQU_0415	1014	family 2 glycosyl transferase	MWXA01000003_1	98480	97467
				DDE84_09675	1044	glycosyltransferase family 2 protein	Bifidobacterium_spec_TMW21764_seq10	67554	68597
				DDF78_01920	1044	glycosyltransferase family 2 protein	lcl_Bifidobacterium_spec_TMW22057_seq2	67554	68597
DMG_44	100	no	36	BAQU_0437	786	alpha_beta hydrolase	MWXA01000003_1	123828	123043

				DDE84_03850	825	alpha_beta hydrolase	Bifidobacterium_spec_TMW21764_seq3	181380	180556
				DDF78_03640	825	hypothetical protein	lcl_Bifidobacterium_spec_TMW22057_seq3	181379	180555
DMG_45	100	no	37	BAQU_0511	1197	hydroxymethylglutaryl-CoA synthase	MWXA01000003_1	212771	213967
				DDE84_07585	1170	hydroxymethylglutaryl-CoA synthase	Bifidobacterium_spec_TMW21764_seq7	110369	111538
				DDF78_09550	1170	hydroxymethylglutaryl-CoA synthase	lcl_Bifidobacterium_spec_TMW22057_seq10	97140	95971
DMG_46	100	no	23	BAQU_0516	264	50S ribosomal protein L31	MWXA01000003_1	217579	217842
				DDE84_01620	273	type B 50S ribosomal protein L31	Bifidobacterium_spec_TMW21764_seq1	355732	356004
				DDF78_00030	273	50S ribosomal protein L31	lcl_Bifidobacterium_spec_TMW22057_seq1	2334	2062
DMG_47	100	no	26	BAQU_0517	123	50S ribosomal protein L36	MWXA01000003_1	217917	218039
				DDE84_01625	123	50S ribosomal protein L36	Bifidobacterium_spec_TMW21764_seq1	356069	356191
				DDF78_00025	123	50S ribosomal protein L36	lcl_Bifidobacterium_spec_TMW22057_seq1	1997	1875
DMG_48	100	no	36	BAQU_0534	1374	MFS permease	MWXA01000003_1	235767	234394
				DDE84_03655	1404	MFS transporter	Bifidobacterium_spec_TMW21764_seq3	125274	126677
				DDF78_03445	1404	MFS transporter	lcl_Bifidobacterium_spec_TMW22057_seq3	125274	126677
DMG_49	100	yes	29	BAQU_0565	3276	DEAD_DEAH box helicase	MWXA01000003_1	268344	271619
				DDE84_07250	3255	type I restriction endonuclease subunit R	Bifidobacterium_spec_TMW21764_seq7	31472	34726
				DDF78_11460	3255	type I restriction endonuclease subunit R	lcl_Bifidobacterium_spec_TMW22057_seq15	31472	34726
DMG_50	100	yes	131	BAQU_0569	1629	type I restriction-modification protein subunit M	MWXA01000003_1	273802	275430
				DDE84_07260	1623	type I restriction-modification system subunit M	Bifidobacterium_spec_TMW21764_seq7	36024	37646
				DDF78_11600	1623	type I restriction-modification system subunit M	lcl_Bifidobacterium_spec_TMW22057_seq17	9763	8141
DMG_51	100	no	63	BAQU_0574	750	hypothetical protein	MWXA01000003_1	283481	284230
				DDE84_10380	750	peroxide stress protein YaaA	Bifidobacterium_spec_TMW21764_seq11	117315	116566
				DDF78_10260	750	peroxide stress protein YaaA	lcl_Bifidobacterium_spec_TMW22057_seq11	116981	116232
DMG_52	100	no	48	BAQU_0589	1377	NAD_FAD_-dependent dehydrogenase	MWXA01000003_1	297079	298455
				DDE84_07235	1371	FAD-dependent oxidoreductase	Bifidobacterium_spec_TMW21764_seq7	27891	29261
				DDF78_11445	1371	FAD-dependent oxidoreductase	lcl_Bifidobacterium_spec_TMW22057_seq15	27891	29261
DMG_53	100	yes	41	BAQU_0629	1029	chromosome replication initiation inhibitor protein	MWXA01000003_1	353934	352906

				DDE84_09780	1071	ArgP_LysG family DNA-binding transcriptional regulator	Bifidobacterium_spec_TMW21764_seq10	96445	95375
				DDF78_02025	1071	hypothetical protein	lcl_Bifidobacterium_spec_TMW22057_seq2	96445	95375
DMG_54	100	no	47	BAQU_0634	1299	4-aminobutyrate aminotransferase	MWXA01000003_1	358054	359352
				DDE84_06240	1272	aminotransferase class III-fold pyridoxal phosphate-dependent enzyme	Bifidobacterium_spec_TMW21764_seq5	220410	219139
				DDE84_08635	1323	aminotransferase class III-fold pyridoxal phosphate-dependent enzyme	Bifidobacterium_spec_TMW21764_seq8	174295	175617
				DDF78_05105	1272	aspartate aminotransferase family protein	lcl_Bifidobacterium_spec_TMW22057_seq5	3405	4676
				DDF78_07620	1323	aspartate aminotransferase family protein	lcl_Bifidobacterium_spec_TMW22057_seq7	174295	175617
