TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Technische Mikrobiologie

Metabolic activity and symbiotic interaction of bacteria and yeasts in water kefir

Jasmin Stadie

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: Univ.-Prof. Dr. W. Liebl

Prüfer der Dissertation:

1. Univ.-Prof. Dr. R. F. Vogel
2. Univ.-Prof. Dr. S. Scherer

Die Dissertation wurde am 20.06.2013 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 12.11.2013 angenommen.

STATEMENTS BELONGING TO THE DISSERTATION

I. Independent of the origin of the consortium and the related different distribution of microbes the resulting beverages are similar in their content of major metabolic compounds. *(this thesis)*

II. Grain EPS is not used as storage compound, but rather to ensure a stable order inside the consortium. *(this thesis)*

III. Based on limited nitrogen content in water kefir medium, isolates compete for it and rely on support of each other. *(this thesis)*

IV. Co-cultivation experiments with yeasts and lactobacilli isolated from water kefir delineates mutualism in water kefir. *(this thesis)*

V. Enhanced formation of metabolites and glycolytic proteins demonstrates the increasing metabolic activity of single organisms during co-cultivation. *(this thesis)*

VI. Lactobacilli optimize the physical environment for *Z. florentina* while acidifying the milieu. *(this thesis)*

VII. Co-cultivated yeasts are induced to supply amino acids and vitamins, which are essential for lactobacilli. It appears that lactobacilli "domesticate" yeasts to enable a better living. *(this thesis)*

VIII. Wenn das Wohl der Gemeinschaft über dem Leben des Einzelnen steht…

IX. „Einer für alle, alle für einen" (Die drei Musketiere)

VORWORT UND DANKSAGUNG

Viele Menschen haben mich auf dem Weg zu dieser Arbeit unterstützt. Meiner gesamten Familie, allen Freunden, Arbeitskollegen und Bekannten sei an dieser Stelle gedankt.

Die vorliegende Arbeit entstand im Rahmen von aus Haushaltsmitteln des BMWi über die Arbeitsgemeinschaft industrieller Forschungsvereinigungen, Otto von Guericke Stiftung und der Wissenschaftsförderung der Deutschen Brauwirtschaft geförderten Projektes (AiF 16454 N).

Mein größter Dank gilt meinem Doktorvater Prof. Dr. Rudi F. Vogel, der mir die Möglichkeit bot die Arbeit an seinem Lehrstuhl anzufertigen. Ich danke für die Anregungen, Ideen, Diskussionen, Bereitstellung der finanziellen Mittel, wertvollen Tips, Unterstützung zu Verzweiflungszeiten und für seine stetige Verfügbarkeit. Das entgegengebrachte Vertrauen auch bezüglich organisatorischer Aufgaben war eine ideale Vorbereitung auf das „echte“ Arbeitsleben.

Weiterhin möchte ich mich bei Prof. Dr. Siegfried Scherer für die Begutachtung meiner Arbeit und Prof. Dr. Wolfgang Liebl für die Übernahme des Prüfungsvorsitzes bedanken.

Prof. Dr. Matthias Ehrmann danke ich für zahlreiche anregende Diskussionen. Seine Hilfsbereitschaft war ein wichtiger Faktor bei der Entstehung dieser Arbeit.

Besonderer Dank ist zu richten an Dr. Jürgen Behr. Vielen Dank für die unermüdliche Hilfsbereitschaft, für die stetige Annahme der Telefonanrufe und für die Weitergabe seines umfangreichen Wissens im Bereich der HPLC-Analytik.

Desweiteren möchte ich mich bei der gesamten TMW-Gemeinschaft für das kollegiale und angenehme Betriebsklima bedanken. Angela Seppeur, Monika Hadek und Sabine Dummert danke ich für die Übernahme jeglicher organisatorischer Arbeiten.

Ein herzliches Dankeschön richte ich an Patrick Preissler, Anna Gulitz, Carola Kern, Julia Usbeck, Juliane Schnabel, Benjamin Schurr, Christian Lenz, Ekaterina Minenko, Frank Jakob und Claudia Specker. Als Arbeitskollegen trugen sie durch wertvolle

Diskussionen zum Gelingen der Arbeit bei. Viel wichtiger ist mir aber der Dank an Euch als wundervolle Freunde. Mit Eurem seelischen und moralischen Rückhalt war die, zu mancher Zeit Nerven aufreibende, Arbeit so weit von der Heimat um so vieles einfacher.

Besonderer Dank gilt dabei Anna: Einen besseren Symbionten im Konsortium hätte ich mir nicht vorstellen können.

Meiner gesamten Familie möchte ich für ihre Unterstützung während meiner Studiums- und Promotionszeit danken. Besonders danke ich meiner Mama, meinem Papa und Onkel Horst für ihre stetige Hilfsbereitschaft in allen Lebenslagen.

Ganz besonders möchte ich Patrick danken, der mir zu jeder Tages- und Nachtzeit bei fachlichen Fragen zur Verfügung stand und in schwierigen Phasen mich stetig emotional unterstützte. Selbst an angespannten Tagen schaffte er es mich mit seinem Humor zu ermuntern.

Eine Mischung aus Rot und Blau.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

2D	two-dimensional
A	ampere
A.	*Acetobacter*
AAB	acetic acid bacteria
APS	ammonium persulfate
ATCC	American type culture collection, Manassas, Virginia, USA
ATP	adenosintriphosphate
BLAST	basic local alignment search tool
B.	*Bifidobacterium*
Da	Dalton
dH_2O	demineralized water
DNA	desoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EPS	exopolysaccharide
eV	electron volt
FD	fast digestion
Fig.	figure
FMOC	9-fluorenylmethyl chloroformate
g	g-force
G.	*Gluconobacter*
GC-MS	gas chromatography mass spectrometry
h	hour
HePS	heteropolysaccharide
HoPS	homopolysaccharide
HPLC	high-performance liquid chromatography
IEF	iso-electrical focusing
IPG	immobilized pH gradient
l	liter
LAB	lactic acid bacteria
Lb.	*Lactobacillus*

Lc.	*Leuconostoc*
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
m	milli (10^{-3}), meter
M	mega (106), molar
min	minute
Mw	molecular weight
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
NCBI	National center for Biotechnology Information
OD	optical density
OPA	o-phtalaldehyd-3-mercaptopropionic acid
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pI	isoelectric point
s	second
SDS	sodium n-dodecylsulfate
SPME	solid phase microextraction
TE	Tris, EDTA
TEMED	Tetramethylethylendiamin
THF	Tetrahydrofuran
TMW	Technische Mikrobiologie Weihenstephan
Tris	tris (hydroxymethyl) aminomethan
V	volt
µ	Micro (10^{-6})

1 INTRODUCTION

1.1 Water kefir

Water kefir is a stable microbial multispecies community of different microorganisms, which is used for preparing a homemade fermented beverage. For beverage preparation grains are plunged into a sucrose solution (8%) supplemented with dried or fresh fruits, best figs (Reiß, 1990) and fermented at room temperature for two or three days. Lemon slices can be added to receive a fresh flavor but they are not necessary for the growth of the consortium. After fermentation the grains can be removed by sieving and the supernatant is potable. Subsequently, grains are washed with tap water and reused for the next fermentation step. The resulting beverage is fizzy and cloudy, carbonated, low acid, somewhat sweet and slightly alcoholic, depending on how long it was fermented. The origin of water kefir is unknown. First description of similar grains called “Ginger-beer Plant” was made by Ward in 1892 (Ward, 1892). He reported that the grains were brought back by British soldiers from the Crimean war in 1855. Other descriptions are linking the grains (called Tibi) to the Mexican cacti of the genus “*Opuntia*” where they were taken off the leaves (Lutz, 1899). Until today various synonyms are known, thus this symbiosis is also called “California bees”, “African bees”, “Ale nuts”, “Balm of Gilead”, “Japanese Beer Seeds” or “Sugary kefir grains” (Kebler, 1921; Pidoux et al., 1988). A few studies determined the microbial species that are located inside the grains as a stable consortium of several lactobacilli, acetic acid bacteria and yeasts (Franzetti et al., 1998; Horisberger, 1969; Lutz, 1899; Moinas et al., 1980; Neve & Heller, 2002; Pidoux, 1989; Stadelmann, 1957; Ward, 1892). Gulitz et al. (2011) could illustrate that the consortium of water kefir is comprised of 10^8 lactobacilli, 10^6 – 10^8 acetic acid bacteria and 10^6 – 10^7 yeasts per gram grains. Additionally, yet uncultivable bifidobacteria could be detected in several water kefir grains from different origin (Gulitz et al., 2013). These uncultivable organisms display obligate synergism between microbiota in the water kefir consortium. Microorganisms are embedded in transparent, crushed-ice-shaped grains which mainly consist of an insoluble dextran with α-1,6-linked glucose and α-1,3-branching (Horisberger, 1969; Pidoux et al., 1988). Pidoux et al. (1988) and Waldherr et al.

(2010) displayed *Lb. hilgardii* as the important species for EPS and therefore grain formation during water kefir fermentation.

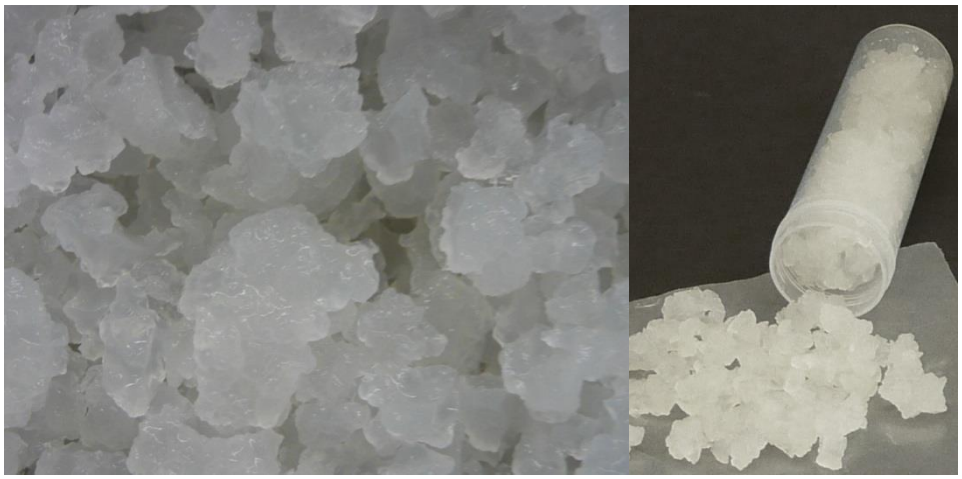


Fig. 1: Water kefir grains that can be used as starter cultures for water kefir fermentation

Fig. 2: Ingredients for water kefir fermentation (from left: mineral water, figs, water kefir grains, sugar) and the resulting beverage (right))

Leroi and Pidoux (Leroi & Pidoux, 1993a) determined as first the synergism of water kefir isolates, namely the interaction of *Lb. hilgardii* and *S. florentinus* (reclassified as *Zygotorulaspora (Z.) florentina* (Kurtzman, 2003)). In mixed culture *Lb. hilgardii* was supported in better survival and lactic acid production, but growth of *S. florentinus* was drastically reduced, so they found a parasitism interaction between these water kefir organisms. They could show that CO_2, pyruvate, propionate, acetate and succinate, thus metabolites of the yeast were responsible for the benefits of *Lb. hilgardii*. On the other hand, they could display that the combination of *Lb. hilgardii* and *Candida lambica* did not reveal a

stimulation, quite the contrary, immobilized in calcium alginate beads bacterial growth and lactic acid production was inhibited (Leroi & Pidoux, 1993a, 1993b).

1.2 Sugar metabolism of water kefir organisms

1.2.1 Lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) are Gram-positive bacteria, which use carbohydrates as energy source by producing lactic acid. The genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Carnobacterium*, *Enterococcus*, *Lactococcus* and *Vagococcus* belong to the group of lactic acid bacteria (Jay, 1992a). In water kefir several species of the first two genera could be found (Gulitz et al., 2013). Metabolism of sugars is divided into two groups, homofermentative and heterofermentative LAB. Homofermentative LAB catabolize glucose via the Emden-Meyerhof pathway with lactate as their main end product (at least 85% lactate from 100% glucose; see Fig. 3). Due to the lack of aldolase and triose phosphate isomerase heterofermentative LAB metabolize glucose by the production of equimolar amounts of lactate, ethanol and carbon dioxide via the pentose phosphate pathway. During these reactions only one mol ATP results out of one mol glucose (pathway [A] Fig. 4), whereas while homolactic fermentation 2 mol ATP are produced. In the presence of electron accepting substances like fructose, citrate, malate, fumarate, oxygen or unsaturated fatty acids (Stolz, Böcker et al., 1995) and at a given enzyme equipment (acetate kinase) acetate and ATP can be built instead of ethanol (pathway [B] Fig. 4). Under these conditions heterolactic fermentation of glucose also results in 2 mol ATP. Facultative heterofermentative lactobacilli ferment hexoses to lactate as homofermentative bacteria, but they are additionally able to produce ethanol and lactate without gas formation out of pentoses. This kind of fermentation was not detected for water kefir organisms. Table 1 diplays homo- and heterofermentative species of LAB those are included in the water kefir consortium.

Sucrose transport into the cells can occur mainly by a phosphotransferase system (PTS) with simultaneous phosphorylation of sucrose to sucrose-6-phosphate by phosphoenolpyruvate (PEP) or alternatively via a cation symport system (Kaditzky, 2008).

Table 1: Homo- and heterofermentative species in water kefir (Gulitz et al., 2013, 2011)

homofermentative LAB in water kefir	heterofermentative LAB in water kefir
Lb. hordei	*Lc. mesenteroides*
Lb. nagelii	*Lc. citreum*
Lb. casei	*Lb. hilgardii*
Lb. satsumensis	

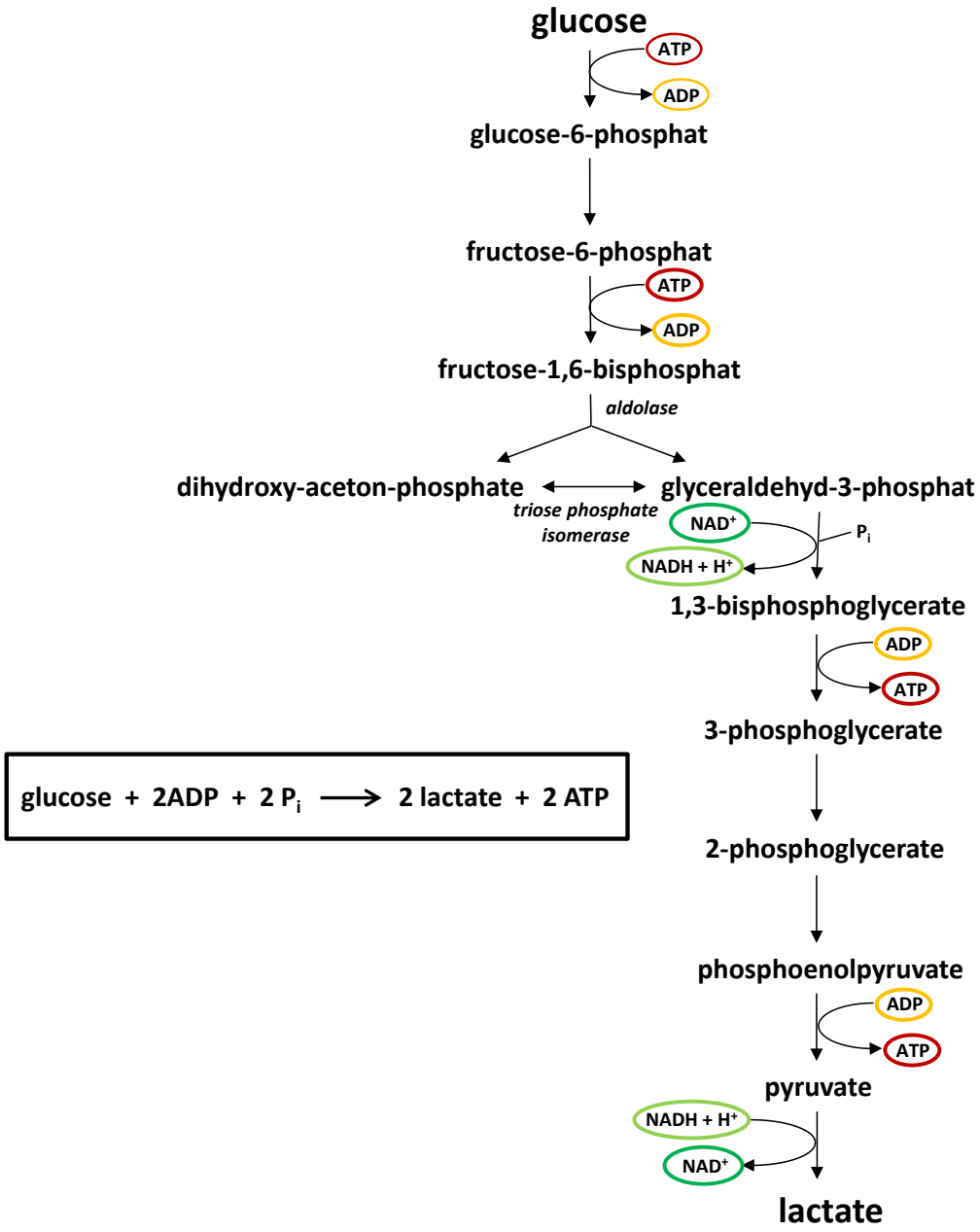
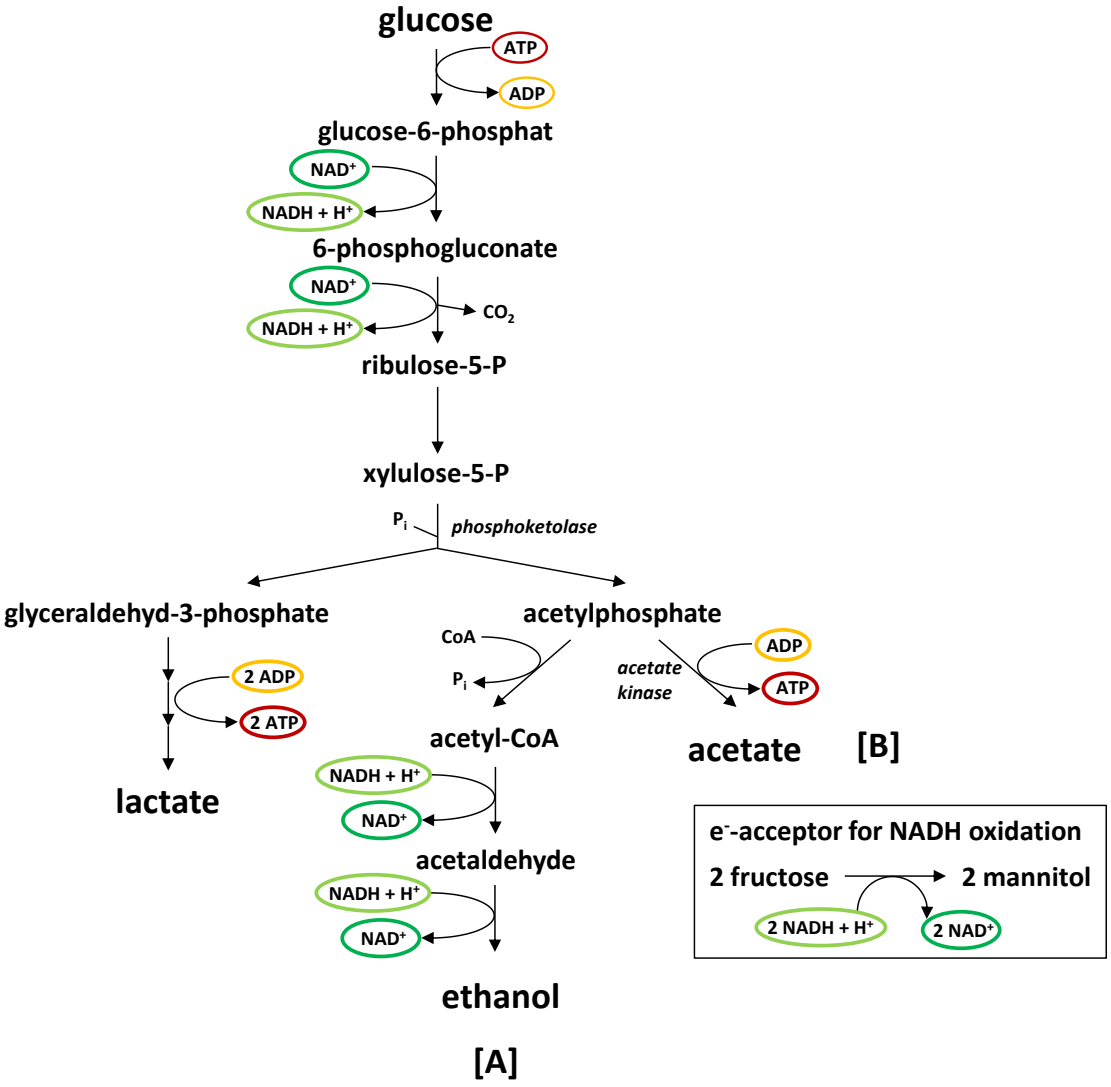


Fig. 3: Lactic acid fermentation of homofermentative LAB

(Doenecke et al., 2005; Goyal, 1999; modified)



[A] glucose + ADP + P_i ⟶ lactate + ethanol + CO_2 + ATP

[B] glucose + 2 fructose + 2 ADP + 2 P_i ⟶ lactate + acetate + CO_2 + 2 ATP + 2 mannitol

Fig. 4: Lactic acid fermentation of heterofermentative LAB

(Goyal, 1999; modified)

1.2.2 Acetic acid bacteria (AAB)

Acetic acid bacteria are Gram-negative, acid resistant (up to pH 2.6) and obligate aerobes, including the Genera of *Acetobacter* and *Gluconobacter*. *Gluconobacter* generate their energy via the incomplete oxidation of carbohydrates or alcohols resulting in the corresponding acid forms (e.g. gluconic acid out of glucose and acetid acid out of ethanol) (Jakob et al., 2012; Krämer, 2007). *Acetobacter sp.* are further able to oxidize acetic acid to CO_2 and water. AAB can be naturally found in carbohydrate or ethanol-rich plant habitats. Today AAB are very important for the commercial production of acetic acid (Gullo & Giudici, 2008; Krämer, 2007) whereas this is exactly why they are unwanted during wine fermentation. Many AAB strains could be identified as fructan producers, their EPS only consists of fructose monomers (Jakob et al., 2013, 2012).