DMG_55	100	no	33	BAQU_0635	951	ABC transporter permease	MWXA01000003_1	359398	360348
				DDE84_06235	951	ABC transporter permease	Bifidobacterium_spec_TMW21764_seq5	219086	218136
				DDF78_05110	951	ABC transporter permease	lcl_Bifidobacterium_spec_TMW22057_seq5	4729	5679
DMG_56	100	no	38	BAQU_0636	879	peptide ABC transporter permease	MWXA01000003_1	360351	361229
				DDE84_06230	942	ABC transporter permease	Bifidobacterium_spec_TMW21764_seq5	218134	217193
				DDF78_05115	942	ABC transporter permease	lcl_Bifidobacterium_spec_TMW22057_seq5	5681	6622
DMG_57	100	yes	58	BAQU_0637	1002	oligopeptide_dipeptide ABC transporter ATPase	MWXA01000003_1	361237	362238
				DDE84_06225	1002	ABC transporter ATP-binding protein	Bifidobacterium_spec_TMW21764_seq5	217187	216186
				DDF78_05120	1002	peptide ABC transporter ATP-binding protein	lcl_Bifidobacterium_spec_TMW22057_seq5	6628	7629
DMG_58	100	no	126	BAQU_0638	828	ABC transporter ATP-binding protein	MWXA01000003_1	362315	363142
				DDE84_06220	828	ABC transporter ATP-binding protein	Bifidobacterium_spec_TMW21764_seq5	216189	215362
				DDF78_05125	828	peptide ABC transporter ATP-binding protein	lcl_Bifidobacterium_spec_TMW22057_seq5	7626	8453
DMG_59	100	no	50	BAQU_0639	1566	ABC transporter periplasmic protein	MWXA01000003_1	363190	364755
				DDE84_06215	1575	hypothetical protein	Bifidobacterium_spec_TMW21764_seq5	215318	213744
				DDF78_05130	1575	hypothetical protein	lcl_Bifidobacterium_spec_TMW22057_seq5	8497	10071
DMG_60	100	no	31	BAQU_0640	2157	asp-trnaasn_glu-trnagln amidotransferase	MWXA01000003_1	364789	366945
				DDE84_08640	2022	amidase	Bifidobacterium_spec_TMW21764_seq8	175812	177833
				DDF78_07625	2022	amidase	lcl_Bifidobacterium_spec_TMW22057_seq7	175812	177833

DMG_61	100	no	31	BAQU_0641	1644	amidohydrolase	MWXA01000003_1	367051	368694
				DDE84_06210	1650	amidohydrolase	Bifidobacterium_spec_TMW21764_seq5	213476	211827
				DDF78_05135	1650	amidohydrolase	lcl_Bifidobacterium_spec_TMW22057_seq5	10339	11988
DMG_62	100	no	35	BAQU_0642	1323	permease of the major facilitator superfamily	MWXA01000003_1	368794	370116
				DDE84_07170	1446	MFS transporter	Bifidobacterium_spec_TMW21764_seq7	15062	16507
				DDF78_11380	1446	MFS transporter	lcl_Bifidobacterium_spec_TMW22057_seq15	15062	16507
DMG_63	100	no	83	BAQU_0643	1044	alcohol dehydrogenase	MWXA01000003_1	370186	371229
				DDE84_06200	1044	IMP dehydrogenase	Bifidobacterium_spec_TMW21764_seq5	210934	209891
				DDF78_05145	1044	IMP dehydrogenase	lcl_Bifidobacterium_spec_TMW22057_seq5	12881	13924
DMG_64	100	no	37	BAQU_0645	1380	4-aminobutyrate aminotransferase	MWXA01000003_1	372399	373778
				DDE84_06190	1401	aminotransferase class III-fold pyridoxal phosphate-dependent enzyme	Bifidobacterium_spec_TMW21764_seq5	208683	207283
				DDF78_05155	1401	aspartate aminotransferase family protein	lcl_Bifidobacterium_spec_TMW22057_seq5	15132	16532
DMG_65	100	no	30	BAQU_0646	336	hypothetical protein	MWXA01000003_1	374201	373866
				DDE84_09490	342	hypothetical protein	Bifidobacterium_spec_TMW21764_seq10	19228	19569
				DDF78_01735	342	hypothetical protein	lcl_Bifidobacterium_spec_TMW22057_seq2	19228	19569
DMG_66	100	yes	300	BAQU_0687	1446	sugar phosphate permease	MWXA01000004_1	46711	45266
				DDE84_05265	1398	MFS transporter	Bifidobacterium_spec_TMW21764_seq4	216779	218176
				DDF78_05055	1398	MFS transporter	lcl_Bifidobacterium_spec_TMW22057_seq4	216779	218176
DMG_67	100	no	37	BAQU_0688	804	oxidoreductase	MWXA01000004_1	48011	47208
				DDE84_04115	765	SDR family NAD_P_-dependent oxidoreductase	Bifidobacterium_spec_TMW21764_seq3	246922	246158
				DDF78_03905	765	oxidoreductase	lcl_Bifidobacterium_spec_TMW22057_seq3	246921	246157
DMG_68	100	no	35	BAQU_0744	774	radical SAM protein	MWXA01000004_1	127739	128512
				DDE84_10295	771	radical SAM protein	Bifidobacterium_spec_TMW21764_seq11	94155	94925
				DDF78_10175	771	glycyl-radical enzyme activating protein	lcl_Bifidobacterium_spec_TMW22057_seq11	93821	94591
DMG_69	100	no	45	BAQU_0745	2469	PFL2_glycerol dehydratase family glycyl radical enzyme	MWXA01000004_1	128804	131272

				DDE84_10300	2403	formate C-acetyltransferase_glycerol dehydratase family glycyl radical enzyme	Bifidobacterium_spec_TMW21764_seq11	95423	97825
				DDF78_10180	2403	formate C-acetyltransferase_glycerol dehydratase family glycyl radical enzyme	lcl_Bifidobacterium_spec_TMW22057_seq11	95089	97491
DMG_70	100	no	32	BAQU_0746	1197	Proline racemase	MWXA01000004_1	132527	131331
				DDE84_10305	1179	proline racemase	Bifidobacterium_spec_TMW21764_seq11	99304	98126
				DDF78_10185	1179	proline racemase	lcl_Bifidobacterium_spec_TMW22057_seq11	98970	97792
DMG_71	100	no	23	BAQU_0747	579	transcriptional regulator	MWXA01000004_1	133102	132524
				DDE84_10310	579	TetR_AcrR family transcriptional regulator	Bifidobacterium_spec_TMW21764_seq11	100033	99455
				DDF78_10190	579	TetR_AcrR family transcriptional regulator	lcl_Bifidobacterium_spec_TMW22057_seq11	99699	99121
DMG_72	100	no	31	BAQU_0748	729	D-proline reductase _dithiol_ protein PrdB	MWXA01000004_1	133951	133223
				DDE84_10315	729	D-proline reductase _dithiol_ protein PrdB	Bifidobacterium_spec_TMW21764_seq11	101013	100285
				DDF78_10195	729	D-proline reductase _dithiol_ protein PrdB	lcl_Bifidobacterium_spec_TMW22057_seq11	100679	99951
DMG_73	100	no	29	BAQU_0749	189	hypothetical protein	MWXA01000004_1	134173	133985
				DDE84_10320	189	hypothetical protein	Bifidobacterium_spec_TMW21764_seq11	101328	101140
				DDF78_10200	189	hypothetical protein	lcl_Bifidobacterium_spec_TMW22057_seq11	100994	100806
DMG_74	100	no	31	BAQU_0750	1779	D-proline reductase _dithiol_ proprotein PrdA	MWXA01000004_1	136044	134266
				DDE84_10325	1785	D-proline reductase _dithiol_ proprotein PrdA	Bifidobacterium_spec_TMW21764_seq11	103188	101404
				DDF78_10205	1785	D-proline reductase _dithiol_ proprotein PrdA	lcl_Bifidobacterium_spec_TMW22057_seq11	102854	101070
DMG_75	100	no	31	BAQU_0751	1206	Respiratory-chain NADH dehydrogenase family protein	MWXA01000004_1	137274	136069
				DDE84_10330	1293	proline reductase-associated electron transfer protein PrdC	Bifidobacterium_spec_TMW21764_seq11	104589	103297
				DDF78_10210	1293	proline reductase-associated electron transfer protein PrdC	lcl_Bifidobacterium_spec_TMW22057_seq11	104255	102963
DMG_76	100	no	34	BAQU_0753	861	amino acid ABC transporter substrate-binding protein	MWXA01000004_1	139343	138483

				DDE84_04665	864	amino acid ABC transporter substrate-binding protein	Bifidobacterium_spec_TMW21764_seq4	73882	74745
				DDF78_04455	864	amino acid ABC transporter substrate-binding protein	lcl_Bifidobacterium_spec_TMW22057_seq4	73882	74745
DMG_77	100	no	40	BAQU_0754	633	amino acid ABC transporter permease	MWXA01000004_1	140110	139478
				DDE84_04660	660	amino acid ABC transporter permease	Bifidobacterium_spec_TMW21764_seq4	73059	73718
				DDF78_04450	660	amino acid ABC transporter permease	lcl_Bifidobacterium_spec_TMW22057_seq4	73059	73718
DMG_78	100	no	32	BAQU_0755	642	amino acid ABC transporter permease YckA1	MWXA01000004_1	140785	140144
				DDE84_04655	642	amino acid ABC transporter permease	Bifidobacterium_spec_TMW21764_seq4	72418	73059
				DDF78_04445	642	amino acid ABC transporter permease	lcl_Bifidobacterium_spec_TMW22057_seq4	72418	73059
DMG_79	100	yes	41	BAQU_0756	1239	acetylornithine aminotransferase	MWXA01000004_1	142462	141224
				DDE84_04650	1227	aspartate aminotransferase family protein	Bifidobacterium_spec_TMW21764_seq4	70562	71788
				DDF78_04440	1227	aspartate aminotransferase family protein	lcl_Bifidobacterium_spec_TMW22057_seq4	70562	71788
DMG_80	100	no	32	BAQU_0759	1083	aminotransferase	MWXA01000004_1	147002	145920
				DDE84_03040	1080	aminotransferase class I_II-fold pyridoxal phosphate-dependent enzyme	Bifidobacterium_spec_TMW21764_seq3	2723	1644