During this work, AAB played a minor role, since they could only be found in low numbers in water kefir grains, probably because oxygen is limited in this environment and only offered in the washing and re-starting procedure (Gulitz et al., 2013).

1.2.3 Bifidobacteria

Bifidobacteria are Gram-positive, saccharolytic microorganisms. Lactic acid is one of their major metabolites, therefore, they were classified as lactic acid bacteria for long time. Due to their phylogenetic and metabolic differences to lactic acid bacteria they were separated in 1974 (reviewed by Ballongue, 1993). Monosaccharides are metabolized within the so called “bifidus shunt” that is different to the homo- and heterofermentative pathway of lactic acid bacteria (Fig. 5). Instead of aldolase and glucose-6-phosphat dehydrogenase bifidobacteria produce fructose-6-phosphate phosphoketolase, the key enzyme in their metabolic pathway and the taxonomic marker for *Bifidobacteriaceae*. Similar to the homofermentative pathway the bifidus shunt starts with the convertion of G-6-P to F-6-P by G-6-P isomerase. Afterwards F-6-P is divided into acetylphosphate and erythrose-4-phosphate. At the end the bifidus shunt yields in 1.5 mol acetate, 1 mole lactate and 2.5 mol ATP out of 1 mol glucose (Kaditzky, 2008; Pokusaeva et al., 2011; de Vries and Stouthamer, 1967). Based on the ability to utilize different types of oligosaccharides bifidobacteria are adapted to specific niches and therefore they are able to survive in demanding habitats. Bifidobacteria are part of the bacterial colonization of human and animal gastrointestinal tract. Especially high amounts of bifidobacteria could be found in faeces of infants fed with breast milk due to their

metabolism of non-digestible human milk oligosaccharides (reviewed by Pokusaeva et al., 2011). Beside their utilization of oligosaccharides, bifidobacteria are able to inhibit pathogens by acid production resulting in a pH lowered milieu, by the production of bacteriocins and additionally by blocking the adhesion receptors for pathogens and toxins. Thus, bifidobacteria are combined with beneficial health effects and often used as probiotics in foods (Collado et al., 2005; Macfarlane and Englyst, 1986; Rastall et al., 2005; de Vries and Stouthamer, 1968).

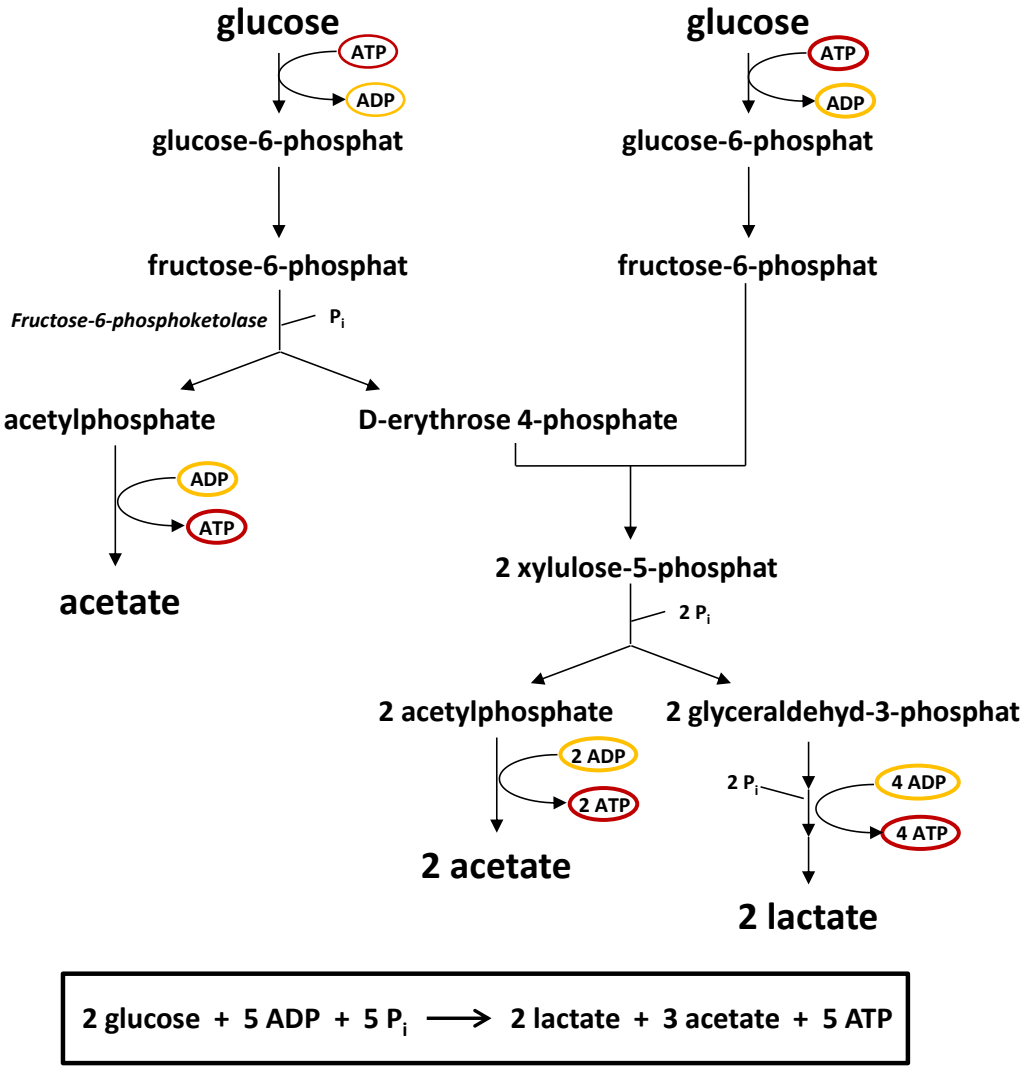


Fig. 5: Glucose metabolism of bifidobacteria, so called "bifidus shunt"

Most *Bifidobacterium* species could be isolated out of the human and animal intestine and they usually grow strict anaerobically at 37°C, as it is usual for this habitat. With culture independent detection methods some *Bifidobacterium* sp. could be detected in milk kefir as well as water kefir grains (Dobson et al., 2011; Gulitz et al., 2013). *B. psychraerophilum*, the single species that was yet cultivable out of water kefir grains (Gulitz et al., 2013) is an uncommon *Bifidobacterium* species. As this species is able to grow at low temperatures (until 4°C) and under aerobic conditions it is called *psychraerophilum* ("cold- and air-loving"). Still it shows its growth optimum anaerobically at 37°C (Simpson et al., 2004).

1.2.4 Yeasts

As major energy source yeasts catabolize glucose in the general glycolysis pathway to pyruvate. In the absence of oxygen NADH has to be re-oxidized during ethanolic fermentation. Upon fermentation pyruvate is converted to acetaldehyde and CO_2 and afterwards acetaldehyde is reduced to ethanol catalyzed by alcohol dehydrogenase (Dickinson & Kruckeberg, 2006). In the presence of oxygen and absence of repression pyruvate can be respirated to CO_2 and energy in form of ATP. Therefore, pyruvate is transported into the mitochondria, converted to acetyl-CoA and oxidized via the citric acid cycle. Received reducing equivalents during glycolysis and the citric acid cycle, namely NADH and $FADH_2$, are re-oxidized while respiratory chain in order to produce energy in form of ATP (Dickinson & Kruckeberg, 2006; Feldmann, 2005). The citric acid cycle is not only a catabolic pathway for energy production but also an anabolic part of the production of intermediates for amino acid and nucleotide formation (Feldmann, 2005). *Saccharomyces (S.) cerevisiae*, a member of water kefir grains, is a facultative anaerobic yeast. These yeasts are able to catabolize glucose aerobically and anaerobically. Anaerobically they ferment glucose to ethanol as described above. Respiration in a glucose-containing medium (more than 0.1 %) from *S. cerevisiae* is limited and counts less than 10% of glucose catabolism because of the so called Crabtree effect or glucose repression. In the presence of glucose respiratory and gluconeogenic enzymes are not synthesized anymore and emerging pyruvate is therefore channeled to ethanol even in the presence of oxygen (Barnett & Entian, 2005; Gancedo & Serrano, 1989).

The formation of succinate by yeasts can be caused in different ways. On the one hand the oxidative pathway of the citric acid cycle is interrupted at the level of succinate within fermenting yeasts under aerobic conditions (reviewed by de Klerk, 2010; Gancedo and

Serrano, 1989). Reduced equivalents can be recycled e.g. by the production of mannitol while electron upload to fructose (Lee et al., 2003). On the other hand in the absence of oxygen the citric acid cycle can function in the reductive pathway from oxaloacetate over malate and fumarate to succinate (reviewed by De Klerk, 2010).

The uptake of hexoses into the cells is regulated by permeases induced hexose transport systems (Dickinson & Kruckeberg, 2006; Gancedo & Serrano, 1989). Water kefir medium contains of glucose and fructose but the main sugar used for the preparation is sucrose. The disaccharide sucrose is hydrolyzed extracellularly by invertase of yeast to fructose and glucose (Dickinson & Kruckeberg, 2006; Feldmann, 2005). Afterwards, these hexoses are transported to the cells as described above.

Zygosaccharomyces species are osmotolerant yeasts and therefore they are able to grow on substrates with high sugar concentrations (Dickinson & Kruckeberg, 2006). Water kefir medium is with its high sugar (approx. 90 g/l in total) and low amino acid concentration a demanding habitat for microorganisms. *Zygotorulaspora florentina*, the main representative yeast in water kefir grains (Gulitz et al., 2011) was called *Zygosaccharomyces florentinus* before it was reclassified in 2003 (Kurtzman, 2003: reclassification along multigene sequence analysis, in contrast to the previous phenotype classification), thus it is known as an osmotolerant yeast. These species can cause food spoilage but during water kefir fermentation they ferment desirably.

1.3 Exopolysaccharides (EPS)

Polysaccharides are high-molecular-weight compounds, which consist of glycosidically linked monosaccharides. In nature, polysaccharides are wide spread as structure forming substances (e.g. cellulose or chitin), as reserve materials (e.g. starch or glycogen) and as water-binding substances (e.g. agar or pectin). Functional properties depend on different linkage types and branching as well as their weight and sugar monomers (Belitz et al., 2001).

The first description of microbial polysaccharides was made in 1839, where Kircher examined organisms that form slimy structures when they were grown on sucrose-containing medium (Jay, 1992b). Microbial polysaccharides that are excreted extracellularly are called exopolysaccharides (EPS). EPS in use as nutritional storage seemed to be unlikely because most bacteria are not able to metabolize their own EPS (Cerning, 1990). More probably, EPS are produced as protective agents against dehydration, different attacks like toxins, antibiotics or phages, predation by protozoans and osmotic stress. Another function is that bacteria can

adhere to solid surfaces with EPS to build biofilm formations (De Vuyst and Degeest, 1999). For example dental plaque is a complex biofilm out of different EPS. Fructans can be used as energy store for other bacteria in the consortium and glucans are important as adhesion- and aggregation factors for plaque bacteria colonization (Russel, 2009).

EPS that are associated to the cell surface display a capsular appearance whereas free diffusible EPS are slimy. In some cases both types can be produced by the same organism (Cerning, 1990). Additionally EPS can be classified along their sugar monomer composition. Heteropolysaccharides (HePS) consist of different sugar monomers with repeating precursor units. These units are synthesized intracellularly and polymerized extracellularly. This is an energy-dependent process (De Vuyst and Degeest, 1999). HePS are important for the mouthfeel and rheology in fermented milk products, for example kefiran (50 % glucose and 50 % galactose) in milk kefir (La Rivière & Kooiman, 1967).

In contrast, Homopolysaccharides (HoPS) consist of one type of monomer. EPS composed of glucose are called glucans, those out of fructose fructans. Furthermore, HoPS can be differentiated along their dominant linkage types. α-D-glucans with α-1,6-linkages and branches at positions 3, 2 and 4 are called dextrans, whereas branching is strain specific. Strains of several *Leuconostoc sp*. are known to produce dextrans. Mutans that are produced by streptococci, mainly display α-1,3-linkages, and alternan that is produced by different strains of *Leuconostoc mesenteroides* contains both linkage types alternating. Representatives of the fructans are levans, with β-2,6-linkages and β-2,1 branching, and inulin with β-2,1 as main linkage type (De Vuyst and Degeest, 1999; Waldherr, 2009). Homopolysaccharides are synthesized by glycosyltransferases, whereas glucansucrases catalyze the production of glucans and fructansucrases these of fructans. The enzymes can occur cell wall-bound or extracellular. Substrates for polymerization can be sucrose or raffinose, whereas raffinose can only be used by fructansucrases (Van Geel-Schutten et al., 1999). Energy that is needed for the transfer reaction of sugar monomers is released upon cleavage of the glycosidic bond of sucrose. One sugar unit (fructose or glucose) is then transferred to an acceptor molecule while the other one is released to the medium. If water reacts as acceptor molecule glycolsyltransferases show their hydrolysis activity resulting in free glucose and fructose. Oligosaccharides are produced if small sugar units (maltose, sucrose) react as acceptor molecules, whereas sucrose can only be used by fructosyltransferases resulting in kestose or nystose. This function of glycosyltransferases is called acceptor reaction. The transferase activity of glycosyltransferases leads to EPS because sugar monomers are transferred to growing polysaccharide chains (Kaditzky, 2008; Waldherr, 2009).

Strains of several species of lactic acid bacteria are able to produce EPS during fermentation. While milk fermentation, produced EPS affect texture, mouthfeel, taste perception and stability of the end product (e.g. yoghurt, milk kefir, Nordic ropy milk or cheese) (reviewed by Jolly et al., 2002). Jakob et al. (2012) could demonstrate in baking experiments with bacterial fructans an improvement on wheat bread in a larger volume, softer bread and decelerated senescence. But not in all cases EPS are admired. For example, slime producing *Leuconostocaceae* or acetic acid bacteria cause tube closing and loss of sugar in sugar industry or spoilage of sugared soft drinks resulting in a slimy structure of the drink (De Vuyst and Degeest, 1999; Korakli and Vogel, 2006).

1.4 Symbiosis

Symbiosis was firstly defined in 1879 by the German mycologist Heinrich Anton de Bary, who defined it as "the living together of unlike organisms". It generally includes mutualism, commensalism and parasitism (Görtz, 1988). Organisms live in mutualism when both partners benefit from each other. The association of mutual organisms which benefit from each other but are also able to live alone is also called proto-cooperation, whereas the opposite is an obligate mutualism where participating organisms are not able to survive singly cultivated (Fredrickson, 1977). Commensalism describes an association of organisms where only one symbiont benefits and the other one is not adversely affected. During a parasitical consortium one organism benefits while the other one is harmed. The benefitting organism is called parasite and is well adapted to its host (harmed organism). Furthermore, synergistic associations of two or more populations are based on the demanding habitat and can be reduced from mutualism to commensalism or even abolished if limiting nutrients are supplemented to the medium (Fredrickson, 1977). Interactions between different organisms can emerge in different spatial ways. Physical associations can occur in loose communities which are based on special signaling molecules (quorum sensing) or in close symbiotic associations with adhesion factors like proteins or polysaccharides (biofilm). These associations can be based on different molecular interactions like the adjustment of the physiochemical environment (change of pH), trophic interactions (organisms benefit from metabolic agents of the other), metabolite exchange of different organisms resulting in molecules that neither partner can produce alone (cooperative metabolism), protein secretion and gene transfer (Frey-Klett et al., 2011).

Many varieties of symbiosis are known in nature. Of utmost agricultural importance is for example the synergism between *Fabaceae* (legumes) and bacteria of the genus *Rhizobium/Bradyrhizobium*. These bacteria interact with the roots of the plant resulting in so called root nodules. These mutualistic nodules are able to fix nitrogen, which none of the partners is able to do alone (Lüttge et al., 2005; Werner, 1987). Furthermore, other well common symbiosis are the mycorrhiza of higher plants and fungi. Participating fungi support the plant with phosphate and mineral compounds whereas the fungi are supported with assimilates of the plant (Lüttge et al., 2005). To mention are also the lichen (mutualism between algae and fungi) and the interaction between intestinal bacteria and their animal and human host, respectively.

1.5 Symbiosis while food fermentations

Best taste of fermented foods is ensured with a mixture of starter cultures producing all desired aroma active compounds. Different microorganisms growing together can lead to interaction among themselves. Symbiosis in the forms of mutualism or commensalism is wide-spread in fermented foods, for example in yogurt, milk kefir or sourdough. Backgrounds of interactions are hard to determine, especially since there are a variety of microorganisms in the different consortia (De Vuyst and Neysens, 2005; Farnworth, 2005; Gulitz et al., 2011).

One of the best investigated synergism in food fermentation is the interaction of yogurt cultures. Yogurt is manufactured through the fermentation of milk with two thermophilic lactic acid bacteria in planktonic association. The mutualism (proto-corporation) of yogurt cultures *Streptococcus (St.) thermophilus* and *Lactobacillus (Lb.) delbrueckii* subsp. *bulgaricus* appears to be well established. While *Streptococcus* is provided with branched chain amino acids (namely valin) containing peptides by the proteolytic activity of *Lb. bulgaricus*, in return the *Lactobacillus* is stimulated by production of formic and pyruvic acid caused by *St. thermophilus* (Courtin & Rul, 2003; Zourari et al., 1992). Another example of symbiosis during food fermentation is sourdough where microorganisms are versatile through the viscous dough. A mixture of flour and water standing for two days at room temperature is usually necessary for sourdough production. The natural microbiota of flour and the environment ferments the dough until a stable consortium is adjusted. To ensure sourdough production with desirable flavor, a well-known existing sourdough is used as starter culture. Lactic acid bacteria and yeasts in sourdough are well adapted to their

environment in respect to pH, temperature and organic acids (reviewed by De Vuyst and Neysens, 2005). All heterofermentative sourdough lactobacilli are able to ferment maltose using maltose phosphorylases combined with glucose expression. Due to glucose repression yeasts are fermenting glucose and do not compete for maltose with sourdough lactobacilli (Stolz, Vogel, et al., 1995). Trophic interaction in the consortium sourdough could be found between *Candida humilis* and *Lb. sanfransiscensis*. The acetate-tolerant and maltose-negative yeast cleaves gluco-fructosans in the flour and releases fructose to the environment. In the presence of fructose *Lb. sanfransiscensis* is able to produce acetate instead of ethanol and gains additional ATP (Stolz, Böcker et al., 1995). Gobbetti et al. studied the interaction between yeast and lactobacilli of sourdough microbiota. They found commensalism in co-culture, where final yields and growth rates of lactobacilli increased, however, yeasts were unaffected (Gobbetti et al., 1994a, 1994b).

An example for the symbiosis of microorganisms in a biofilm community is milk kefir, where the organisms live in grains based on the polysaccharide kefiran (La Rivière & Kooiman, 1967; Lopitz-Otsoa et al., 2006; Yokoi et al., 1991). The interactions of participating microbes are not fully understood. Until today, a reconstruction of milk kefir grains out of a mixture of isolates has not been possible, yet. Certain enlightenments were presented by Cheirsilp et al., who described the interaction between *Lb. kefiranofaciens* and *Saccharomyces (S.) cerevisiae* in mixed culture concerning the enhancement of kefiran production, especially capsular kefiran (Cheirsilp, Shimizu et al., 2003; Cheirsilp, Shoji et al., 2003). Cheirsilp, Shimizu, et al. (2003) could demonstrate that *Lb. kefiranofaciens* is supported in growth and kefiran production due to the lactic acid consumption of *S. cerevisiae*. Lopitz-Otsoa et al. (2006) speculated in their review that lactose-negative but galactose-positive yeasts in milk kefir benefit from galactose released by lactic acid bacteria after lactose hydrolysation.

1.6 Objectives of the work

The food and beverage industry is intense in the development of innovative products, especially to those who object an additional benefit for the consumer. Fermented drinks such as “Bionade®” show large consumer consent. Waterkefir is only known as a household fermented beverage, yet. Due to its freshness and slight sweetness waterkefir is a beverage with attractive sensory characteristics and linked with the presence of several lactic acid and bifidobacteria it could also show health functionality. The consortium waterkefir is still poorly understood. A few studies describe occurring organisms in water kefir (Franzetti et al., 1998; Horisberger, 1969; Lutz, 1899; Moinas et al., 1980; Neve & Heller, 2002; Pidoux, 1989; Stadelmann, 1957; Ward, 1892). Less works were concerned with the metabolic activity of the consortium (Reiß, 1990) and the interaction of individual organisms (Leroi & Pidoux, 1993a, 1993b). Leroi and Pidoux (1993 a, b) determined a parasitical interaction between *Lb. hilgardii* and *Sc. florentinus* with a benefit for the lactobacilli disadvantaging yeasts. Gulitz et al. (2011, 2013) examined the composition of the water kefir consortia used in this work and could display that the microbiome previously described by culture dependent methods only was incomplete.

In this work, interactions between members of the water kefir microbiome should be determined. The examination of the metabolic activity of the consortium under standard conditions (fig extract and fermentation at 21 °C) and with changing parameters should form the basis for understanding the system. The study of metabolism and growth of single water kefir isolates in the habitat water kefir medium (WKM) should display information about their potential use as starter cultures.

Water kefir organisms live in a biofilm of insoluble EPS, called grains. Since combined cultivation of water kefir isolates does neither lead to a stable consortium nor to grain formation, single isolates should be investigated for their EPS production. Characterization of grain EPS and EPS produced by single water kefir isolates should provide information about production of grains and the reason for their insolubility. The influence of EPS, formed by water kefir isolates, on growth of water kefir organisms should provide information about their possible function.

WKM is a demanding habitat due to its slight nitrogen and high carbohydrate concentration, where several water kefir organisms are hardly able to grow singly cultivated. In this ecological niche interaction must play an important role. Co-cultivation of several main representative water kefir organisms should therefore provide information about the

synergism of the consortium. Analysis of fermentation broth should reveal metabolic interaction whereas comparison of whole cell protein could show insights into adaption mechanisms of individual organisms. An emphasis should be given to the interaction of lactobacilli and yeasts, which are the numerically predominant members of the water kefir consortium.

2 MATERIAL AND METHODS

2.1 Material

2.1.1 Equipment

Table 2: Overview of used devices

devices	model	manufacturer
2D gel electrophoresis chamber	SE 900-1.0	Hoefer, San Francisco, USA
Agarose gel chamber 25 x 20 cm	Easy Cast electrophoresis system	Owl Separation Systems, Portsmouth, NH, USA
Autoclaves	2540 ELV	Systec GmbH, Wettenberg, Germany
	Varioklav	H + P Labortechnik, Oberschleißheim, Ger-many
Breeding/incubation	Certomat BS-1	Systec GmbH, Wettenberg, Germany
	Hereaus B5042E	Hereaus Instruments, Hanau, Germany
	Memmert INB series	Memmert GmbH & Co. KG, Schwabach, Germany
	Memmert ICP500	
	WiseCube®WIS-ML02	Witeg Labortechnik GmbH, Wertheim, Germany
Centrifuges	Hermle Z216 MK	Hermle Labortechnik, Wehningen, Germany
	Hermle Z382 K	
	Hermle Z383 K	
	Mini Centrifuge MCF-1350	Laboratory Medical Supplies, Hongkong
	Sigma 1 K 15	Sigma Labortechnik, Osterrode am Harz, Germany
	Sigma 6-16K	
Focusing chamber	IEF 100	Hoefer, San Francisco, USA
Laminar flow sterile work bench	HERA safe	Heraeus Instruments, Hanau, Germany
MALDI-TOF MS	microflex LT	Bruker Daltonics GmbH, Bremen
Microscope	Axiolab	Carl Zeiss MicroImaging GmbH, Germany

devices	model	manufacturer
PCR-Cycler	Primus 96 plus	MWG Biotech, AG, Ebersberg, Germany
	Mastercycler gradient	Eppendorf AG, Hamburg, Germany
pH determination (electrode)	InLab 412, pH 0-14	Mettler-Toledo, Gießen, Germany
pH determination (measuring device)	Knick pH 761 Calimatic	Knick elektronische Geräte, Berlin, Germany
Photometer	NovaspeIIq	Pharmacia Biotech, Cambridge, England
Pipettes	Pipetman	Gilson-Abomed, Langenfeld, Germany
Plate readers	TECAN SPECTRAFluor	TECAN Deutschland GmbH, Crailsheim, Germany
	TECAN SUNRISE	TECAN Deutschland GmbH, Crailsheim, Germany
Power supplies	Electrophoresis Power Supply EPS 3501 XL	Pharmacia Biotech, Cambridge, England
Pure water	Euro 25 and RS 90-4/UF pure water system	SG Wasseraufbereitung GmbH, Barsbüttel, Germany
Shaking	Vortex 2 Genie	Scientific Industries Inc., Bohemia, NY, USA
Stirring	Wise Stir MSH-20A	Witeg Labortechnik GmbH, Wertheim, Germany
Thermo block	Techne DRI-Block DB3	Thermo-Dux Gesellschaft für Laborgerätebau mbH, Wertheim, Germany
Ultra sonic water bath	Sonorex Super RK 103H	Bandelin electronic, Berlin, Germany
Ultra sonification	UP 200S	Dr. Hielscher GmbH, Teltow, Germany
	SONOPLUS/SH70G	Bandelin electronic, Berlin, Germany
UV table	Herolab UVT 28M	Herlab GmbH Laborgeräte, Wiesloch, Germany
Water bath	Lauda BD	LAUDA Dr. D. Wobser GmbH & Co., Lauda-Königshofen, Germany

2.1.2 Chemicals

Table 3: Overview about used chemicals

Chemical	Purity	Manufacturer
6 x DNA loading dye	-	Fermentas GmbH, St. Leon-Rot, Germany
Acetic acid	100 %	Merck, Darmstadt, Germany
Acetonitril	HPLC grade	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Acrylamid-Bis solution	(19:1); 30 % (w/v)	SERVA, Heidelberg, Germany
Agar	17uropean agar	Difco, BD Sciences, Heidelberg

Chemical	Purity	Manufacturer
Agarose	for electrophoresis	Biozym Scientific GmbH, Oldendorf, Germany
Ampicillin sodium salt	93.3 %	Gerbu Biotechnik GmbH, Gaiberg, Germany
Ammonium persulfat (APS)	electrophoresis grade	SERVA, Heidelberg, Germany
Boric acid	≥99.5 %	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Bromphenol blue	for electrophoresis	SIGMA-Aldrich, Steinheim, Germany
$CaCl_2 * 2H_2O$	p.a.	Merck, Darmstadt, Germany
Dimidium bromide	≥98 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
DTT (1,4 Dithio-D,L-Threitol)	high purity	GERBU Biotechnik, GmbH, Gaiberg, Germany
EDTA	for molecular biology	SIGMA-Aldrich, Steinheim, Germany
Ethanol, absolute	≥99,8 %	VWR, Prolabo, Foutenay-sous-Bois, France
Ethanol, denatured	99 % with 1 % methylethylketone	Chemikalien und Laborbedarf Nierle, Freising, Germany
Fast-AP		Fermentas GmbH, St. Leon-Rot, Germany
FD restriction buffer		Fermentas GmbH, St. Leon-Rot, Germany
FD restriction enzymes		Fermentas GmbH, St. Leon-Rot, Germany
$FeSO_4$	97 %	SIGMA-Aldrich, Steinheim, Germany
FMOC	-	SIGMA-Aldrich, Steinheim, Germany
Fructose	HPLC grade	Merck, Darmstadt, Germany
Glucose	for biochemical use	Merck, Darmstadt, Germany
Glycerol	99.5 %, high purity	GERBU Biotechnik, GmbH, Gaiberg, Germany
Glycine	p.a.	Merck, Darmstadt, Germany
HCl 37 %	p.a.	Merck, Darmstadt, Germany
KH_2PO_4	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$K_2HPO_4 * 3 H_2O$	p.a.	Merck, Darmstadt, Germany
Lactic acid	1 M	SIGMA-Aldrich, Steinheim, Germany
L-amino acids	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Lysozyme	-	SERVA, Heidelberg, Germany
Maltose	for microbiology	GERBU Biotechnik, GmbH, Gaiberg, Germany
Mannitol	98 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Meat extract	for microbiology	Merck, Darmstadt, Germany
Methanol	HPLC-grade	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

Chemical	Purity	Manufacturer
$MgSO_4$ * 7 H_2O	p.a.	Merck, Darmstadt, Germany
$MnSO_4$ * 4 H_2O	p.a.	Merck, Darmstadt, Germany
Na-acetate * 3 H_2O	≥ 99.5 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
NaCl	p.a.	Merck, Darmstadt, Germany
NaH_2PO_4	p.a.	Merck, Darmstadt, Germany
Na_2HPO_4	p.a.	Merck, Darmstadt, Germany
NaOH	50 %	J.T. Baker, Deventer, Netherlands
$(NH_4)_2$H-citrate	≥ 98 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Nucleobases	p.a.	SIGMA-Aldrich, Steinheim, Germany
OPA	-	SIGMA-Aldrich, Steinheim, Germany
Paraffin oil	-	SIGMA-Aldrich, Steinheim, Germany
Peptone from casein	for microbiology	Merck, Darmstadt, Germany
Peptone from soybeans	for microbiology	Oxoid, Hampshire, England
Perchloric acid	70 %	Merck, Darmstadt, Germany
Primer	-	MWG-BiotechAG, Ebersberg, Germany
Raftilose	-	Orafti, Oraye, Belgium
SDS	research grade	SERVA, Heidelberg, Germany
Succinic acid	≥ 99.0 %	SIGMA-Aldrich, Steinheim, Germany
Sucrose	HPLC-grade	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sulfuric acid	95 – 98 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
T4 DNA ligase	-	Fermentas GmbH, St. Leon-Rot, Germany
TEMED	p.a.	Merck, Darmstadt, Germany
THF	HPLC grade	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Tris	ultra-pure	MP Biomedicals Solon, Ohio, USA
Tris-HCl	p.a.	Merck, Darmstadt, Germany
Tween 80	-	Mallinkrodt Baker B. v., Deventer, NL
Vitamins	p.a.	SIGMA-Aldrich, Steinheim, Germany
Yeast extract	for microbiology	Merck, Darmstadt, Germany
$ZnSO_4$ * 7 H_2O	99 %	SIGMA-Aldrich, Steinheim, Germany

2.1.3 Equipment for water kefir preparation

Table 4: Equipment for water kefir preparation

Material	Type	Manufacturer
Dried fruits	figs, apricots, cranberries	Seeberger, Ulm, Germany
Plastic vessel	2 l	
Sieve + spoon	autoclaved	
Still mineral water	naturell	Residenzquelle, Bad Windsheim, Germany
Sucrose	EG-Qualität I	Tip, Düsseldorf, Germany
Water kefir grains		Different vendor, Germany

2.1.4 Consumables

Table 5: Overview of used consumables

Material	Type	Manufacturer
Anaerocult	A, A mini, C mini	Merck, Darmstadt, Germany
IPG stripes	pH 4-7, 240 x 3 x 0.5 mm	SERVA, Heidelberg, Germany
HPLC vials	1.5 ml	Techlab GmbH, Erkerode, Germany
HPLC crimp caps	PTFE	Techlab GmbH, Erkerode, Germany
Microtiter plates	multi well plate 96-well flat bottom with lid	Sarstedt, Nümbrecht, Germany
Reaction tubes	2 ml, 1.5 ml, 200 µl	Eppendorf, Hamburg, Germany
Security guard cartridges	CarboH, 4 x 3.0 mm; CarboPb 4 x 3.0 mm; Gemini C18 4 x 2.0 mm	Phenomenex, Aschaffenburg, Germany
Sterile filter	Filtropur S 0.2 (0.2 µm)	Sarstedt, Nümbrecht, Germany
	Rapid-Flow Bottle Top Filter, 0.2 µm, 500 ml	Nalgene, NY, USA
Sterile ml tubes	5 ml, 15 ml, 50 ml	Sarstedt, Nümbrecht, Germany
Syringe	2 ml, 10 ml, 50 ml, sterile	Braun, Melsungen, Germany
Syringe filter	15 mm, 0.2 µm, RC and Nylon membrane	Phenomenex, Aschaffenburg, Germany
Tissue culture plate	6 well, Flat Bottom	BD Falcon, Franklin Lakes, USA
	Transwell, 24 mm dia inserts, 0.4 µm PC membrane, 6 well	Corning Incorporated, NY, USA
UV cuvette	LCH 8.5 mm, from 220 nm	Sarstedt, Nümbrecht, Germany

2.1.5 Molecular-biological kits

Table 6: Overview of used molecular-biological kits

Kit	Role	Manufacturer
E.Z.N.A. Bacterial DNA Kit	DNA isolation	Omega Bio-Tek Inc., Norcross, GA, USA
QIAquick PCR purification Kit	PCR purification Kit	Qiagen GmbH, Hilden, Germany
Taq Core Kit	DNA polymerase	MP Biomedicals Solon, Ohio, USA

2.1.6 Water kefir consortia

During this work isolates from four water kefir consortia with different origin were used. WkF originated from household of Florian Waldherr. WkA (also called Anja), WkW (also called Willi) and WkI (also called Inka) were delivered from three different ebay providers. For comparative metabolic analysis WkW and WkI were used.

2.1.7 Bacterial strains

A collection of water kefir strains isolated from four different water kefirs were used in this study. Isolation and identification of the single isolates was executed from Anna Gulitz (Technische Mikrobiologie Weihenstephan (TMW)).

Table 7: Water kefir isolates used in this study

Species	Strain [TMW]	origin	Species	Strain [TMW]	origin
Lactobacillus (Lb.) casei	1.1814	WkA	*Acetobacter (Ac.) cerevisiae*	2.1084	WkA
Lb. casei	1.1816	WkA	*Gluconobacter (G.) oxidans*	2.1085	WkA
Lb. hordei	1.1817	WkA	*Ac. aceti*	2.1153	WkW
Lb. hordei	1.1818	WkA	*Ac. Iovaniensis*	2.1154	WkW
Lb. hilgardii	1.1819	WkA	*Ac. ghanensis*	2.1155	WkW
Lb. satsumensis	1.1820	WkA	*Ac. fabarum*	2.1156	WkW
Lb. hordei	1.1821	WkA	*Ac. ghanensis*	2.1157	WkW

Species	Strain [TMW]	origin	Species	Strain [TMW]	origin
Lb. hordei	1.1822	WkF	*Ac. cerevisiae*	2.1158	WkW
Lb. nagelii	1.1823	WkA	*G. albidus*	2.1191	WkA
Lb. nagelii	1.1824	WkF	*Ac. fabarum*	2.1192	WkW
Lb. nagelii	1.1825	WkW	*Leuconostoc (Lc.) mesenteroides*	2.1193	WkW
Lb. nagelii	1.1826	WkF	*Lc. citreum*	2.1194	WkW
Lb. nagelii	1.1827	WkF	*Lc. mesenteroides*	2.1195	WkW
Lb. hilgardii	1.1828	WkA	*Ac. orientalis*	2.1196	WkW
Lb. satsumensis	1.1829	WkF	*Bifidobacterium (B.) psychroerophilum*	2.1395	WkW
Lb. hordei	1.1907	WkW	*B. psychroerophilum*	2.1397	Ebay Kefir Ehrmann
Lc. mesenteroides	2.1073	WkA	*B. psychroerophilum*	2.1398	Ebay Kefir Ehrmann
Lb. casei	2.1074	WkA	*Zygotorulaspora (Z.) florentina*	3.220	WkA
Lc. mesenteroides	2.1075	WkF	*Saccharomyces (S.) cerevisiae*	3.221	WkA
Lc. mesenteroides	2.1076	WkF			

2.2 Methods

2.2.1 Water kefir analysis

2.2.1.1 Fermentation of water kefir

Water kefir grains were propageted in standardized water kefir medium (WKM). WKM is comprised of 100 ml fig extract and a sterile solution of 80 g sucrose per liter still mineral water. For the extract 48 g dried figs were mixed in 100 ml still mineral water by shaking for 20 min. Big solids were removed by sieving and the smaller parts by centrifugation (17000 g, 3h) and sterile filtration (0.2 µm). The usage of still mineral water could ensure a constant mineral content. Lemon slices were not supplemented to minimise potential contamination, besides Reiß (1990) could demonstrate that Lemon did not show an impact on fermentation.

For water kefir fermentation 80 g grains (wet weight) were plunged in one liter WKM and incubated in a 2 l plastic container covered with a cotton cloth at 21°C without shaking for 72 h. For re-propagation the grains were collected in a sterile sieve, washed with tap water and used for new fermentation.

2.2.1.2 Water kefir supernatant analysis

Two water kefirs with different origin (WkW, WkI) were separately cultivated in triplicates as described above (2.2.1.1). After 6, 12, 24, 30, 36, 48, 54, 60 and 72 h 2 ml supernatant were taken for pH, 1 ml for organic and amino acid and 0.5 ml for sugar analysis. Pure medium was analyzed for initial conditions. pH-values were determined with a pH measuring electrode and metabolites were determined per HPLC and IC, respectively.

2.2.1.3 Change of parameters for water kefir fermentation

The influence of temperature on water kefir fermentation was determined with fig extract at 21 °C (standard conditions), 37 °C and 12 °C. For the variation of fruits an extract of apricot and cranberries, respectively, was used at 21 °C fermentation temperature. Extract preparation was similar to fig extract.
Metabolite analysis was measured as described for water kefir supernatant analysis (2.2.1.2).

2.2.1.4 Growth of water kefir grains

Water kefir fermentation was prepared as described above (2.2.1.1). After three days of fermentation grains were washed and weighted (humid grain mass = HGM).

$$Increase\ of\ HGM\ \left(\frac{\%}{d}\right) = \frac{HGM\ after\ fermentation - starting\ HGM}{d * starting\ HGM}$$

Increase of humid grain mass during standard fermentation conditions were compared with water kefir fermentation with dH_2O instead of still mineral water as well as the supplementation of 0.1 % $CaCl_2$

2.2.1.5 Analysis of water kefir grains

1 g grains were mixed with 4 ml water and treated with different enzymes, 100 µl each enzyme. α-glucosidase, β-amylase, dextranase, amyloglucosidase, glucooxidase and a mixture of all of them were added and after 24 h grains were examined.

70 g grains were solved in 140 ml 1M NaOH. The solution was neutralized with acetic acid and transferred to Viskis® dialysis tubing 20/32. Grain EPS was dialyzed at 4°C for two days against 2 l water. The water was replaced regularly. Dialyzed grain EPS solution was freeze dried and hydrolyzed for monomer determination as described for other EPS (2.2.3.2 and 0).

2.2.2 Microbiological methods

2.2.2.1 Media and growth conditions

2.2.2.1.1 Medium for and cultivation of LAB

LAB were cultivated in a modified mMRS medium, the ingredients are listed in Table 8. For agar plates 1.5 % agar was added. The ingredients were dissolved in dH_2O, the pH was adjusted to 5.7 and the solution was autoclaved for 20 min at 121 °C. The sugar solution was autoclaved separately and after cooling the components were mixed together under sterile conditions.

LAB were incubated anaerobically at 30 °C for 48 h. For growth experiments overnight (24 h) liquid cultures were centrifuged (5000 g, 5 min), washed twice with Ringer reagent and afterwards the cells were re-suspended in Ringer reagent to an absorbance at 590 nm

(OD_{590}) of 2.5 (stock culture). OD_{590} 2.5 is equal to 10^9 cfu/ml LAB. All experiments were carried out in biological triplicates and technical duplicates.

The identification of EPS producing strains was determined on sucrose (8%) containing mMRS-Sac agarose plates. Sucrose was used instead of glucose.

Table 8: Ingredients of mMRS medium for LAB

substance	concentration [g/l]
peptone	10
meat extract	2
yeast extract	4
Tween 80	1
K_2HPO_4 * 3 H_2O	2.5
Na-Acetate * 3 H_2O	5
$(NH_4)_2H$-citrate	2
$MgSO_4$ x 7 H_2O	0.2
$MnSO_4$ x H_2O	0.038
glucose	20

2.2.2.1.2 Medium for and cultivation of acetic acid bacteria (AAB)

AAB were cultivated in No5 medium, the ingredients are listed in Table 9. For agar plates 1.5 % agar was added. The ingredients were dissolved in dH_2O, the pH was adjusted to 6.0 and the solution was autoclaved for 20 min at 121 °C.

AAB were incubated aerobically by shaking (180 rpm) in an Erlenmeyer flask at 30 °C for 48 h. For growth experiments cultures were centrifuged (5000 g, 5 min), washed twice with Ringer reagent and afterwards the cells were re-suspended in Ringer reagent to an absorbance at 590 nm (OD_{590}) of 2.5 (stock culture). OD_{590} 2.5 is equal to 10^9 cfu/ml AAB. All experiments were carried out in biological triplicates and technical duplicates.

Table 9: Ingredients of No5 medium for AAB

substance	concentration [g/l]
mannitol	10
yeast extract	15
$MgSO_4$	2.5
Glycerol	0.5

2.2.2.1.3 Medium for and cultivation of Bifidobacteriaceae

Bifidobacteria were cultivated in a tryptone phytone medium (TP), the ingredients are listed in Table 10. For agar plates 1.5 % agar was added. The ingredients were dissolved in dH_2O, the pH was adjusted to 7.0 and the solution was autoclaved for 20 min at 121 °C. The sugar solution was autoclaved separately and after cooling the components were mixed together under sterile conditions.

Bifidobacteria were incubated anaerobically at 30 °C for 72 h. For growth experiments 48 h incubated liquid cultures were centrifuged (5000 g, 5 min), washed twice with Ringer reagent and afterwards the cells were re-suspended in Ringer reagent to an absorbance at 590 nm (OD_{590}) of 2.5 (stock culture). OD_{590} 2.5 is equal to 10^8 cfu/ml bifidobacteria. All experiments were carried out in biological triplicates and technical duplicates.

Table 10: Ingredients of TP medium for *Bifidobacteriaceae*

substance	concentration [g/l]
Peptone from casein	10
Peptone from soybeans	10
yeast extract	6
NaCl	5
K_2HPO_4	2.5
glucose	2.5
raftilose	2.5

2.2.2.1.4 Medium for and cultivation of yeasts

Yeasts were cultivated in YPG medium; the ingredients are listed in Table 11. For agar plates 1.5 % agar was added. The ingredients were dissolved in dH_2O, the pH was adjusted to 6.0 and the solution was autoclaved for 20 min at 121 °C. The sugar solution was autoclaved separately and after cooling the components were mixed together under sterile conditions.
Yeasts were incubated aerobically by shaking (180 rpm) in an Erlenmeyer flask at 30 °C for 48 h. For growth experiments overnight (24 h) cultures were centrifuged (5000 g, 5 min), washed twice with Ringer reagent and afterwards the cells were re-suspended in Ringer reagent to an absorbance at 590 nm (OD_{590}) of 2.5 (stock culture). OD_{590} 2.5 is equal to 10^8 cfu/ml yeasts. All experiments were carried out in biological triplicates and technical duplicates.