				DDF78_02830	1080	aminotransferase	lcl_Bifidobacterium_spec_TMW22057_seq3	2723	1644
DMG_81	100	no	47	BAQU_0811	519	transcriptional regulator	MWXA01000005_1	41261	40743
				DDE84_06485	558	ArsR family transcriptional regulator	Bifidobacterium_spec_TMW21764_seq6	56287	55730
				DDF78_06275	558	MarR family transcriptional regulator	lcl_Bifidobacterium_spec_TMW22057_seq6	56287	55730
DMG_82	100	no	39	BAQU_0812	1455	MFS transporter permease	MWXA01000005_1	41451	42905
				DDE84_06490	1425	MFS transporter	Bifidobacterium_spec_TMW21764_seq6	56574	57998
				DDF78_06280	1425	MFS transporter	lcl_Bifidobacterium_spec_TMW22057_seq6	56574	57998
DMG_83	100	no	36	BAQU_0846	930	periplasmic solute binding protein	MWXA01000005_1	86904	87833
				DDE84_04895	936	cation ABC transporter substrate-binding protein	Bifidobacterium_spec_TMW21764_seq4	129644	128709
				DDF78_04685	936	cation ABC transporter substrate-binding protein	lcl_Bifidobacterium_spec_TMW22057_seq4	129644	128709
DMG_84	100	no	87	BAQU_0847	912	ABC transporter	MWXA01000005_1	87830	88741
				DDE84_04890	978	metal ABC transporter ATP-binding protein	Bifidobacterium_spec_TMW21764_seq4	128702	127725

				DDF78_04680	978	ABC transporter ATP-binding protein	lcl_Bifidobacterium_spec_TMW22057_seq4	128702	127725
DMG_85	100	no	40	BAQU_0848	885	ABC transporter	MWXA01000005_1	88835	89719
				DDE84_04885	990	metal ABC transporter permease	Bifidobacterium_spec_TMW21764_seq4	127738	126749
				DDF78_04675	990	metal ABC transporter permease	lcl_Bifidobacterium_spec_TMW22057_seq4	127738	126749
DMG_86	100	no	27	BAQU_0849	771	ABC transporter	MWXA01000005_1	89730	90500
				DDE84_04880	792	metal ABC transporter permease	Bifidobacterium_spec_TMW21764_seq4	126756	125965
				DDF78_04670	792	metal ABC transporter permease	lcl_Bifidobacterium_spec_TMW22057_seq4	126756	125965
DMG_87	100	no	52	BAQU_0913	705	peptidase S24	MWXA01000005_1	165669	166373
				DDE84_00930	390	peptidase S24	Bifidobacterium_spec_TMW21764_seq1	220729	221118
				DDF78_00725	390	peptidase S24	lcl_Bifidobacterium_spec_TMW22057_seq1	137337	136948
DMG_88	100	no	23	BAQU_0941	1029	hypothetical protein	MWXA01000005_1	201745	202773
				DDE84_05195	1035	YdcF family protein	Bifidobacterium_spec_TMW21764_seq4	199120	200154
				DDF78_04985	1035	YdcF family protein	lcl_Bifidobacterium_spec_TMW22057_seq4	199120	200154
DMG_89	100	no	30	BAQU_0945	474	glutathione peroxidase	MWXA01000005_1	205288	204815
				DDE84_07450	567	glutathione peroxidase	Bifidobacterium_spec_TMW21764_seq7	78544	77978
				DDF78_09685	567	glutathione peroxidase	lcl_Bifidobacterium_spec_TMW22057_seq10	128965	129531
DMG_90	100	no	228	BAQU_0955	945	4-amino-4-deoxychorismate lyase	MWXA01000005_1	218096	219040
				DDE84_06860	963	aminodeoxychorismate lyase	Bifidobacterium_spec_TMW21764_seq6	148285	149247
				DDF78_06650	963	aminodeoxychorismate lyase	lcl_Bifidobacterium_spec_TMW22057_seq6	148285	149247
DMG_91	100	no	27	BAQU_0961	351	cysteine methyltransferase	MWXA01000005_1	226197	225847
				DDE84_11475	345	methylated-DNA--[protein]-cysteine S-methyltransferase	Bifidobacterium_spec_TMW21764_seq15	16631	16287
				DDF78_11525	345	cysteine methyltransferase	lcl_Bifidobacterium_spec_TMW22057_seq16	16631	16287
DMG_92	100	no	39	BAQU_1066	699	TENA_THI-4 family protein	MWXA01000005_1	344385	345083
				DDE84_03405	681	transcriptional regulator_ TENA_THI-4 family protein	Bifidobacterium_spec_TMW21764_seq3	68505	69185
				DDF78_03195	681	transcriptional regulator_ TENA_THI-4 family protein	lcl_Bifidobacterium_spec_TMW22057_seq3	68505	69185

DMG_93	100	no	38	BAQU_1067	819	Peptidase M50B-like	MWXA01000005_1	345073	345891
				DDE84_00830	804	M50 family peptidase	Bifidobacterium_spec_TMW21764_seq1	194507	195310
				DDF78_00825	804	M50 family peptidase	lcl_Bifidobacterium_spec_TMW22057_seq1	163559	162756
DMG_94	100	no	147	BAQU_1183	1083	permease	MWXA01000005_1	503959	505041