Table 11: Ingredients of YPG medium for yeasts

substance	concentration [g/l]
peptone from casein	10
yeast extract	5
glucose	20

2.2.2.2 Analysis of growth in general

Growth experiments were executed in a 96 well plate with 250 µl medium per well, an inoculation of 10 µl stock culture (2.2.2.1) and a cover of 75 µl sterile paraffin oil for anaerobiosis for growth determination of LAB and bifidobacteria, and a cover of 25 µl paraffin oil for growth determination of yeasts and AAB to avoid dehydration. Measurements were done in a photometer every 30 min for 48 h or 72 h at 590 nm.

2.2.2.3 Co-cultivation experiments

Co-cultivation experiments were executed in the Corning Transwell® culture system (Corning, Lowell, USA; (Gobbetti et al., 1994b)). This system consists of a 6 well plate with two separated parts in every well. The lower compartment (reservoir) is related with the upper compartment (insert) by a polycarbonate membrane (0.4 µm), which ensures diffusion of metabolic products but prevents mixture of cells. The reservoir resp. insert of the system were filled with 2.5 ml WKM inoculated with 4 % stock culture (2.2.2.1) of yeast,

Bifidobacterium, *Leuconostoc* resp. *Lactobacillus* (start OD 0.1) and incubated at 30 °C. Furthermore each organism was singly cultured in 5 ml WKM in 6-well plates with the same inoculation ratio as in co-culture. Pure WKM was filled in a 6 well plate as a sterile control and as a blank for OD measurement. After 24, 48 and 72 h 100 µl of each re-suspended culture was mixed with 400 µl WKM and measured at 590 nm. Each possible combination between yeast and *Lactobacillus* and *Bifidobacterium* and yeast resp. *Lactobacillus* was tested. Preliminary tests showed the best effects with yeasts cultivated in the reservoir and bacteria in the insert. An acid-base diffusion assay with bromphenol blue as indicator could demonstrate metabolic diffusion after 10 min incubation time.

After OD measurement of single organisms at 72 h liquids of insert and reservoir were mixed, the OD of this mixture was measured and 1 ml and 0.5 ml solution were taken for chromatographic metabolite and sugar analysis. Evaluation of sugar consumption resp. increase and metabolite production values was referred to OD 1 for better comparison of the metabolic activity of the little "consortia". Values of respective single cultures were pooled and declared as "calculated co-cultures".

2.2.2.4 Mixed-culture experiments

Always 130 ml WKM were inoculated with 4 % stock culture of *Z. florentina*, *Lb. hordei*, *Lb. nagelii* and *B. psychraerophilum*, respectively, as single cultures. For mixed-cultures 4% stock culture of *Z. florentina* and additional 4 % stock culture of one of the bacteria were used as inoculation. Each solution was divided in three tubes, 40 ml per tube. One tube was used for sugar, metabolite and OD measurement and the other two for proteome analysis. Each experiment was carried out in biological triplicates. The solutions were closed and incubated at 30 °C without shaking. At time zero starting OD of the solutions was checked. After 24 h, 48 h and 72 h samples were taken for OD, microscopy, sugar and metabolite analysis. Additional, after 24 and 48 h always one tube with 40 ml fermentation broth was used for proteome analysis. First the OD of these solutions was measured and afterwards cells were centrifuged (5000 g, 5 min). Following steps are described for whole-cell protein extraction (2.2.5.1).

2.2.2.5 Modifikation of WKM

Metabolic interaction of water kefir isolates was determined in modified WKM.

Water kefir produces grains during fermentation. Therefore, the influence of EPS from different water kefir isolates on growth of other isolates was tested. EPS of *Lb. hilgardii* (TMW 1.1828), *Lc. mesenteroides* (TMW 2.1073) and *Lc. citreum* (TMW 2.1194) were solved in dH_2O (10 g/l) and autoclaved (121°C, 20 min). For growth experiments WKM was supplemented with 10 % sterile dH_2O and with 10 % of each EPS solution, respectively. In these four media growth (2.2.2.2) of different water kefir isolates (Table 12) was compared.

Table 12: Strains used for growth experiments in WKM supplemented with EPS

species	strain	origin	species	strain	origin
Lb. casei	TMW 1.1814	WkA	*Lb. satsumensis*	TMW 1.1829	WkF
Lb. hordei	TMW 1.1817	WkA	*Lb. hordei*	TMW 1.1907	WkW
Lb. hordei	TMW 1.1821	WkA	*Ac. fabarum*	TMW 2.1192	WkW
Lb. hordei	TMW 1.1822	WkF	*Lc. mesenteroides*	TMW 2.1193	WkW
Lb. nagelii	TMW 1.1823	WkA	*Lc. mesenteroides*	TMW 2.1195	WkW
Lb. nagelii	TMW 1.1824	WkF	*Ac. orientalis*	TMW 2.1196	WkW
Lb. nagelii	TMW 1.1825	WkW	*B. psychraerophilum*	TMW 2.1395	WkW
Lb. nagelii	TMW 1.1826	WkF	*Z. florentina*	TMW 3.220	WkA
Lb. nagelii	TMW 1.1827	WkF	*S. cerevisiae*	TMW 3.221	WkA

Since yeasts showed an improvement in growth for lactobacilli, it was to find out if they always produce growth factors. Therefore the influence of pre-fermented WKM with yeasts was tested. 15 ml WKM was inoculated with 4 % stock solution of *Z. florentina* and *S. cerevisiae*, respectively, and incubated aerobically as well as anaerobically at 30 °C. After 24 h of fermentation, fermentation broth was centrifuged (5000 g, 5 min) and the supernatant was sterile filtrated and used for further growth experiments with *Lb. hordei* (TMW 1.1822) and *Lb. nagelii* (TMW 1.1825).

To determine the influence of the physiochemical environment on yeasts the growth in media with different starting pHs was examined. The pH of WKM was adjusted with hydrochloric acid, lactic acid and acetic acid, respectively to pH 8.0, 7.0, 6.0, 5.0, 4.0 and 3.0. Growth of *Z. florentina* and *S. cerevisiae* was determined as described above (2.2.2.2).

The influence of lactic acid on *Z. florentina* was tested with growth experiments in pure WKM and in WKM supplemented with 10 mM and 40 mM lactic acid at constant pH 7.8.

2.2.2.6 Determination of essential nutrients for water kefir isolates

Essential nutrients for *Lb. hordei* (TMW 1.1822), *Lb. naglelii* (TMW 1.1825), *Z. florentina* (TMW 3.220) and *S. cerevisiae* (TMW 3.221) were identified in simplified chemically defined medium (SCDM) (Hebert et al., 2000). This medium contains 20 proteinogenic amino acids, vitamins and bases as single substances instead of an extract base. Glucose was used as carbon source. Growth behavior in full medium was compared to medium with one nutrient omitted. Furthermore, growth of *Lb. nagelii* was determined in a medium with ornithine instead of arginine.

Table 13: Ingredients of simplified chemically defined medium (SCDM)

substance	concentration [g/l]	substance	concentration [g/l]
sodium acetate	5	L-serine	0.1
KH_2PO_4	3	L-threonine	0.1
K_2HPO_4	3	L-tryptophan	0.1
$MgSO_4 \cdot 7\ H2O$	0.2	L-tyrosine	0.1
$MnSO_4 \cdot 4\ H2O$	0.05	L-valine	0.1
$FeSO_4 \cdot 7\ H2O$	0.02	niacin	0.001
Tween 80	1	pantothenic acid	0.001
L-alanine	0.1	pyridoxal	0.002
L-arginine	0.1	riboflavin	0.001
L-asparagine	0.2	*p*-aminobenzoic acid	0.01
L-aspartic acid	0.2	folic acid	0.001
L-cysteine	0.2	cobalamin	0.001
L-glutamine	0.2	D-biotin	0.01
L- glutamic acid	0.2	thiamin	0.001
glycine	0.1	adenine	0.01
L-histidine	0.1	guanine	0.01
L-isoleucine	0.1	inosine	0.01

substance	concentration [g/l]	substance	concentration [g/l]
L-leucine	0.1	xanthine	0.01
L-lysine	0.1	orotic acid	0.01
L-methionine	0.1	uracil	0.01
L-phenylalanine	0.1	thymine	0.01
L-proline	0.1	glucose	10

2.2.2.7 Modification of SCDM

Co-cultivation experiments in the model system (2.2.2.3) were executed with *Lb. hordei* (TMW 1.1822), *Lb. nagelii* (TMW 1.1825), *Z. florentina* (TMW 3.220) and *S. cerevisiae* (TMW 3.221) in SCDM without pyridoxal (SCDM-VitB6), SCDM without L-arginine (SCDM-Arg) and SCDM without L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-tryptophan, L-tyrosine and L-valin (SCDM-7AS), respectively. Lactobacilli and yeasts were additionally single cultivated in the prepared media and SCDM as control. Since *Z. florentina* could not grow in SCDM-7AS this experiment was executed in seven different media where only one of the amino acids mentioned was omitted.

Subsequent growth experiments were performed with *Lb. nagelii* in SCDM-Arg in mixture (1:1) with sterile filtrated supernatant of pre-fermented SCDM-Arg. Pre-fermentation was performed with singly cultivated *Z. florentina*, with *Z. florentina* and *Lb. nagelii* in mixed-culture with cell contact (inoculation with 4% stock culture of each strain), with *Z. florentina* and dead cells of *Lb. nagelii* (cell death induced by pasteurization, 10 min 78°C), each in 6 well plates for 24 h. Additional media were SCDM-Arg with 10% yeast cell extract resp. yeast cell debris (OD590 0.2). For yeast cell extract stationary phase cells of *Z. florentina* were washed with water, disrupted with a FastPrep-24 and glass beads, pasteurized (10 min, 78°C) and centrifuged (14000 g, 30 min). The supernatant was used as yeast cell extract, the pellet as cell debris.

2.2.2.8 Aggregation assay

Cells of *Z. florentina* (TMW 3.220), *Lb. hordei* (TMW 1.1822), *Lb. nagelii* (TMW 1.1825) and *B. psychraerophilum* (TMW 2.1195) were pre-cultured as described above (2.2.2.1). Cells were washed twice with PBS-buffer (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 * $2H_2O$ 1.44 g, KH_2PO_4 0.2 g ad 1 liter, pH 7.4) and re-suspended in PBS to OD 1. 8 ml of each cell

suspension was used for auto-aggregation (AAg) of the individual organisms and 4 ml of the yeast cell suspension was mixed with each bacterium for mixed-aggregation (MAg). Cell suspension were mixed again for 20s and let stand. After 24 h 200 µl sample from the top of the suspension were taken and the OD_{590} was measured in a 96 well plate. This experiment was carried out in biological triplicates and technical duplicates.

Calculation of auto- and mixed-aggregation were based on Kos et al. (2003):

$$aggregation\ (\%) = 1 - (A_{24}/A_0)*100$$

A_{24} = absorbance after 24 h
A_0 = absorbance at the beginning

2.2.2.9 Experiments for reconstitution of water kefir grains

Water kefir isolates were pre-cultured in their respective medium (2.2.2.1) and mixed proportional to their appearance in water kefir grains investigated by Gulitz et al. (2011). 400 ml WKM was inoculated with 4% of this water kefir organism mixture and incubated for 30 days in an Erlenmeyer flask covered with a cotton cloth at 21°C. Every three days grain formation was controlled.
Since Gulitz et al. (2013) found uncultivable organisms in water kefir grains, 50 g grains were mixed with 50 ml still mineral water and disrupted by a stomacher for 1 min. The supernatant of the disrupted grains should contain all water kefir organisms without a cultivation step. 400 ml WKM were inoculated with 4% of the grain supernatant and incubated as described above. To induce grain formation glass beads and autoclaved water kefir grains, respectively, were added as crystallization seeds.
20 ml of the grain supernatant were mixed with 20 ml sodium alginate solution (1 g/100 ml) and added drop wise to a calcium chloride solution (10 g/100 ml). 32 g water kefir grain organisms embedded in alginate beads were incubated in 400 ml WKM for three days. After three days the beads were washed, weighted and re-cultivated. This procedure was carried out five times.

2.2.3 EPS isolation and characterisation

2.2.3.1 Screening for EPS producers

Water kefir isolates (2.1.6) were screened for EPS production on sucrose containing agar plates. Therefore, agar plates were prepared as described before (2.2.2.1) with 80 g/l sucrose as single carbon source. As control the organisms were simultaneously plated on their normal medium without sucrose. Shiny and slimy colonies were identified as EPS-producers in different levels, very strong (+++), strong (++), slight (+) and non EPS producer.

2.2.3.2 Production and isolation of EPS

Very strong and strong EPS producing water kefir isolates were cultivated in 40 ml liquid medium with sucrose (8%) as single carbon source with an inoculation of OD 0.1. *Leuconostoc* and lactobacilli were cultivated anaerobically, *Acetobacter* aerobically and with shaking, at 30 °C for 48 h. To determine EPS production in the natural environment of the isolates, organisms were cultivated in normal WKM as described.

Fermentation broth was centrifuged (8000 g; 15 min) and EPS in the supernatant was precipitated with two sample volumes of ethanol for 24 h at 4°C. Afterwards, EPS was centrifuged (10 000 g; 15 min) and the supernatant was discarded. Remaining ethanol was vaporized during 1 h at 60 °C. Precipitated EPS was solved in as few water as possible and dialyzed in dialysis tubings against 2 l water for 48 h at 4°C with smoothly stirring. Water was replaced at least five times. Dialyzed EPS solution was filled into a round-bottom flask and frozen while shaking in an ethanol cooling bath (-40°C). Subsequently, frozen EPS solution was vacuum freeze-dried at -80°C and 0.02 mbar. Used round-bottom flasks were pre-weighted and EPS concentration of fermentation broth could be gravimetrically determined while weighting the dried EPS in the flask. Dried EPS could be stored at room temperature.

EPS concentration in water kefir supernatant was determined in 40 ml supernatant after 72 h of fermentation. EPS precipitation and drying was executed as described above.

2.2.3.3 EPS hydrolysis

For identification of sugar monomer compounds of isolated EPS, EPS had to be hydrolyzed. 1 ml of a solution of 10 g/l EPS in water was added with 25 µl perchloric acid (70 %) for slight hydrolysis and 75 µl for strong hydrolysis. Solutions were treated at 100 °C for 1 h (slight) and 5 h (strong), respectively. Fructans had to be slightly hydrolyzed since emerged fructose further reacts under these conditions. Bonds in glucans could only be splitted under stronger conditions into their glucose monomers. Since structure of produced EPS was not known at the beginning both hydrolysis conditions were prepared for each EPS. After heat treatment samples were cooled down and filtrated (0.2 mm, regenerated cellulose membrane) for chromatographic analysis.

2.2.4 Analytical methods

2.2.4.1 Chromatographic analysis of sugars and metabolites

Water kefir supernatants and fermentation broths were chromatographically analyzed along their changes in sugar and metabolite concentrations.

2.2.4.1.1 Sugars and sugar alcohols

Before chromatographic analysis proteins of the samples had to be removed to save chromatographic columns. For sugar analysis acid precipitation of proteins was not possible because of the inversion of sucrose under these conditions. Therefore 500 µl of a sample were added with 250 µl $ZnSO_4$-solution (10 %) and mixed thoroughly for 1 min. Afterwards addition of 250 µl 0.5 M NaOH, mixing for one minute and incubation for 20 min led to a voluminous precipitation that pulled proteins down. The supernatant after centrifugation (15 min; 14 000 g) was diluted if necessary, filtrated (0.2 µm; regenerated cellulose (RC) membrane) and used for chromatographic analysis.

Mono-, disaccharides and sugar alcohols were quantified with the ion-exclusion HPLC column Rezex RPM-Monosaccharide Pb^{2+} (8% cross-linked resin). Quantification was executed employing calibration adjustment.

Table 14: Conditions for sugar and sugar alcohol quantification

Column	Rezex RPM-Monosaccharide Pb^{2+} (8% cross-linked resin)
Dimensions	300 x 7.8 mm
Mobile phase	dH_2O, filtrated (0.2 µm) and degassed (helium)
Flow rate	0.6 ml/min
Detection	RI (Gynkotek RI 71)
Temperature	85 °C
Injection volume	20 µl
System	Pump: dionex P680; autosampler: Gynkotek GINA 50
Evalution software	Chromeleon 6.60

2.2.4.1.2 Organic acids and ethanol

For the quantification of organic acids and ethanol samples were treated with perchloric acid (50 µl/ml sample) and incubated over night at 4 °C for protein precipitation. Afterwards samples were centrifuged (14 000 g, 15 min, 4 °C) and the supernatant was filtrated (0.2 µm, RC membrane). Organic acids and ethanol were quantified with the ion-exclusion HPLC column Rezex ROA-Organic Acid H^+ (8% cross-linked resin). Quantification was executed employing calibration adjustment.

Table 15: Conditions for organic acid and ethanol quantification

Column	Rezex ROA-Organic Acid H^+ (8% cross-linked resin)
Dimensions	300 x 7.8 mm
Mobile phase	2.5 mM sulfuric acid, filtrated 0.2 µm and degassed (helium)
Flow rate	0.6 ml/min
Detection	RI (Gynkotek RI 71)
Temperature	85 °C
Injection volume	10 µl
System	Pump: dionex P680; autosampler: Gynkotek GINA 50
Evalution software	Chromeleon 6.60

2.2.4.1.3 Amino acids

Qualification and quantification of amino acids was performed by reversed phase high performance liquid chromatographie (RP-HPLC). Protein precipitation was prepared as described for organic acid determination, but samples were filtered through a nylon membrane. Amino acid could be UV-detected after pre-column derivatisation with o-phthalaldehyde-3-mercaptopropionic acid (OPA) and 9-fluorenylmethyl chloroformate (FMOC) following Bartóak et al. (1994). Quantification was executed employing calibration adjustment.

Table 16: Conditions for amino acid determination

Column	Gemini 5 µm C18 110 Å
Dimensions	150 x 4.6 mm
Mobile phase	A) 20 mM NaH_2PO_4 + 20 mM Na_2HPO_4 + 0.8 % THF B) 30 % acetonitrile + 50 % methanol + 20 % dH_2O
Flow rate	0.8 ml/min
Gradient	0 min 0 % B, 16 min 64 % B, 19 min 100 % B, 22 min 100 % B, 22.25 min 0 % B
Detection	UV 338 nm, 269 nm
Temperature	40 °C
Injection volume	5 µl
System	Dionex Ultimate 3000
Evalution software	Chromeleon 6.80

2.2.4.2 Volatile compounds in water kefir supernatant determined by Headspace GC-MS

Analysis of volatile compounds in water kefir supernatant was carried out by Headspace GC-MS with solid phase microextraction (SPME). GC-vials were filled with 10 ml supernatant. Solvent-free adsorption of volatile compounds in the headspace to SPME fiber has been achieved by incubation for 30 min in the agitator (30 °C, 250 rpm). Afterwards compounds were desorbed for 10 min at 250 °C in the injector block. Fractionation was carried out using a ZB-WAX column as solid phase and helium for mobile phase. With the coupled mass spectrometer (MS) including ionization (70 eV) and fractionation using a mass charge ratio

(Quadrupol) and the comparison of mass spectral data with the Nist 2002 Mass Spectral Database identification of volatile substances was possible.

Table 17: Headspace GC-MS conditions for qualification of volatile compounds

SPME fiber	Carboxen/polydimethylsiloxane (CAR/PDMS), Assembly 85 nm, 23 ga
Column	ZB-WAX, diameter 0.25 mm, coat thickness 0.25 µm, 60 m
Mobile phase	helium
Temperature program	30 °C/15 min//3 °C/min//50 °C//4 °C/min//110 °C//5°C/min //150 °C//10°C/min//250°C/10 min
System	Agilent Technologies 7890 A GC system
Detection	MS Agilent Technologies 5975 C VL MSD
Evaluation software	Agilent MSD ChemStation E.02.00.493

2.2.4.3 NMR analysis of water kefir grains

Linkage types of water kefir grain EPS were analyzed by NMR. Lyophilized grain EPS was sent to Lehrstuhl für Makromolekulare Chemie II, Universität Bayreuth where Ramón Novoa-Carballal measured the samples according to Jakob et al. (2013).

2.2.5 Molecular-biological methods

2.2.5.1 Whole cell protein extraction

For proteome analysis whole cell protein of mixed- and single-cultures were extracted. Cell pellets (received during 2.2.2.4) were washed twice with TE buffer (10 mM Tris HCl, 10 mM EDTA, pH 8.0) and afterwards re-suspended with a lysozyme buffer (5 mg/ml TE). The suspension was incubated for 1 h at 37 °C. After centrifugation (5000 *g*, 3 min) cells were transferred with 1 ml TE to a 1.5 ml tube and centrifuged again (14 000 *g*, 5 min). Supernatant was discarded and the pellet was suspended in SDS lysis buffer (0.9% SDS, 0.1% Pefabloc, 100 mM Tris base, pH 8.6). The amount of used SDS lysis buffer depended on the volume and the optical density of the primary fermentation broth (600 µl SDS lysis buffer per cell pellet of 50 ml fermentation broth with OD 0.5). Afterwards cells were disrupted by sonication (3 x 30 s; power, 90%; cycle, 70%; on ice). Always 200 µl of the suspension were diluted with 500 µl Chaps lysis buffer (6.10 M urea, 1.79 M thiourea, 65.06

mM Chaps, 1% [wt/vol] DTT, 0.5% [vol/vol] Pharmalyte 3-10) and mixed thoroughly for 20 min at 4 °C. Remaining cell wall fragments were removed by centrifugation (14 000 *g*, 30 min, 4 °C) and 200 µl aliquots of the supernatant were stored at -80 °C.

2.2.5.2 2D-gelelectrophoresis

For 2D-gelelectrophoresis whole cell protein of a mixed-culture was compared with a pool of the whole cell protein of single-cultured cells of the individual organisms. Whole cell proteins of single-cultures were pooled in their respective OD ratio of the single-culture fermentation broth.

Rehydration of IPG stripes (immobilized pH gradient 4 – 7, 24 cm) was carried out overnight (at least for 6 h) in 1 ml rehydration buffer (6.10 M urea, 1.79 M thiourea, 8.13 mM DDM, 0.2% [wt/vol] DTT, 0.2% [vol/vol] Pharmalyte 3-10) per stripe in a reswelling tray. Afterward stripes were washed in water and installed to the IEF 100 system (Hoefer). For analytical gels 200 µl protein extract was applied by sequential anodic cup loading. Within 18 h at 250 V samples were desalted and afterwards proteins were fractionated along the stripe concerning their isoelectric point at 12 000 V (until 60 – 80 kVh). After equilibration (Table 18; 100 ml equilibration buffer supplemented with 2 g DTT for 12 min at 60 rpm and 100 ml equilibration buffer supplemented with 4 g iodacetamid for 12 min at 60 rpm) stripes were loaded on the polyacrylamide gels and covered with bromphenyl blue containing agarose solution for run trace control. SDS-polyacrylamide gel electrophoresis (PAGE) was performed on a vertical system with gels of a total acrylamide concentration of 11% (Table 19) at 15°C. Silver staining visualized proteins on the gels (Blum, Beier, & Gross, 1987).

Expressed proteins of mixed-cultured cells in comparison to the pool of single-cultured cells were analyzed using Progenesis Same Spots (Nonlinear Dynamics Limited, Newcastle, UK). For protein identification gels were stained with Roti Blue. Chosen proteins were picked and send to the Zentrallabor für Proteinanalytik (Ludwig-Maximilians-Universität München, Munich, Germany) for LC MS/MS analysis.

Table 18: Ingredients of equilibration buffer for focused IPG stripes

Substance	Amount
Urea	72 g
Glycerol (99 %)	60 g
SDS	5 g
Buffer T (18.2 g Tris ultra pure, 0.4 g SDS ad 100 ml, pH 8.8)	6.6 ml
dH_2O	ad 200 ml

Table 19: Ingredients for polyacrylamide gels

Substance	Amount
Acrylamid 30 %; 29/1, 3 % crosslinked	179 ml
Buffer D (90.83 g Tris ultra pure; 2 g SDS ad 500 ml; pH 8.6)	125 ml
Water	170 ml
Glycerol (99 %)	28.75 g
TEMED	28 µl
APS (aqueous solution 10 %)	3.5 ml

2.2.5.3 Screening for glucansucrases catalyzing production of water insoluble α-D-glucan

Since Côté and Skory (2012) identified a glucansucrase (YP_819212) from *Lc. mesenteroides* that catalysis the synthesis of a water-insoluble α-D-glucan, water kefir isolated *Leuconostoc* strains and water kefir grains were screened for exactly this gene and genes in general that catalyze the synthesis of α-1,3-linked glucans.

Table 20: PCR conditions for specific primer set for glucansucrase (YP_819212)

Primer forward (5‘-3‘)	TCCAACTCAAGGTGGTTATG
Primer reverse (3´-5´)	AGTTCGTAATTCCCCAACTC
Primer concentration	0.25 µl each primer ad 50 µl
$MgCl_2$ concentration	1.5 mM
Denaturation	2 min; 94 °C
Melting	45 s; 94 °C
Annealing	1 min ; 53 °C
Elongation	1.5 min; 72 °C
Final extension	5 min; 72 °C

Melting, annealing and elongation steps were repeated within 32 cycles.

Table 21: PCR conditions for degenerated primer set for α-1,3-linked glucans

Primer forward (5‘-3‘)	GAYGGZTAYYTZACZGCZG
Primer reverse (3´-5´)	AGCCCARTCYTTZARZAC
Primer concentration	1 µl each primer ad 50 µl
$MgCl_2$ concentration	2 mM
Denaturation	2 min; 94 °C
Melting	45 s; 94 °C
Annealing	2 min ; 46 °C
Elongation	3 min; 72 °C
Final extension	5 min; 72 °C

Melting, annealing and elongation steps were repeated within 32 cycles.

Amplicons were detected by electrophoresis in 1.0 % agarose gels and visualized with dimidium bromide and UV.

2.2.5.4 Fast digestion

6 µl purificated PCR products (degenerated primer set) of WkW and WkI were digested with restriction enzymes EcoRI, HindIII and NdeI, respectively according to the manufacture protocol (Fermentas GmbH, St. Leon-Rot, Germany).

3 RESULTS

3.1 Water kefir supernatant analysis

The supernatant of two water kefir consortia with different origin was determined depending their conformities and differences during the fermentation process under standard fermentation conditions.

3.1.1 Time course of pH during water kefir fermentation

During water kefir fermentation the pH decreased rapidly within 48 h from about 6.5 to 3.5. Both water kefir consortia showed similar pH trends, merely during the first 20 h pH of WkI decreased faster than pH of WkW. After 48 – 72 h of fermentation, where the beverage normally is consumed, supernatant of both water kefir consortia showed final pH-values between 3.7 and 3.5 (Fig. 6).

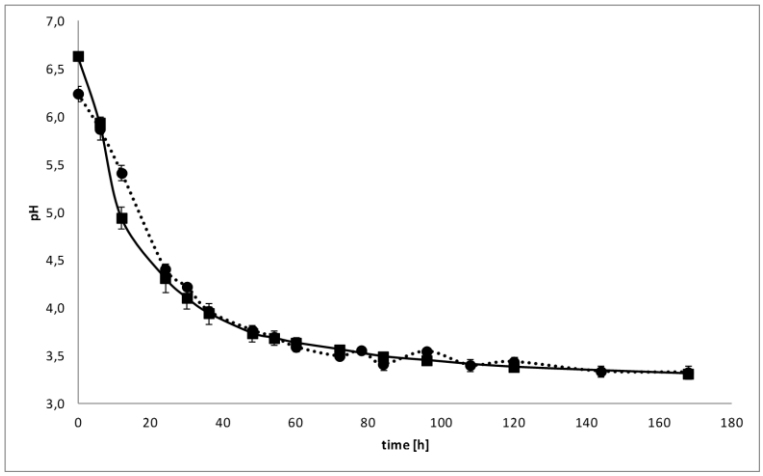


Fig. 6: Change of pH during water kefir fermentation
Squares represent pH of WkI and circles the pH of WkW.

3.1.2 Concentration of sugars and metabolites during water kefir fermentation

The supernatants of two water kefir consortia from different origin were investigated regarding sugar metabolism/utilization as well as production of ethanol and organic acids during fermentation. Within 48 h, the total content of sucrose was fermented and

concentrations of fructose, glucose and mannitol increased. The concentration of released glucose decreased after 36 h, whereas the content of fructose was reduced after 60 h (Fig. 7 and Fig. 8).

Sugar metabolism and utilization, respectively, as well as ethanol and succinate production were similar in both water kefir consortia tested. Differences in both systems could be detected concerning the production of acetate, after 96 h the concentration of acetic acid in the WkW-supernatant rose drastically, whereas WkI showed the same production rate during whole fermentation time (Fig. 8). Lactate concentration increased within 96 h up to 3.7 g/l during fermentation of WkW, whereas lactate concentration of WkI stagnated after 72 h of fermentation at about 2.0 g/l. Additional differences could be seen during the mannitol production, the consortium WkW steadily produced mannitol up to 8.0 g/L, whereas the production of mannitol in the supernatant of WkI stagnated after 72 h at 1.0 g/l (Fig. 8).

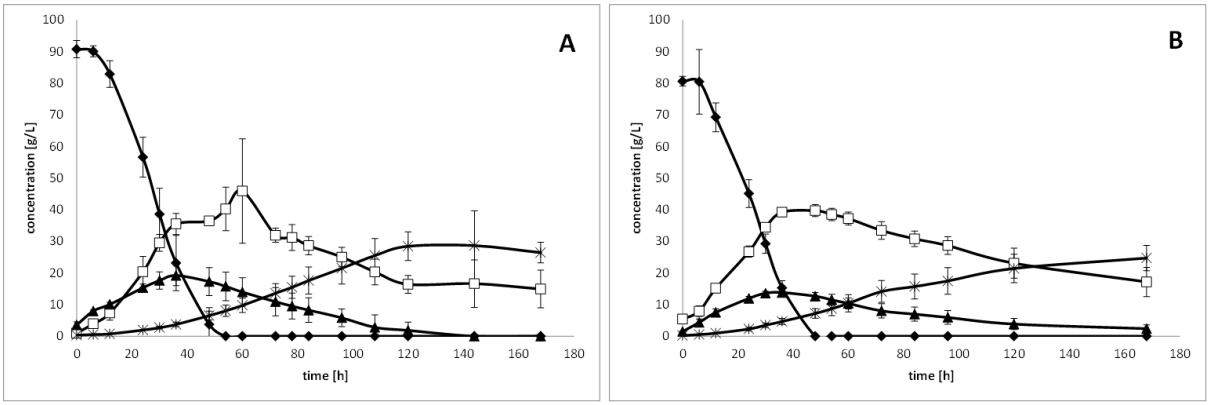


Fig. 7: Sugar and ethanol concentrations during water kefir fermentation

Water kefir supernatants of WkW (A) and WkI (B) were analyzed concerning their sucrose (diamonds), glucose (triangles), fructose (squares) and ethanol (crosses) concentrations.

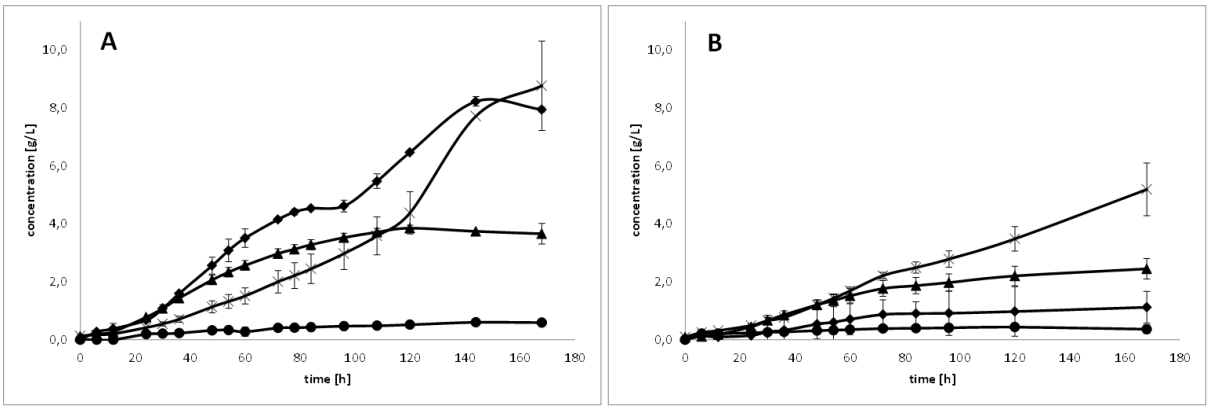


Fig. 8: Metabolite production during water kefir fermentation

Water kefir supernatants of WkW (A) and WkI (B) were analyzed concerning their lactate (triangles), acetate (crosses), succinate (circles) and mannitol (diamonds) concentration.

3.1.3 Volatile compounds of water kefir supernatant

Two water kefirs with different origin but same cultivation procedure showed same pattern of volatile compounds in water kefir supernatant (Fig. 9).

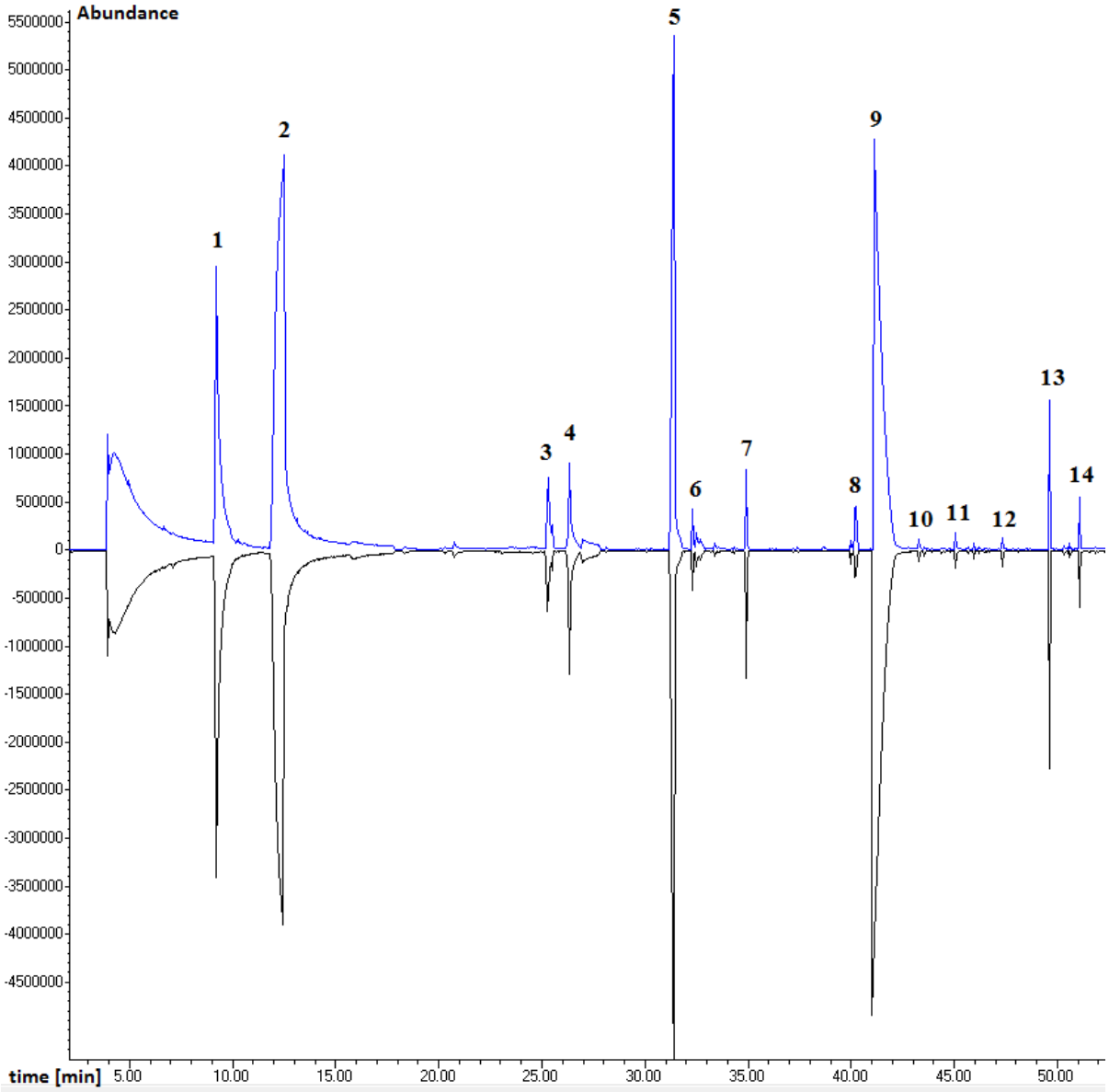


Fig. 9: Spectra of volatile compounds in the supernatant of 48 h fermented WkW (top) and WkI (bottom)

1	ethyl acetate	8	caprylic acid ethyl ester
2	ethanol	9	acetic acid
3	isobutanol	10	benzaldehyd
4	isoamyl acetate	11	isobutyric acid
5	isoamyl alcohol	12	isovaleric acid
6	caproic acid ethyl ester	13	β-phenethyl acetate
7	acetoin	14	2-phenylethanol

3.1.4 Amino acids in water kefir supernatant

Pure WKM contained only low concentrations of free amino acids (Table 22), ammonium chloride (0.024 mmol/l) and GABA (0.047 mmol/l). After three days of water kefir fermentation all these substances had been consumed.

Table 22: Amino acid concentration in WKM

amino acid	concentration in WKM [mmol/l]	amino acid	concentration in WKM [mmol/l]
aspartic acid	< 0.004	tyrosine	< 0.004
glutamic acid	< 0.004	cystine	< 0.004
asparagine	< 0.004	valine	0.006
serine	0.017	methionine	< 0.004
histidine	< 0.004	phenylalanine	< 0.004
glycine	< 0.004	isoleucine	0.005
threonine	0.006	ornithine	< 0.004
arginine	0.005	leucine	< 0.004
alanine	0.024	lysine	0.005

3.1.5 Change of parameters for water kefir fermentation

The influence of fermentation parameters were identified regarding sugar metabolism/utilization as well as production of ethanol and organic acids. Therefore, water kefir supernatant of standard water kefir fermentation (21°C, fig extract) was compared with the supernatant of water kefir fermentations with cranberry and apricot extract (21°C), respectively, as well as under different fermentation temperatures (12°C, 37°C).

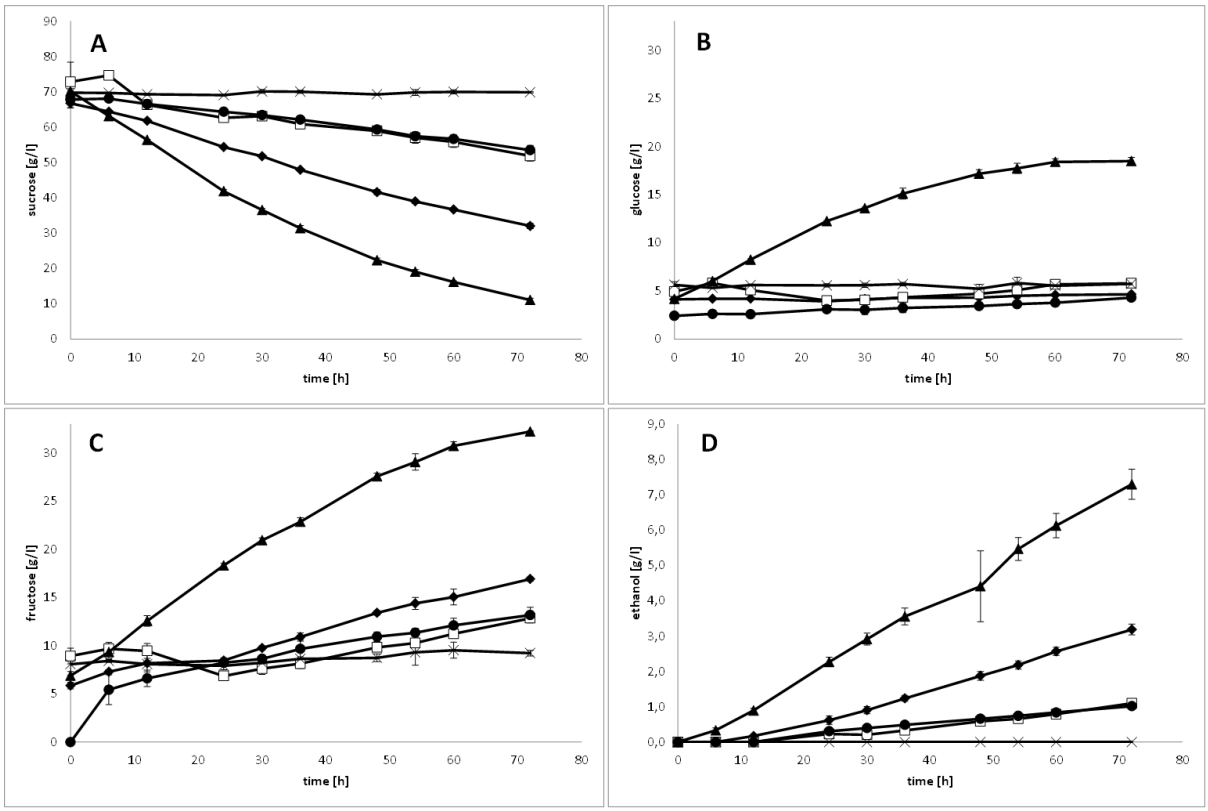


Fig. 10: Comparison of sugar and ethanol concentrations of water kefir fermentations under different conditions

Water kefir supernatant was analyzed under standard conditions (21°C, fig extract; diamonds), at different temperatures, 12 °C (squares) and 37°C (triangles) and with different fruit extracts, cranberry (21°C; crosses) and apricot (21°C; circles) concerning their change in sucrose (A), glucose (B), fructose (C) and ethanol (D) concentration during fermentation.

Sucrose concentration decreased within fermentation under every condition, except in the supernatant with cranberry extract, the sucrose concentration did not change. Highest sucrose consumption could be detected while fermentation at 37 °C (about 70 g/l within 72 h), whereas the concentration under standard conditions diminished about 40 g/l per 72 h (Fig. 10 A). Amounts of glucose rose from the beginning of fermentation at 37°C, whereas under the other conditions tested glucose concentrations did not change during 72 h of fermentation (Fig. 10 B). Similar observations could be made for fructose and ethanol concentrations. However, these contents also increased a bit while fermentation at 12°C and with apricot extract and particularly under standard fermentations conditions (Fig. 10 C and D).