				DDE84_11115	1263	permease	Bifidobacterium_spec_TMW21764_seq13	63157	61895
				DDF78_10805	1263	permease	lcl_Bifidobacterium_spec_TMW22057_seq13	14724	15986
DMG_95	100	no	62	BAQU_1184	741	hypothetical protein	MWXA01000005_1	505045	505785
				DDE84_11110	843	TIGR03943 family protein	Bifidobacterium_spec_TMW21764_seq13	61895	61053
				DDF78_10810	843	TIGR03943 family protein	lcl_Bifidobacterium_spec_TMW22057_seq13	15986	16828
DMG_96	100	no	30	BAQU_1195	252	addiction module antitoxin RelB	MWXA01000005_1	519917	519666
				DDE84_06295	273	hypothetical protein	Bifidobacterium_spec_TMW21764_seq6	8774	9046
				DDE84_07030	252	type II toxin-antitoxin system RelB_DinJ family antitoxin	Bifidobacterium_spec_TMW21764_seq6	194589	194840
				DDF78_06085	273	hypothetical protein	lcl_Bifidobacterium_spec_TMW22057_seq6	8774	9046
				DDF78_06820	252	type II toxin-antitoxin system antitoxin_ RelB_DinJ family	lcl_Bifidobacterium_spec_TMW22057_seq6	194589	194840
DMG_97	100	no	62	BAQU_1238	1467	amidase	MWXA01000005_1	568449	566983
				DDE84_07000	1452	CHAP domain-containing protein	Bifidobacterium_spec_TMW21764_seq6	188151	186700
				DDF78_06790	1452	CHAP domain-containing protein	lcl_Bifidobacterium_spec_TMW22057_seq6	188151	186700
DMG_98	100	no	43	BAQU_1246	552	single-stranded DNA-binding protein	MWXA01000005_1	579048	578497
				DDE84_07065	741	single-stranded DNA-binding protein	Bifidobacterium_spec_TMW21764_seq6	201629	200889
				DDF78_06855	741	single-stranded DNA-binding protein	lcl_Bifidobacterium_spec_TMW22057_seq6	201629	200889
DMG_99	100	no	114	BAQU_1285	1074	acetoin reductase	MWXA01000005_1	626521	627594
				DDE84_07355	774	_S_-acetoin forming diacetyl reductase	Bifidobacterium_spec_TMW21764_seq7	55536	54763
				DDF78_09780	774	_S_-acetoin forming diacetyl reductase	lcl_Bifidobacterium_spec_TMW22057_seq10	151973	152746
DMG_100	100	no	37	BAQU_1292	468	Fur family transcriptional regulator	MWXA01000005_1	633306	632839
				DDE84_01530	870	transcriptional repressor	Bifidobacterium_spec_TMW21764_seq1	338958	339827
				DDF78_00120	870	hypothetical protein	lcl_Bifidobacterium_spec_TMW22057_seq1	19108	18239

DMG_101	100	no	31	BAQU_1293	597	Rubrerythrin	MWXA01000005_1	633409	634005
				DDE84_01525	450	hypothetical protein	Bifidobacterium_spec_TMW21764_seq1	338948	338499
				DDF78_00125	450	hypothetical protein	lcl_Bifidobacterium_spec_TMW22057_seq1	19118	19567
DMG_102	100	no	41	BAQU_1297	939	sugar ABC transporter permease	MWXA01000005_1	639867	638929
				DDE84_05565	957	carbohydrate ABC transporter permease	Bifidobacterium_spec_TMW21764_seq5	65284	66240
				DDF78_05780	957	carbohydrate ABC transporter permease	lcl_Bifidobacterium_spec_TMW22057_seq5	158530	157574
DMG_103	100	no	39	BAQU_1298	924	ABC transporter permease	MWXA01000005_1	640807	639884
				DDE84_05560	939	sugar ABC transporter permease	Bifidobacterium_spec_TMW21764_seq5	64303	65241
				DDF78_05785	939	sugar ABC transporter permease	lcl_Bifidobacterium_spec_TMW22057_seq5	159511	158573
DMG_104	100	no	29	BAQU_1299	1326	sugar-binding protein	MWXA01000005_1	642152	640827
				DDE84_05555	1326	extracellular solute-binding protein	Bifidobacterium_spec_TMW21764_seq5	62963	64288
				DDF78_05790	1326	sugar-binding protein	lcl_Bifidobacterium_spec_TMW22057_seq5	160851	159526
DMG_105	100	yes	80	BAQU_1311	876	6-phosphogluconate dehydrogenase	MWXA01000005_1	656120	656995
				DDE84_01020	876	decarboxylating 6-phosphogluconate dehydrogenase	Bifidobacterium_spec_TMW21764_seq1	241754	240879
				DDE84_07700	879	decarboxylating 6-phosphogluconate dehydrogenase	Bifidobacterium_spec_TMW21764_seq7	156985	157863
				DDF78_00635	876	decarboxylating 6-phosphogluconate dehydrogenase	lcl_Bifidobacterium_spec_TMW22057_seq1	116312	117187
				DDF78_09435	879	decarboxylating 6-phosphogluconate dehydrogenase	lcl_Bifidobacterium_spec_TMW22057_seq10	50524	49646