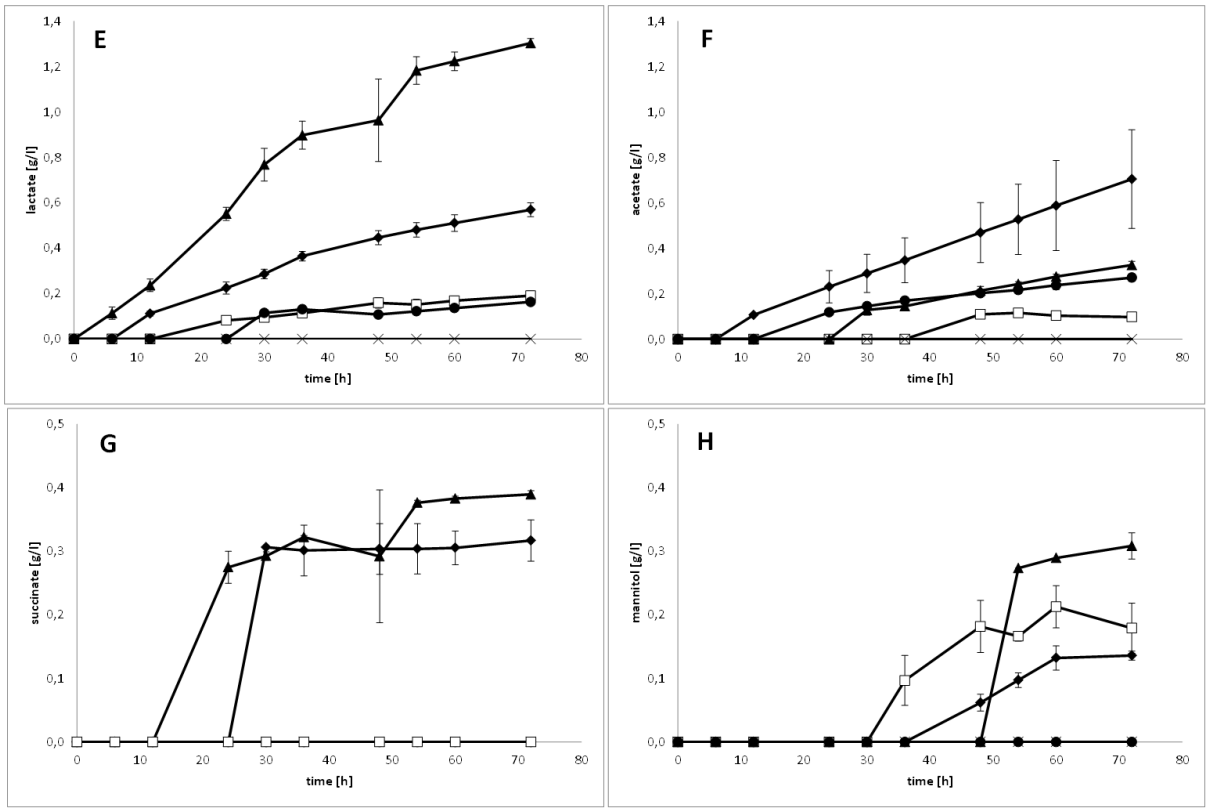


Fig. 11: Comparison of organic acid and mannitol concentrations of water kefir fermentations under different conditions

Water kefir supernatant was analyzed under standard conditions (21°C, fig extract; diamonds), at different temperatures, 12 °C (squares) and 37°C (triangles) and with different fruit extracts, cranberry (21°C; crosses) and apricot (21°C; circles) concerning their production of lactate (E), acetate (F), succinate (G) and mannitol (H) during fermentation.

Amounts of lactate especially rose while fermentation at 37 °C. Equal increases of lactate and acetate could be determined under standard conditions. Lactate production at 12°C started after 10 h, whereas the production of acetate in this system not until 36 h of fermentation. Conversely, in the system with apricot extract the production of acetate (12 h) began before that of lactate (24 h) (Fig. 11 E and F). Contents of succinate could merely be detected under standard conditions and at 37°C in both systems up to 0.3 g/l. First mannitol amounts could be determined at 12 °C after 30 h, whereas under standard conditions the production began after 36 h and at 37°C after 48 h.

In the system with cranberry extract concentrations of the different determined substances did not change during fermentation time.

3.1.6 Soluble EPS in water kefir supernatant

The concentration of EPS in the water kefir supernatant amounted to < 1.0 g/l. Acid hydrolysis and HPLC analysis revealed glucose and fructose as sugar monomers.

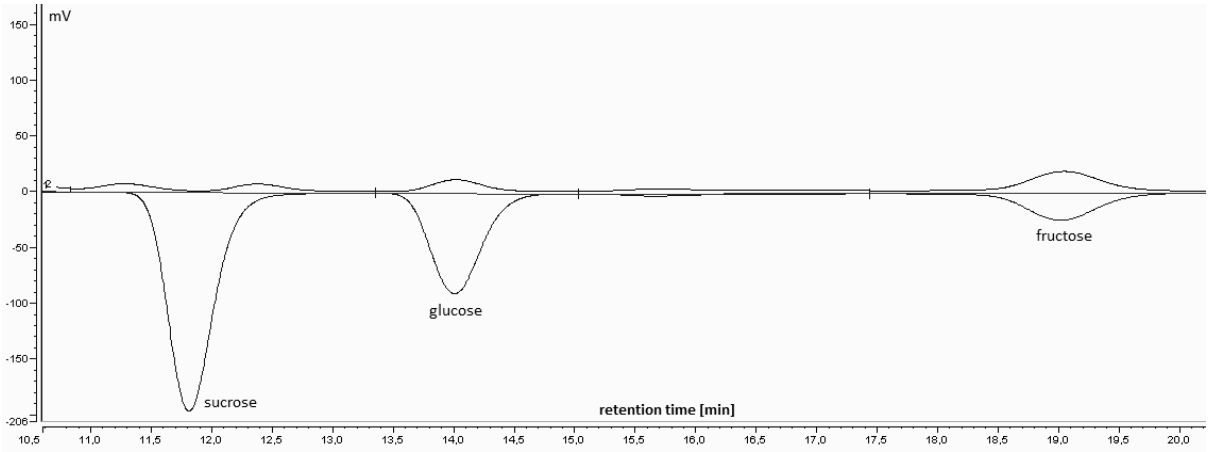


Fig. 12: Comparison of the HPLC chromatogram for hydrolyzed water kefir supernatant EPS (top) and sugar standard substances (bottom)

Small peaks between 11 and 13 minutes display incomplete hydrolyzed glucose-polymer fragments. Stronger hydrolysis of the supernatant EPS resulted in complete cleavage of glucose polymers resulting in a high glucose peak, but fructose did not exist anymore because of its heat and acid sensitivity.

3.2 Analysis of water kefir grains

3.2.1 Growth of water kefir grains during fermentation

Water kefir consumers observed that the grains grew better in hard-water (without scientific background). Therefore, the influence of mineral compounds, especially calcium, was analyzed. Mass increase of water kefir grains was determined under standard conditions (pure WKM) in comparison to WKM that was prepared with dH_2O and WKM supplemented with Ca-ions (supplementation of 0.1 % $CaCl_2$). Fig. 13 displays the percentage increase of water kefir grain mass per fermentation day. After three days (first fermentation step) water kefir grains in all three media showed similar mass increase (about 25 % per day).

The increase of grain mass decreased drastically in the system prepared with dH_2O from the second cultivation step on. Growth of water kefir grains in WKM supplemented with Ca-ions fluctuated between single samples, averaging the increase is less than during fermentation under standard conditions.

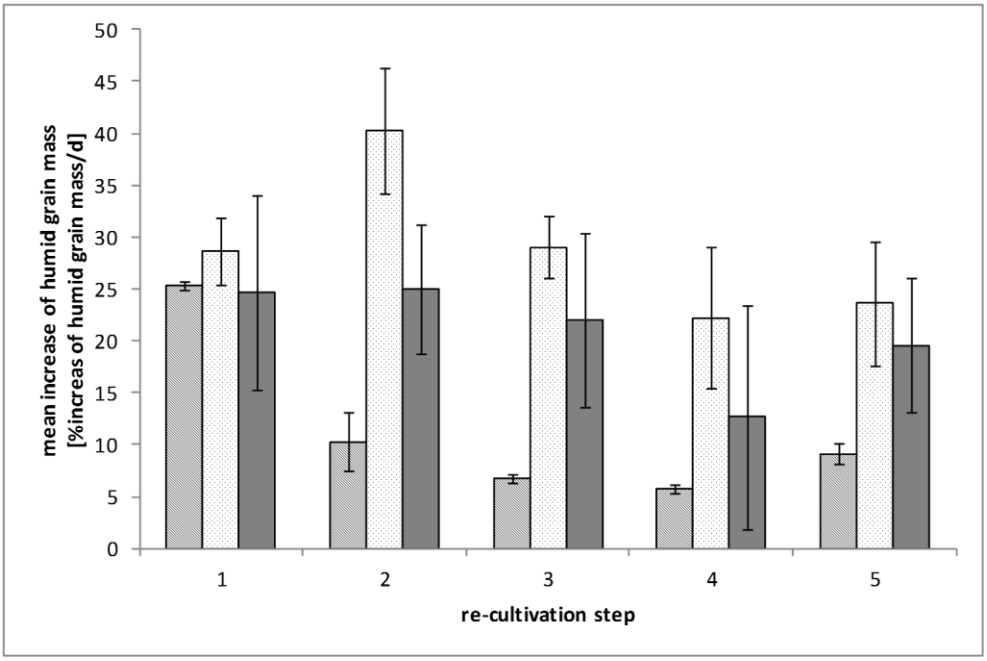


Fig. 13: Percentage increase of humid grain mass per fermentation day

The increase of grain mass was determined within 15 days, each 3 days the grains were weighted and re-cultivated in fresh medium in total of 5 steps. Striped bars represent increase of WkW in WKM prepared with dH_2O, pointed bars the increase under standard conditions (WKM) and grey bars the increase of humid grain mass in WKM supplemented with Ca-ions.

3.2.2 Enzyme treatment of water kefir grains

To learn more about the bond types of water kefir grains, the grains were treated with various enzymes dividing different linkages in polysaccharides. Addition of α-glucosidase, β-amylase, amyloglucosidase and glucooxidase did not show an effect on water kefir grains. Whereas, dextranase displayed a disintegration of water kefir grains, thus the main linkages of water kefir grains are α-1,6 linkages. The cloudy suspension after dextranase treatment showed, that there were still insoluble parts.

3.2.3 Structural analysis of water kefir grain EPS

Water kefir grains could be solved with 1 M NaOH. After purification of water kefir grain EPS, the sugar monomers of the EPS could be determined. HPLC analyses displayed, that grain EPS consisted of glucose monomers.

NMR analysis constituted grain EPS mainly as linear dextran (Table 23). Additional resonances at 5.33 in the ^{1}H-NMR spectrum could be measured. Comparison of peak areas of both anomeric ^{1}H atoms displayed 10 % α(1→3) branching.

Table 23: ^{1}H and ^{13}C chemical shifts of water kefir grain EPS

Atoms	1	2	3	4	5	6
^{1}H	4.99	3.59	3.76	3.52	3.92	4.00
^{13}C	97.65	73.31	71.30	70.11	69.46	65.50

3.3 EPS producing water kefir isolates

Water kefir isolates were screened for their EPS production to identify their impact on the growth of water kefir grains.

3.3.1 EPS production of single water kefir isolates

Thirty-seven water kefir strains were determined for their EPS production on sucrose containing agar plates. 17 isolates produced EPS in different concentrations. Fig. 14 displays EPS production of four strains with different production levels.

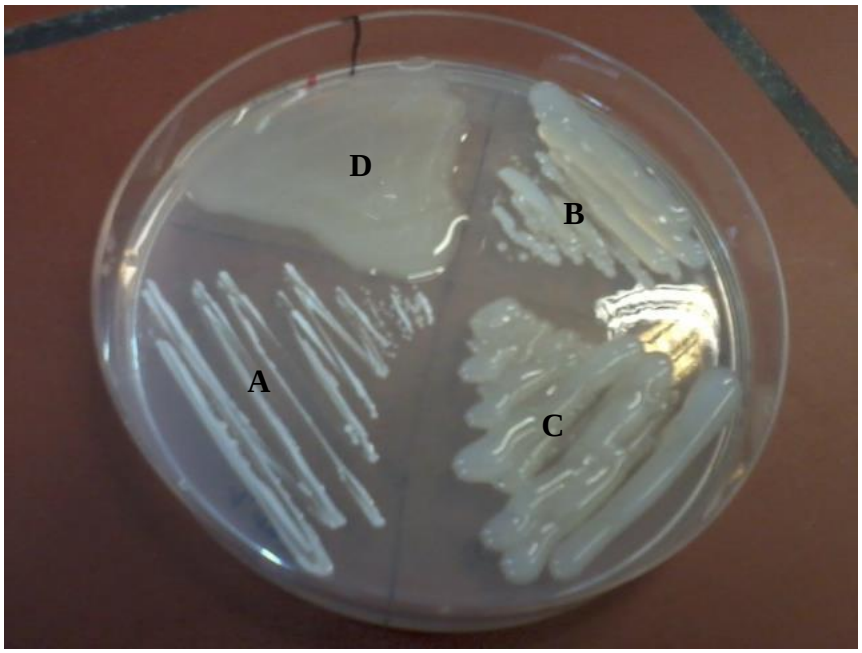


Fig. 14: EPS production of water kefir isolates in different concentrations

A represents a strain that did not produce any EPS (-) (*Lb. nagelii*, TMW 1.1825), **B** slight EPS production (+) (*Lb. nagelii*, TMW 1.1826), **C** strong EPS production (++) (*Lb. hordei*, TMW 1.1907) and **D** very strong EPS production (+++) (*Lc. citreum*, TMW 2.1194) on sucrose containing agar plates.

3.3.2 EPS characterization of single water kefir isolates

Strong and very strong EPS producers were determined regarding the EPS concentration in liquid sucrose containing medium after 48 h of fermentation. The isolated and purified EPS were further characterized along their sugar monomers. 12 of 13 isolated EPS consisted of glucose (Table 24). Only one strain of *G. albidus*, produced a fructan, as indicated by the high fructose monomer content of the EPS.

Interesting is the difference between the assessment of EPS production on agar plates in comparison to the concentration found after 48 h of fermentation in liquid medium. *Lc. mesenteroides* (TMW 2.1073) and *G. albidus* (TMW 2.1191) were identified as very strong EPS producers (+++) on agar plates. In liquid medium the EPS production of these both strains was less, the amounts of produced EPS in liquid medium was similar to other strong EPS producers (++). Conversely the other three very strong EPS producing strains *Lb. hilgardii* (TMW 1.1819 and 1.1828) and *Lc. citreum* (TMW 2.1194) produce amounts of 30 g/l after 48 h of fermentation in liquid medium.

Table 24: EPS production and characterization of single water kefir isolates

microorganisms	strain	origin	EPS-production on agar plates	EPS-concentration in liquid medium [g/l]	Identified monomers after EPS hydrolysis
L. casei	TMW 1.1814	WkA	-		
L. casei	TMW 1.1816	WkA	-		
L. hordei	TMW 1.1817	WkA	++	10.8	glucose
Lb. hordei	TMW 1.1818	WkA	-		
Lb. hilgardii	TMW 1.1819	WkA	+++	32.5	glucose
Lb. satsumensis	TMW 1.1820	WkA	-		
Lb. hordei	TMW 1.1821	WkA	++	10.8	glucose
Lb. hordei	TMW 1.1822	WkF	++	9.2	glucose
Lb. nagelii	TMW 1.1823	WkA	++	23.3	glucose
Lb. nagelii	TMW 1.1824	WkF	++	23.3	glucose
Lb. nagelii	TMW 1.1825	WkW	-		
Lb. nagelii	TMW 1.1826	WkF	+		
Lb. nagelii	TMW 1.1827	WkF	++	17.5	glucose
Lb. hilgardii	TMW 1.1828	WkA	+++	30.8	glucose
Lb. satsumensis	TMW 1.1829	WkF	++	20.0	glucose
Lb. hordei	TMW 1.1907	WkW	++	22.5	glucose
Lc. mesenteroides	TMW 2.1073	WkA	+++	10.8	glucose
Lb. casei	TMW 2.1074	WkA	-		
Lc. mesenteroides	TMW 2.1075	WkF	+		
Lc. mesenteroides	TMW 2.1076	WkF	+		
Ac. cerevisiae	TMW 2.1084	WkA	-		
G. oxidans	TMW 2.1085	WkA	-		
Ac. aceti	TMW 2.1153	WkW	-		
Ac. Iovaniensis	TMW 2.1154	WkW	-		
Ac. ghanensis	TMW 2.1155	WkW	-		

microorganisms	strain	origin	EPS-production on agar plates	EPS-concentration in liquid medium [g/l]	Identified monomers after EPS hydrolysis
Ac. fabarum	TMW 2.1156	WkW	-		
Ac. ghanensis	TMW 2.1157	WkW	-		
Ac. cerevisiae	TMW 2.1158	WkW	-		
G. albidus	TMW 2.1191	WkA	+++	6.7	fructose, glucose
Ac. fabarum	TMW 2.1192	WkW	-		
Lc. mesenteroides	TMW 2.1193	WkW	-		
Lc. citreum	TMW 2.1194	WkW	+++	32.5	glucose
Lc. mesenteroides	TMW 2.1195	WkW	+		
Ac. orientalis	TMW 2.1196	WkW	-		
B. psychroerophilum	TMW 2.1395	WkW	-		
B. psychroerophilum	TMW 2.1397	Ebay Wk Ehrmann	-		
B. psychroerophilum	TMW 2.1398	Ebay Wk Ehrmann	-		

3.3.3 EPS production in water kefir medium

Previous experiments displayed EPS production in MRS-Sac and No5-Sac medium, respectively, depending on the investigated species (MRS for lactobacilli and No5 for acetic acid bacteria). To learn more about the EPS production in the natural water kefir environment, very strong EPS producing water kefir isolates were cultivated in WKM and their EPS production was determined. After 48 h of fermentation in WKM the concentrations of EPS were much lower than cultivating the strains in MRS and No5, respectively (Table 25). *Lb. hilgardii* (TMW 1.1828), a very strong EPS producing strain in MRS-Sac (30.8 g/l), produced hardly any EPS in WKM (< 1.0 g/l). However *Lc. mesenteroides* (TMW 2.1073) is not that affected, this strain produced with 5.7 g/l about 50 % less EPS in comparison to MRS-Sac medium (10.8 g/l).

Chromatographic analysis of hydrolyzed EPS did not show a difference in containing sugar monomers to EPS produced in MRS-Sac and No5-Sac, respectively.

Table 25: EPS production in WKM

species	strain	EPS-concentration in WKM after 48 h [g/l]	Identified monomers after EPS hydrolysis
Lb. hilgardii	TMW 1.1828	< 1.0	glucose
Lc. mesenteroides	TMW 2.1073	5.7	glucose
G. albidus	TMW 2.1191	< 1.0	fructose, glucose
Lc. citreum	TMW 2.1194	4.1	glucose

3.3.4 Glucansucrases of water kefir isolates catalyzing production of water insoluble α-D-glucan

Côté and Skory (2012) identified a glucansucrase (YP_819212) from *Lc. mesenteroides* that catalysis the synthesis of a water-insoluble α-D-glucan. We screened our water kefir isolated *Leuconostoc* strains with a specific primer set for exactly this *Leuconostoc* gene and with a degenerated primer set for genes in general that catalyze the synthesis of α-1,3-linked glucans to identify key players in water kefir grain production.

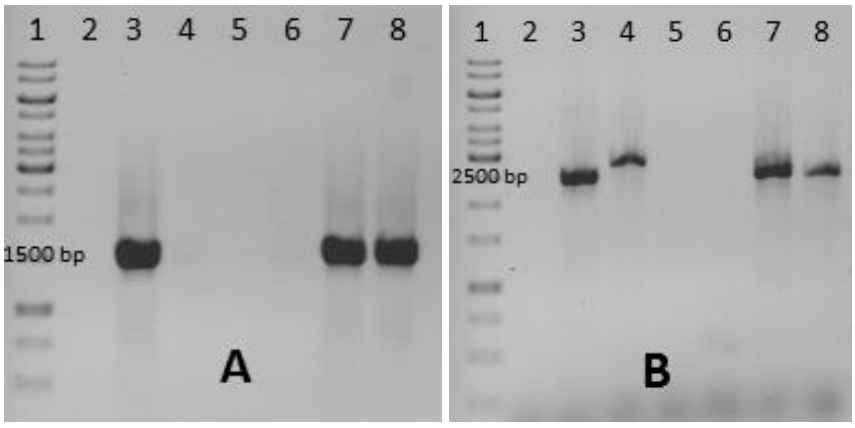


Fig. 15: Screening of water kefir *Leuconostoc* strains with a specific primer set for glucansucrase YP_819212 (A) and with a degenerated primer set for α-1,3-linkaging glucansucrases (B).

Lane 1: 1 kb DNA ladder; lane 2: negative control; lane 3: *Lc. mesenteroides* (TMW 2.1193); lane 4: *Lc. citreum* (TMW 2.1194); lane 5: *Lc. mesenteroides* (TMW 2.1195); lane 6: *Lc. mesenteroides* (TMW 2.1073); lane 7: *Lc. mesenteroides* (TMW 2.1075); lane 8: *Lc. mesenteroides* (TMW 2.1076)

Fig. 15 A shows that three water kefir isolated *Lc. mesenteroides* strains have the gene for glucansucrases ATCC 8293 to express the protein for production of a water-insoluble α-D-glucan. PCR-reactions with the degenerated primer set showed an additional PCR product

with the *Lc. citreum* strain TMW 2.1194. Sequencing of this PCR product revealed a unspecific bond of the primer set.

The same primer sets were tested with water kefir grain DNA of two different water kefirs (WkW, WkI). Both water kefir DNAs showed PCR products with both primer sets. The PCR products received with the degenerated primer set were digested with three different restriction enzymes and compared with the theoretical restriction pattern of the amplified gene fragment of glycosyltransferase of strain ATCC 8293 to determine if other genes for α-1,3-linked glucans are encoded in water kefir DNA. Fig. 16 and Table 26 show that obtained digestion fragments coincided with expected fragments when PCR product of gene from strain ATCC 8293 had been digested.

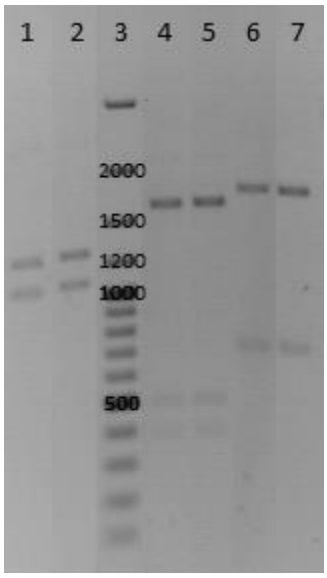


Fig. 16: Fast Digestion pattern of amplicons with the degenerated primer set from water kefir DNA

Table 26: Figure caption for Fig. 16 and comparison with theoretical results

lane	Tested DNA	restriction enzyme	expected DNA fragments [bp]	obtained DNA fragments [bp]
1	WkW	Eco RI	1123, 1324	ca. 1100, 1350
2	WkI			
3		100 bp DNA ladder		
4	WkW	Hind III	1608, 473, 366	ca. 1600, 500, 400
5	WkI			
6	WkW	Nde I	699, 1748	ca. 1800, 750
7	WkI			

3.4 Single cultivation of water kefir isolates in WKM

3.4.1 Growth of different water kefir isolates in water kefir medium

During water kefir fermentation microorganisms live in a community. The individual behavior of single water kefir isolates was examined during growth experiments in WKM. Different water kefir isolates of one species showed different growth behavior in WKM. Fig. 17 displays growth of *Lb. hordei* isolates during 72 h of fermentation. Lag phase of strain TMW 1.1821 is elongated the other strains did not show a lag phase. Strains TMW 1.1821 and TMW 1.1907 reached stationary phase after ca. 20 h at OD_{590} 0.2, whereas strains TMW 1.1822 (after 70 h) and TMW 1.1817 (after 28 h) obtained OD_{590} 0.45. Strain TMW 1.1818 displayed highest growth rate of *Lb. hordei* strains in WKM, though after reaching stationary phase cells lysed and OD_{590} decreased immediately. Growth rate of strains TMW 1.1817 and TMW 1.1822 was similar until 17 h of fermentation. TMW 1.1817 grew to its stationary phase and slowly lysed from this point on, whereas TMW 1.1822 grew on with a lower growth rate to the end of measurement.

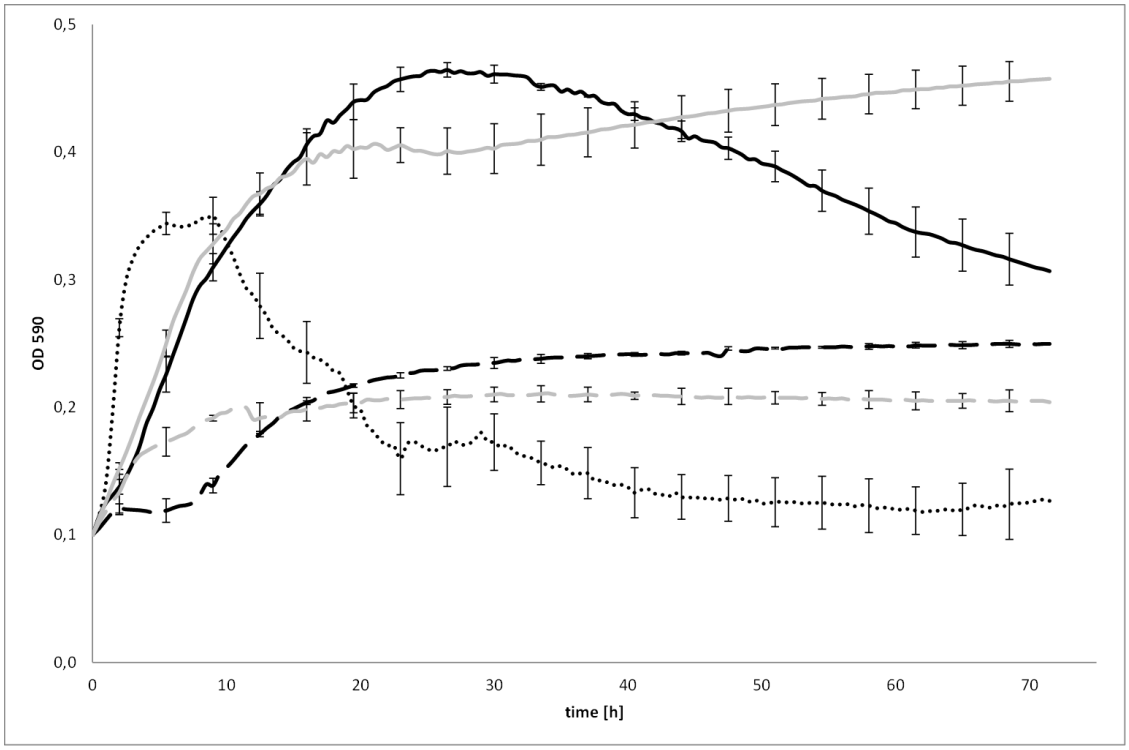


Fig. 17: Growth of different *Lb. hordei* strains isolated from water kefir in WKM

Growth of strain TMW 1.1817 (black line), TMW 1.1818 (black dotted), TMW 1.1821 (black dashed), TMW 1.1822 (grey line) and TMW 1.1907 (grey dashed) in WKM.

3.4.2 Growth of water kefir isolates at different temperatures

Sugar and metabolite concentrations in water kefir supernatant during fermentation were compared at different temperatures (3.1.5). For interpretation of results of the whole consortium growth behavior of single water kefir isolates in WKM was determined at different temperatures. Growth of LAB was not very well in WKM, therefore differences in growth at 12, 21, 30 and 37°C was not clearly distinguishable. *B. psychraerophilum* grew best at 37 °C whereas the other temperatures did not really show a difference in growth (Fig. 18). *Z. florentina* displayed best growth at 21 °C. At 12 °C this yeast showed a short lag phase as against 30 °C, whereas after 60 h OD_{590} at 12 °C rose higher than at 30 °C. *Z. florentina* did not grow very well at 37 °C, after 60 h at this temperature cells seemed to lyse because OD_{590} decreased from this time on (Fig. 19 A). Until 60 h growth of *S. cerevisiae* at 21, 30 and 37 °C was similar. After 60 h at 37 °C cells seemed to lyse because OD_{590} decreased. At 12 °C this yeast displayed a very short lag phase and grew a bit worse than at the other temperatures (Fig. 19 B).

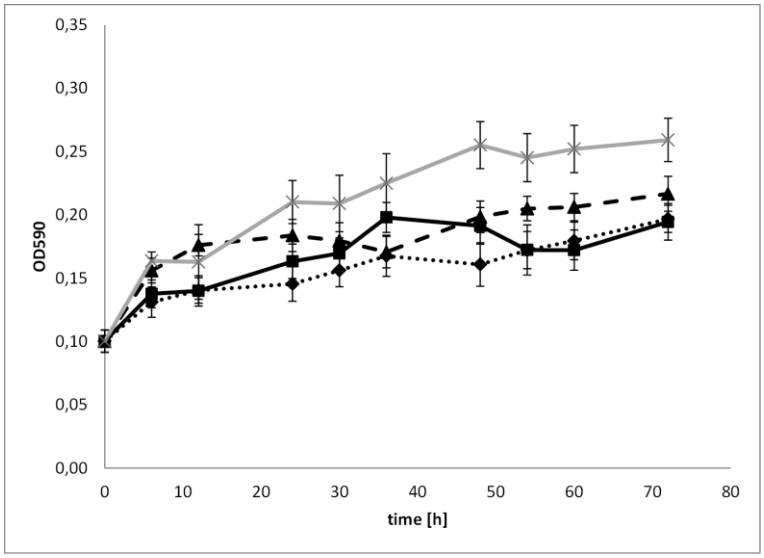


Fig. 18: Growth of *B. psychraerophilum* (TMW 2.1395) at different temperatures in WKM
12 °C (diamonds), 21 °C (squares), 30 °C (triangle) and 37 °C (crosses)

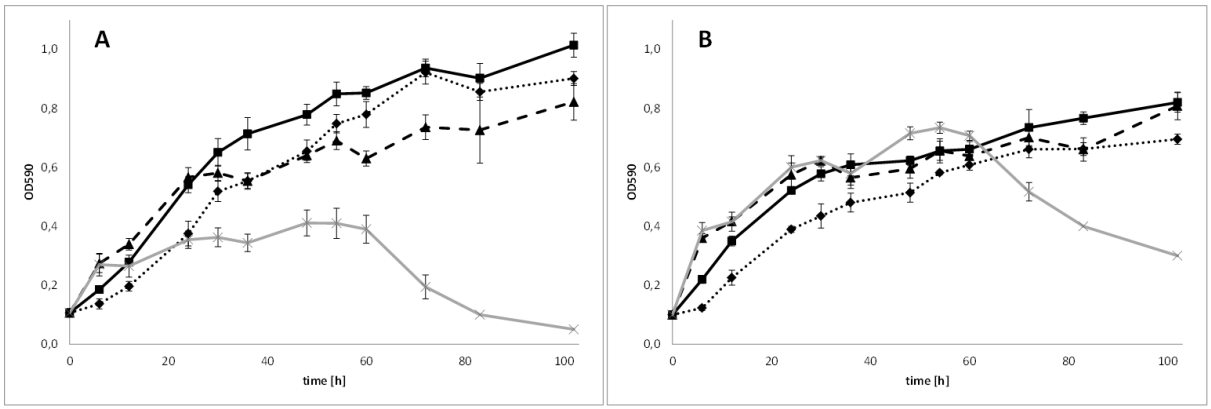


Fig. 19: Growth of *Z. florentina* (A) and *S. cerevisiae* (B) at different temperatures in WKM
12 °C (diamonds), 21 °C (squares), 30 °C (triangle) and 37 °C (crosses)

3.4.3 Growth of water kefir isolates in WKM supplemented with EPS

Normal WKM and MRS without another carbon source were supplemented with different EPS from water kefir isolates. Growth of 18 different water kefir isolates was determined in these prepared media to get to know if water kefir isolates are able to ferment EPS of the consortium.
Growth experiments in supplemented WKM could show that EPS did not promote growth of water kefir isolates. Absent growth in MRS with EPS as single carbon source displayed that water kefir isolates were not able to ferment EPS.

3.5 Interaction of water kefir isolates

3.5.1 Experiments for reconstitution of water kefir grains

After isolation of cultivable water kefir isolates (Gulitz et al., 2011) the isolates were reassembled in order to rebuilt water kefir grains. Already after 48 h the smell of the suspension implied that yeasts overgrew the fermentation broth. The typical odor of water kefir supernatant did not emerge. Even after three month of fermentation no grains were built. Previous investigation only included cultivable water kefir isolates. Since water kefir grains could also contain uncultivable organisms the grains were destroyed and the supernatant, containing theoretically all grain organisms, was used for further grain reconstruction experiments. Fermentation of this suspension in WKM as well as the usage of crystallization seeds in terms of glass beads or autoclaved granules did not cause grain formation.

It had been suggested that grain organisms needed close proximity to build grains. Therefore, suspension of destroyed grains was embedded in alginate beads. WKM fermentation with these alginate beads produced a beverage that smelled similar to normal water kefir fermentation broth, but the beads did not grow.
Grain reconstruction could not be achieved during this work.

3.5.2 Co-cultivation experiments in the model system

Metabolic interactions between single water kefir isolates without cell-cell contact were investigated in a model system (Transwell®).

3.5.2.1 Comparison of growth

Each co-cultivation of yeasts and lactobacilli tested showed an improvement of growth compared with single cultivation of the individual organisms (Fig. 20 and Fig. 21). Both lactobacilli showed equal positive effects in growth for the two yeasts (Fig. 20 A and B), whereas the co-cultivation of *Lb. hordei* with *Z. florentina* showed a better improvement than the co-cultivation with *S. cerevisiae* (Fig. 21 A). OD_{590} of stationary phase of *Lb. nagelii* was similar in co- and in single cultivation but growth rate in the exponential phase was higher in co-cultivation than in single cultivation (Fig. 21 B).

Also the co-cultivation of other *Lactobacillus* and *Leuconostoc* strains showed equal results to these presented for lactobacilli.

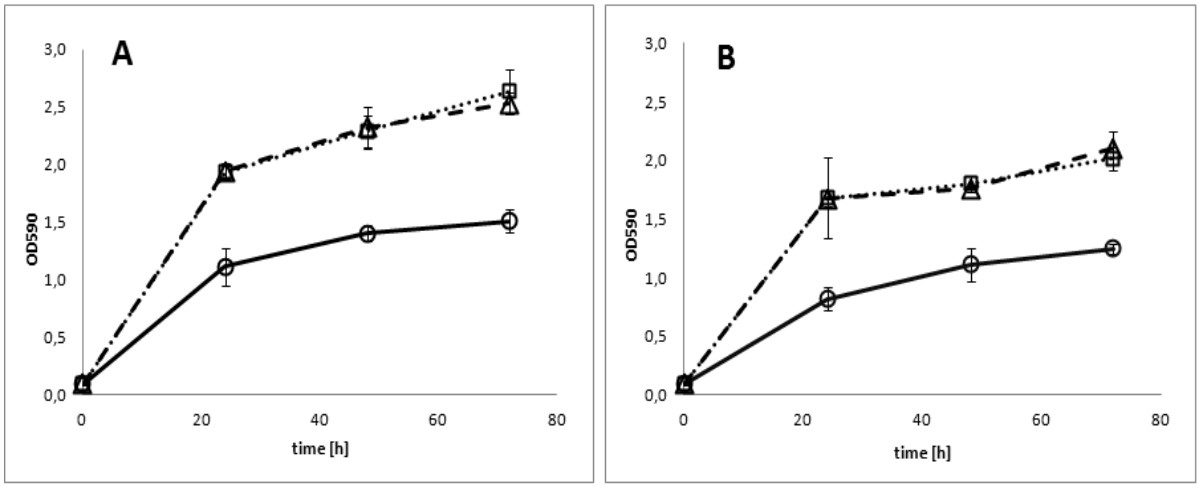


Fig. 20: Difference in growth of water kefir isolated yeasts in single- and in co-culture with lactobacilli in WKM (Stadie et al., 2013)

Circles represent the single cultivation of *Z. florentina* (A) and *S. cerevisiae* (B), respectively. Dashed lines show growth of *Z. florentina* (A) and *S. cerevisiae* (B) in co-cultivation with *Lb. hordei*, dotted line the co-cultivation with *Lb. nagelii*

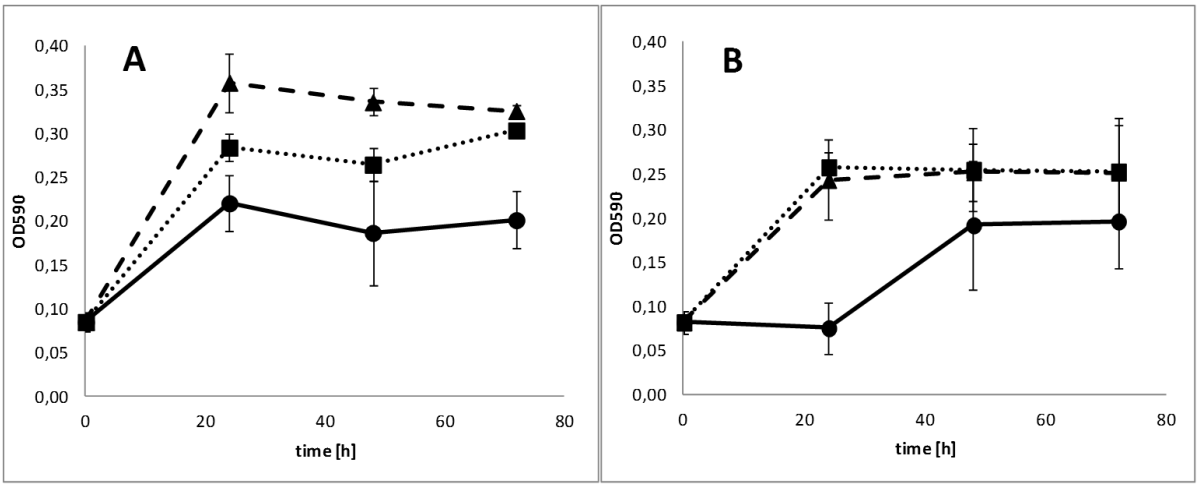


Fig. 21: Difference in growth of water kefir isolated lactobacilli in single- and in co-culture with yeasts in WKM (Stadie et al., 2013)

Circles represent the single cultivation of *Lb. hordei* (A) and *Lb. nagelii* (B), respectively. Dashed lines show growth of *Lb. hordei* (A) and *Lb. nagelii* (B) in co-cultivation with *Z. florentina*, dotted line the co-cultivation with *S. cerevisiae*

The co-cultivation of water kefir yeasts with water kefir isolated *B. psychraerophilum* resulted in the increase of growth for both yeasts (Fig. 22 B and C). *B. psychraerophilum* was not affected in co-cultivation with water kefir yeasts (Fig. 22 A).

Co-cultivation experiments with water kefir lactobacilli and *B. psychraerophilum* did not show a difference to single cultivation of the individual organisms.

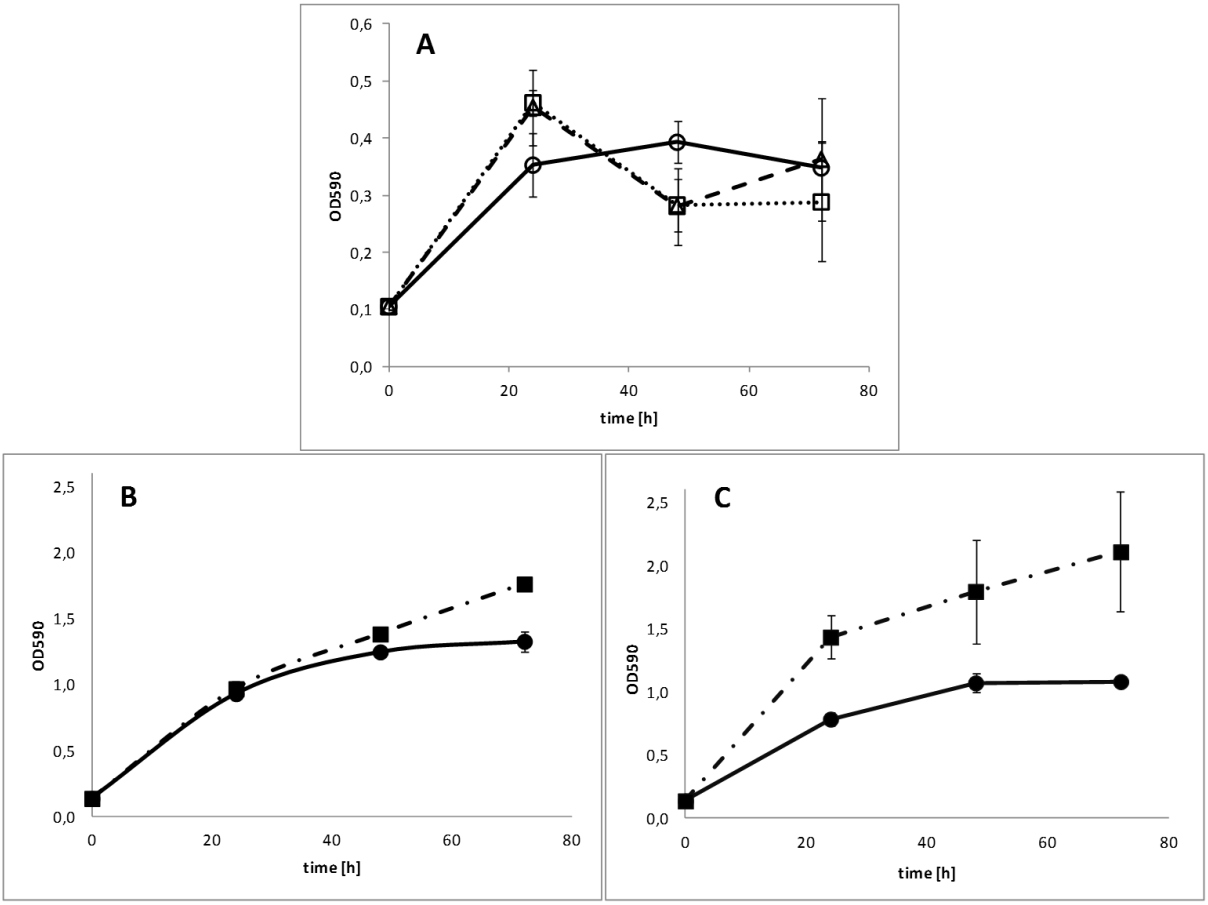


Fig. 22: Difference in growth of water kefir isolated yeasts in single- and in co-culture with *B. psychraerophilum* in WKM

Circles represent the single cultivation of *B. psychraerophilum* (A), *Z. florentina* (B) and *S. cerevisiae* (C), respectively. Dashed line (A) represents growth of *B. psychraerophilum* in co-cultivation with *Z. florentina*, dotted line in co-cultivation with *S. cerevisiae*. Squares show growth of *Z. florentina* (B) and *S. cerevisiae* (C) in co-cultivation with *B. psychraerophilum*.