DMG_106	100	no	26	BAQU_1365	378	Camphor resistance CrcB protein	MWXA01000006_1	4237	4614
				DDE84_06305	561	CrcB family protein	Bifidobacterium_spec_TMW21764_seq6	10023	9463
				DDF78_06095	561	CrcB family protein	lcl_Bifidobacterium_spec_TMW22057_seq6	10023	9463
DMG_107	100	no	23	BAQU_1366	390	camphor resistance protein CrcB	MWXA01000006_1	4611	5000
				DDE84_06300	360	CrcB family protein	Bifidobacterium_spec_TMW21764_seq6	9466	9107
				DDF78_06090	360	camphor resistance protein CrcB	lcl_Bifidobacterium_spec_TMW22057_seq6	9466	9107
DMG_108	100	no	40	BAQU_1399	1848	Pyruvate oxidase	MWXA01000006_1	36463	38310

				DDE84_05060	1848	pyruvate oxidase	Bifidobacterium_spec_TMW21764_seq4	169249	171096
				DDF78_04850	1848	pyruvate oxidase	lcl_Bifidobacterium_spec_TMW22057_seq4	169249	171096
DMG_109	100	no	41	BAQU_1434	819	polar amino acid ABC transporter permease	MWXA01000006_1	82408	81590
				DDE84_09615	816	amino acid ABC transporter permease	Bifidobacterium_spec_TMW21764_seq10	51951	51136
				DDF78_01860	816	amino acid ABC transporter permease	lcl_Bifidobacterium_spec_TMW22057_seq2	51951	51136
DMG_110	100	no	27	BAQU_1435	654	amino acid ABC transporter permease	MWXA01000006_1	83052	82399
				DDE84_09620	654	amino acid ABC transporter permease	Bifidobacterium_spec_TMW21764_seq10	52601	51948
				DDF78_01865	654	amino acid ABC transporter permease	lcl_Bifidobacterium_spec_TMW22057_seq2	52601	51948
DMG_111	100	no	60	BAQU_1436	975	ABC transporter substrate-binding protein	MWXA01000006_1	84187	83213
				DDE84_09625	1008	ABC transporter substrate-binding protein	Bifidobacterium_spec_TMW21764_seq10	53977	52970
				DDF78_01870	1008	ABC transporter substrate-binding protein	lcl_Bifidobacterium_spec_TMW22057_seq2	53977	52970
DMG_112	100	no	46	BAQU_1456	1782	ABC transporter	MWXA01000006_1	106674	108455
				DDE84_05020	1896	ABC transporter ATP-binding protein	Bifidobacterium_spec_TMW21764_seq4	163070	161175
				DDF78_04810	1896	ABC transporter ATP-binding protein	lcl_Bifidobacterium_spec_TMW22057_seq4	163070	161175
DMG_113	100	yes	276	BAQU_1457	1797	multidrug ABC transporter ATP-binding protein	MWXA01000006_1	108452	110248
				DDE84_05015	1863	ABC transporter ATP-binding protein	Bifidobacterium_spec_TMW21764_seq4	161178	159316
				DDF78_04805	1863	multidrug ABC transporter ATP-binding protein	lcl_Bifidobacterium_spec_TMW22057_seq4	161178	159316
DMG_114	100	no	43	BAQU_1477	1410	N-formimino-L-glutamate deiminase	MWXA01000007_1	23099	21690
				DDE84_03835	1455	formimidoylglutamate deiminase	Bifidobacterium_spec_TMW21764_seq3	177210	175756
				DDF78_03625	1455	formimidoylglutamate deiminase	lcl_Bifidobacterium_spec_TMW22057_seq3	177209	175755
DMG_115	100	no	32	BAQU_1590	798	haloacid dehalogenase	MWXA01000008_1	4132	3335
				DDE84_09315	873	HAD family hydrolase	Bifidobacterium_spec_TMW21764_seq9	147606	148478
				DDF78_07770	873	TIGR01457 family HAD-type hydrolase	lcl_Bifidobacterium_spec_TMW22057_seq8	33083	32211
DMG_116	100	yes	412	BAQU_1595	1308	serine hydroxymethyltransferase	MWXA01000008_1	9625	8318
				DDE84_05680	1353	serine hydroxymethyltransferase	Bifidobacterium_spec_TMW21764_seq5	89280	90632
				DDF78_05665	1353	serine hydroxymethyltransferase	lcl_Bifidobacterium_spec_TMW22057_seq5	134534	133182
DMG_117	100	no	28	BAQU_1621	621	superoxide dismutase	MWXA01000008_1	41141	41761
				DDE84_00075	603	superoxide dismutase	Bifidobacterium_spec_TMW21764_seq1	18437	17835

				DDF78_01580	603	superoxide dismutase	lcl_Bifidobacterium_spec_TMW22057_seq1	339629	340231
DMG_118	100	yes	1635	BAQU_1624	2625	Hydroxymethylpyrimidine transport ATP-binding protein	MWXA01000008_1	45987	43363
				DDE84_10890	2664	ATP-binding cassette domain-containing protein	Bifidobacterium_spec_TMW21764_seq13	7927	10590
				DDF78_11030	2664	ABC transporter ATP-binding protein	lcl_Bifidobacterium_spec_TMW22057_seq13	69954	67291
DMG_119	100	no	30	BAQU_1698	867	DNA-binding protein	MWXA01000008_1	134434	133568
				DDE84_08495	888	KilA-N domain-containing protein	Bifidobacterium_spec_TMW21764_seq8	140185	141072
				DDF78_07480	888	KilA-N domain-containing protein	lcl_Bifidobacterium_spec_TMW22057_seq7	140185	141072
DMG_120	100	no	26	BAQU_1770	951	alcohol dehydrogenase	MWXA01000008_1	204213	205163
				DDE84_11120	960	aldo_keto reductase	Bifidobacterium_spec_TMW21764_seq13	63156	64115
				DDF78_10800	960	aldo_keto reductase	lcl_Bifidobacterium_spec_TMW22057_seq13	14725	13766
DMG_121	100	no	28	BAQU_1813	486	ArsC family transcriptional regulator	MWXA01000009_1	25361	25846
				DDE84_06795	372	arsenate reductase family protein	Bifidobacterium_spec_TMW21764_seq6	134752	135123
				DDF78_06585	372	arsenate reductase family protein	lcl_Bifidobacterium_spec_TMW22057_seq6	134752	135123
DMG_122	100	no	33	BAQU_1815	711	SDR family oxidoreductase	MWXA01000009_1	27888	27178
				DDE84_06320	711	SDR family NAD_P_-dependent oxidoreductase	Bifidobacterium_spec_TMW21764_seq6	13928	13218
				DDF78_06110	711	hypothetical protein	lcl_Bifidobacterium_spec_TMW22057_seq6	13928	13218
DMG_123	100	yes	249	BAQU_1816	1779	ABC transporter	MWXA01000009_1	29795	28017
				DDE84_06325	1722	ATP-binding cassette domain-containing protein	Bifidobacterium_spec_TMW21764_seq6	15681	13960
				DDF78_06115	1722	ABC transporter	lcl_Bifidobacterium_spec_TMW22057_seq6	15681	13960
DMG_124	100	yes	59	BAQU_1817	1812	ABC transporter permease	MWXA01000009_1	31611	29800
				DDE84_06330	1812	ABC transporter permease	Bifidobacterium_spec_TMW21764_seq6	17498	15687
				DDF78_06120	1812	ABC transporter permease	lcl_Bifidobacterium_spec_TMW22057_seq6	17498	15687
DMG_125	100	no	66	BAQU_1818	1176	ABC transporter substrate-binding protein	MWXA01000009_1	32895	31720
				DDE84_06335	1155	ABC transporter substrate-binding protein	Bifidobacterium_spec_TMW21764_seq6	18749	17595
				DDF78_06125	1155	ABC transporter substrate-binding protein	lcl_Bifidobacterium_spec_TMW22057_seq6	18749	17595
DMG_126	100	no	32	BAQU_1819	1038	uroporphyrinogen decarboxylase	MWXA01000009_1	33995	32958
				DDE84_06340	1041	uroporphyrinogen decarboxylase	Bifidobacterium_spec_TMW21764_seq6	19880	18840

				DDF78_06130	1041	uroporphyrinogen decarboxylase	lcl_Bifidobacterium_spec_TMW22057_seq6	19880	18840
DMG_127	100	no	41	BAQU_1826	792	DNA polymerase III subunit epsilon	MWXA01000009_1	40208	40999
				DDE84_08795	753	DNA polymerase III subunit epsilon	Bifidobacterium_spec_TMW21764_seq9	30160	29408
				DDF78_08290	753	DNA polymerase III subunit epsilon	lcl_Bifidobacterium_spec_TMW22057_seq8	150529	151281
DMG_128	100	no	24	BAQU_1828	1245	carboxylate--amine ligase	MWXA01000009_1	42852	44096
				DDE84_08785	1245	carboxylate--amine ligase	Bifidobacterium_spec_TMW21764_seq9	27703	26459
				DDF78_08300	1245	carboxylate--amine ligase	lcl_Bifidobacterium_spec_TMW22057_seq8	152986	154230
DMG_129	100	no	33	BAQU_1829	711	aspartate racemase	MWXA01000009_1	44093	44803
				DDE84_08780	723	amino acid racemase	Bifidobacterium_spec_TMW21764_seq9	26462	25740
				DDF78_08305	723	aspartate racemase	lcl_Bifidobacterium_spec_TMW22057_seq8	154227	154949
DMG_130	100	no	29	BAQU_1864	714	phenazine biosynthesis protein PhzF	MWXA01000009_1	81750	81037
				DDE84_05605	840	PhzF family phenazine biosynthesis protein	Bifidobacterium_spec_TMW21764_seq5	76453	75614
				DDF78_05740	840	phenazine biosynthesis protein PhzF	lcl_Bifidobacterium_spec_TMW22057_seq5	147361	148200
DMG_131	100	no	31	BAQU_1884	876	3-hydroxybutyryl-CoA dehydrogenase	MWXA01000009_1	96229	97104
				DDE84_07070	849	3-hydroxyacyl-CoA dehydrogenase	Bifidobacterium_spec_TMW21764_seq6	202703	201855
				DDF78_06860	849	3-hydroxyacyl-CoA dehydrogenase	lcl_Bifidobacterium_spec_TMW22057_seq6	202703	201855
DMG_132	100	no	37	BAQU_1901	1371	sugar_proton symporter	MWXA01000009_1	119985	118615
				DDE84_03115	1350	L-fucose_H+ symporter permease	Bifidobacterium_spec_TMW21764_seq3	17756	16407
				DDF78_02905	1350	L-fucose_H+ symporter permease	lcl_Bifidobacterium_spec_TMW22057_seq3	17756	16407
DMG_133	100	no	49	BAQU_1905	963	polyprenyl synthetase	MWXA01000009_1	122707	123669
				DDE84_05190	990	polyprenyl synthetase family protein	Bifidobacterium_spec_TMW21764_seq4	198940	197951
				DDF78_04980	990	hypothetical protein	lcl_Bifidobacterium_spec_TMW22057_seq4	198940	197951
DMG_134	100	yes	75	BAQU_1906	1884	thiol reductant ABC exporter subunit CydC	MWXA01000009_1	125599	123716
				DDE84_05185	1908	thiol reductant ABC exporter subunit CydC	Bifidobacterium_spec_TMW21764_seq4	196012	197919
				DDF78_04975	1908	thiol reductant ABC exporter subunit CydC	lcl_Bifidobacterium_spec_TMW22057_seq4	196012	197919
DMG_135	100	yes	58	BAQU_1907	1839	cydD_ cytochrome D ABC transporter ATP-binding and permease component	MWXA01000009_1	127406	125568
				DDE84_05180	1797	thiol reductant ABC exporter subunit CydD	Bifidobacterium_spec_TMW21764_seq4	194199	195995

				DDF78_04970	1797	thiol reductant ABC exporter subunit CydD	lcl_Bifidobacterium_spec_TMW22057_seq4	194199	195995
DMG_136	100	no	26	BAQU_1908	1026	cytochrome C oxidase assembly protein	MWXA01000009_1	128528	127503
				DDE84_05175	1044	cytochrome d ubiquinol oxidase subunit II	Bifidobacterium_spec_TMW21764_seq4	192970	194013
				DDF78_04965	1044	cytochrome d ubiquinol oxidase subunit II	lcl_Bifidobacterium_spec_TMW22057_seq4	192970	194013
DMG_137	100	no	38	BAQU_1909	1470	cydA_ cytochrome d ubiquinol oxidase_ subunit 1	MWXA01000009_1	129994	128525
				DDE84_05170	1542	cytochrome ubiquinol oxidase subunit I	Bifidobacterium_spec_TMW21764_seq4	191432	192973
				DDF78_04960	1542	cytochrome ubiquinol oxidase subunit I	lcl_Bifidobacterium_spec_TMW22057_seq4	191432	192973
DMG_138	100	no	31	BAQU_1910	1260	pyridine nucleotide-disulfide oxidoreductase	MWXA01000009_1	131304	130045
				DDE84_05165	1176	NAD_P_FAD-dependent oxidoreductase	Bifidobacterium_spec_TMW21764_seq4	190068	191243
				DDF78_04955	1176	NAD_P_FAD-dependent oxidoreductase	lcl_Bifidobacterium_spec_TMW22057_seq4	190068	191243
DMG_139	100	no	21	BAQU_1920	852	beta-hydroxyacid dehydrogenase_ 3-hydroxyisobutyrate dehydrogenase	MWXA01000010_1	2869	3720
				DDE84_02820	972	NAD_P_-dependent oxidoreductase	Bifidobacterium_spec_TMW21764_seq2	277009	277980
				DDE84_05755	837	NAD_P_-dependent oxidoreductase	Bifidobacterium_spec_TMW21764_seq5	107831	106995
				DDF78_05590	837	NAD_P_-dependent oxidoreductase	lcl_Bifidobacterium_spec_TMW22057_seq5	115983	116819
				DDF78_09040	972	NAD_P_-dependent oxidoreductase	lcl_Bifidobacterium_spec_TMW22057_seq9	130391	131362
DMG_140	100	no	87	BAQU_1946	936	aldo_keto reductase	MWXA01000010_1	37344	36409
				DDE84_09945	939	aldo_keto reductase	Bifidobacterium_spec_TMW21764_seq10	136625	135687
				DDF78_02190	939	aldo_keto reductase	lcl_Bifidobacterium_spec_TMW22057_seq2	136625	135687
DMG_141	100	no	23	BAQU_1947	1905	asparagine synthetase B	MWXA01000010_1	39436	37532
				DDE84_06780	1884	asparagine synthase _glutamine-hydrolyzing_	Bifidobacterium_spec_TMW21764_seq6	132822	130939
				DDF78_06570	1884	asparagine synthase _glutamine-hydrolyzing_	lcl_Bifidobacterium_spec_TMW22057_seq6	132822	130939
DMG_142	100	no	36	BAQU_1971	597	chromosome partitioning protein parA	MWXA01000014_1	552	1148
				BAQU_2008	597	chromosome partitioning protein parA	MWXA01000014_1	33365	33961
				DDE84_04325	597	ParA family protein	Bifidobacterium_spec_TMW21764_seq4	7097	7693
				DDF78_04115	597	ParA family protein	lcl_Bifidobacterium_spec_TMW22057_seq4	7097	7693
DMG_143	100	no	38	BAQU_1973	378	hypothetical protein	MWXA01000014_1	1513	1890
				DDE84_04335	360	hypothetical protein	Bifidobacterium_spec_TMW21764_seq4	8130	8489

	DDF78_04125	372	hypothetical protein	lcl_Bifidobacterium_spec_TMW22057_seq4	8118	8489