3.5.2.2 Comparison of metabolites

This chapter displays sugar consumption and metabolite production of different co-cultivation systems in comparison to the pooled single culture values, declared as "calculated co-culture", of the individual organisms. All values are referred to a specific cell concentration (OD 1). Single cultured yeasts produce ethanol and succinate, *Z. florentina* additionally produced mannitol. Lactate and acetate are metabolites of the bacteria tested.

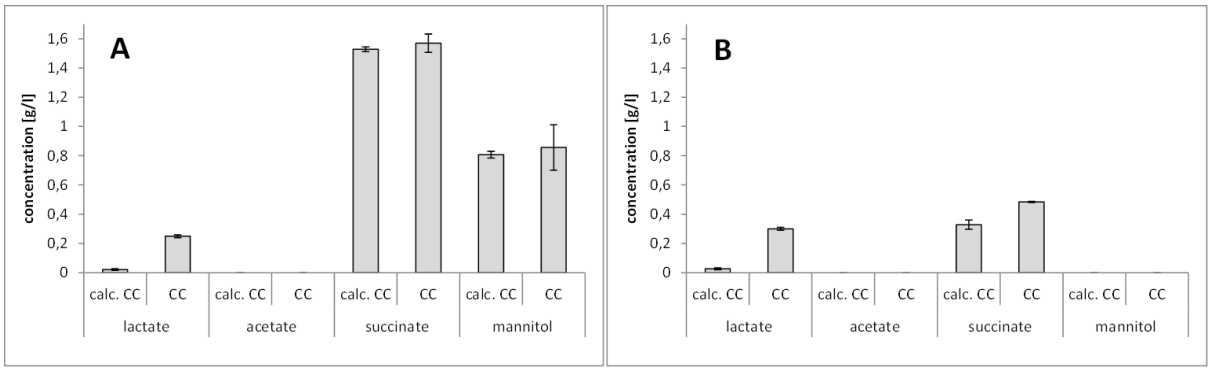


Fig. 23: Metabolite concentrations after 72 h fermentation of *Lb. nagelii* in co-cultivation (CC) and as calculated co-culture (calc. CC) with *Z. florentina* (A) and *S. cerevisiae* (B), respectively.

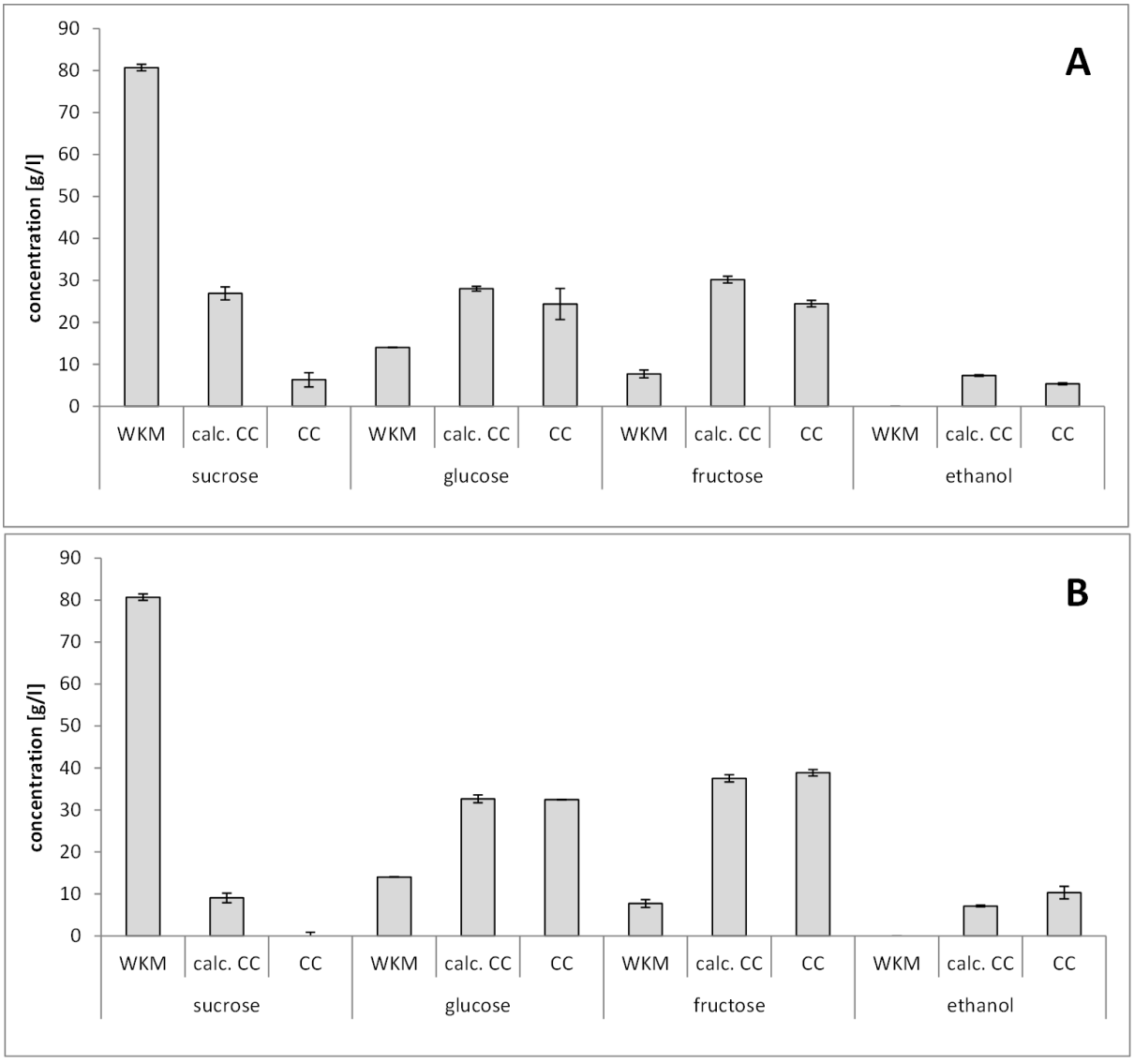


Fig. 24: Sugar and ethanol concentrations after 72 h fermentation of *Lb. nagelii* in co-cultivation (CC) and as calculated co-culture (calc. CC) with *Z. florentina* (A) and *S. cerevisiae* (B), respectively

Values for WKM represent sugar and ethanol concentration of the pure medium.

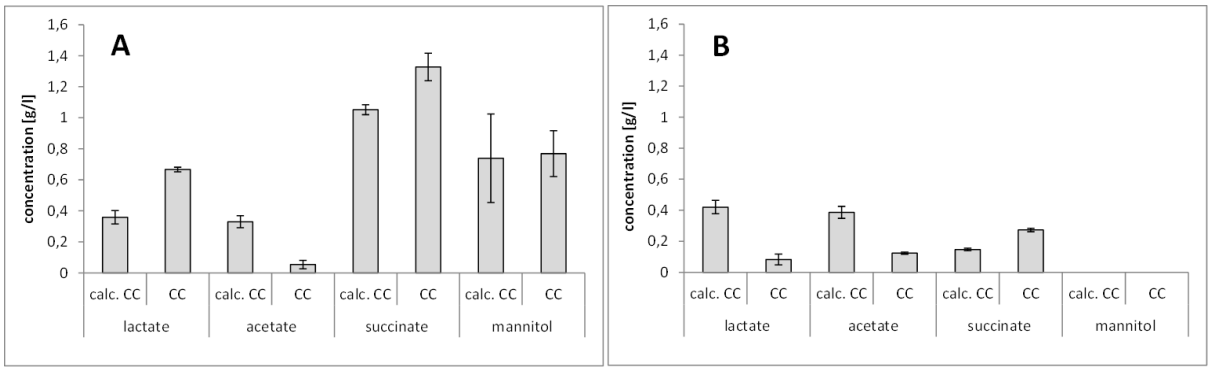


Fig. 25: Metabolite concentrations after 72 h fermentation of *B. psychraerophilum* in co-cultivation (CC) and as calculated co-culture (calc. CC) with *Z. florentina* (A) and *S. cerevisiae* (B), respectively

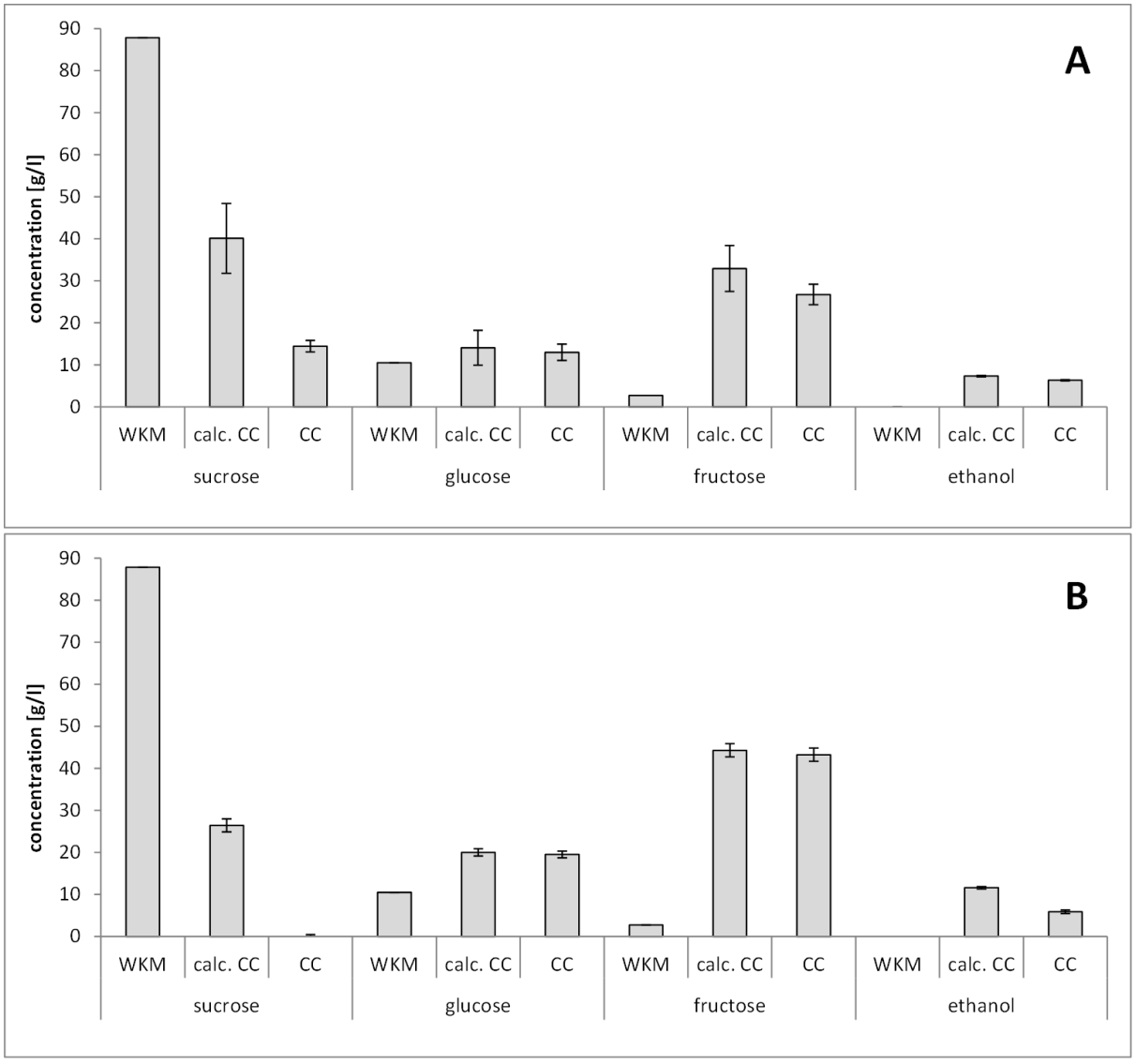


Fig. 26: Sugar and ethanol concentrations after 72 h fermentation of *B. psychraerophilum* in co-cultivation (CC) and as calculated co-culture (calc. CC) with *Z. florentina* (A) and *S. cerevisiae* (B), respectively

Values for WKM represent sugar and ethanol concentration of the pure medium.

In co-cultivation metabolism of organisms was more active because in all of the three systems shown more sucrose was consumed in co-cultivation than in calculated co-cultivation. Likewise more glucose and fructose was metabolized since due to the decrease of sucrose and its inversion to glucose and fructose the concentrations of fructose and glucose did not raise, conversely in the systems with *Z. florentina* both concentrations decreased. Concentrations of ethanol in co-cultivation systems with *Z. florentina* slightly decreased (about 1 g/l). More distinct differences could be determined in co-cultivation with *Lb. nagelii* and *S. cerevisiae* the amount of ethanol rose about 3 g/l compared with its calculated co-cultivations. Conversely, in co-cultivation with *B. psychraerophilum* and *S. cerevisiae* concentrations of ethanol decreased about 50% (from 11.6 g/l in calc. CC to 5.8 g/l in CC) (Fig. 24, Fig. 26, Fig. 39).

The concentrations of lactate in all co-cultivation systems with lactobacilli distinctly rose about tenfold (Fig. 23 and Fig. 39). In co-cultivation of *B. psychraeophilum* and *Z. florentina* amount of lactate only rose about twofold, conversely the content of lactate in the system with *S. cerevisiae* decreased drastically from 0.42 to 0.08 g/l (Fig. 25). The concentration of acetate is decreased in both systems with the *Bifidobacterium* in co-cultivation with yeast. Lactobacilli tested did not produce any detectable acetate.
Concentrations of succinate rose in all co-cultivation systems, except in the system with *Lb. hordei* and *Z. florentina* the amount of succinate decreased about 0.2 g/l (Fig. 40 A). Contents of mannitol in the systems with *Z. florentina* did not differ between calculated co- and co-cultivation, *S. cerevisiae* did not produce any mannitol.

3.5.3 Mixed culture experiments

During water kefir fermentation the participating organisms live in a close community. Therefore, growth, sugar, metabolic products and proteome were investigated in mixed culture with cell-cell contact in comparison to singly cultivated organisms.
Since organisms were mixed-cultured during the experiments of this chapter, growth, concentrations of different substances and proteome are always stated for the little prepared "consortia" and compared with mean pooled amounts of their corresponding single cultures (declared as calculated mixed-culture). All concentration values are referred to a specific cell concentration (OD 1).

3.5.3.1 Comparison of growth during mixed- and calculated mixed-culture fermentation

Mixed culture of *Z. florentina* with predominant water kefir lactobacilli in WKM did not show a difference in growth to the calculated mixed-cultivated strains (Fig. 27 A and B). Conversely, mixed culture of the yeast with *B. psychraerophilum* showed that growth in calculated mixed-culture reached with OD_{590} 0.8 higher end cell concentrations than mixed-cultured (OD_{590} 0.55) (Fig. 27 C).

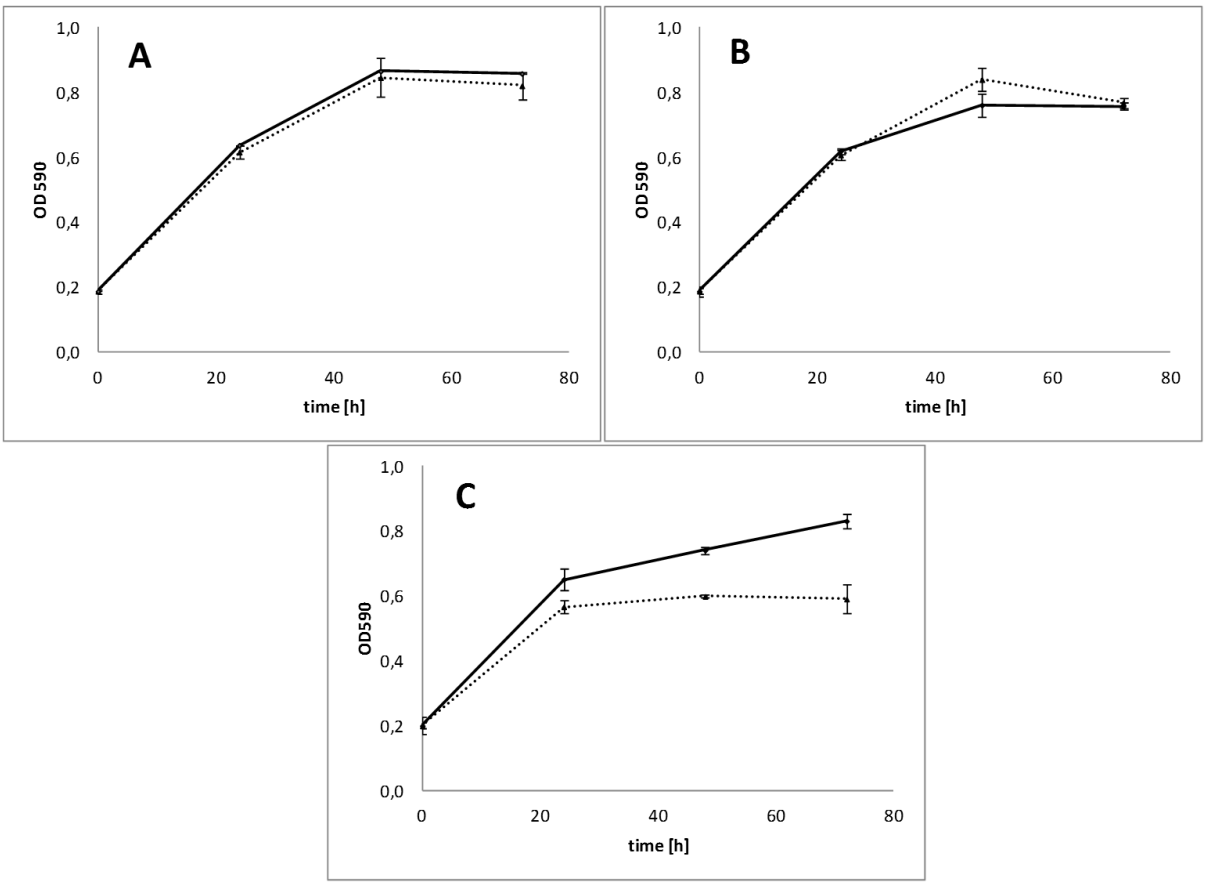


Fig. 27: Growth of mixed- and calculated mixed-cultures water kefir organisms fermented in WKM.

Growth of mixed cultures (pointed line) of *Z. florentina* with *Lb. hordei* (A), *Lb. nagelii* (B) and *B. psychraerophilum* (C) compared with mean pooled amounts of their corresponding single cultures (declared as calculated mixed-culture) (solid line).

3.5.3.2 Comparison of sugar and metabolite concentrations during mixed- and calculated mixed-culture fermentation

Concentration of sucrose was completely degraded within 72 h of fermentation with all fermentation partners tested in calculated mixed-culture as well as in real mixed-culture. Amounts of fructose and glucose as well as the metabolites lactate, acetate, succinate, mannitol and ethanol rose during fermentation. A particularly high increase of lactate could be detected in mixed-culture. The concentration was twofold higher than that in calculated mixed-culture. Also the amount of acetic acid rose in mixed-culture with *Lb. nagelii* to 0.18 g/l, in calculated mixed-culture no acetate was determinable (< 0.01 g/l). Table 27 displays sugar and metabolite concentration while fermentation of *Z. florentina* and *Lb. nagelii* in mixed- as well as in calculated mixed-culture. Tendential, change of sugar and metabolite concentration during fermentation of *Z. florentina* with *Lb. hordei* and *B. psychraerophilum*, respectively, proceeded similarly to the example mentioned. Distinct differences could be shown for acetate concentrations. The combination of *Z. florentina* with *Lb. hordei* did not produce any acetate. The system with *B. psychraerophilum* produced acetic acid in calculated mixed-culture as well as in mix-culture, whereas the concentration decreased in mixed-culture from 0.87 to 0.35 g/l (Table 35). 80 g/l sucrose was inverted to 40 g/l glucose and 40 g/l fructose. Thus, in mixed- and in calculated mixed-culture with *Lb. nagelii* about 10 g/l glucose were metabolized, in the system with *Lb. hordei* and *B.psychraerophilim*, respectively, about 15 g/l. With 5 g/l in the system with *Lb. nagelii* and *Lb. hordei* (Table 27 and Table 34), respectively, and in calculated mixed-culture in the system with *B. psychraerophilum* less fructose was metabolized than glucose. The only exception showed the mixed-culture with *B. psychraerophilum*, there the concentration of fructose decreased about 15 g/l (Table 35).

Table 27: Sugar and metabolite concentration during mixed- and calculated mixed-cultivation of *Lb. nagelii* and *Z. florentina* fermented in WKM

time [h]	sucrose [g/l per OD 1]				glucose [g/l per OD 1]				fructose [g/l per OD 1]				mannitol [g/l per OD 1]			
	cMC*		MC**		cMC		MC		cMC		MC		cMC		MC	
	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD
0	83.2	0.7	83.2	0.7	4.6	0.4	4.6	0.4	4.3	0.4	4.3	0.4	< 0.06		< 0.06	
24	54.3	0.9	47.0	0.5	15.9	1.0	17.9	0.1	18.2	0.4	20.2	0.2	0.32	0.01	0.25	0.01
48	22.3	2.4	20.8	1.0	27.5	0.7	26.2	0.4	29.1	1.1	30.3	1.2	0.46	0.02	0.37	0.01
72	< 0.07		< 0.07		35.3	0.5	33.2	0.5	39.4	0.5	39.8	0.5	0.61	0.01	0.55	0.02

time [h]	lactate [g/l per OD 1]				acetate [g/l per OD 1]				succinate [g/l per OD 1]				ethanol [g/l per OD 1]			
	cMC		MC		cMC		MC		cMC		MC		cMC		MC	
	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD
0	< 0.02		< 0.02		< 0.01		< 0.01		< 0.03		< 0.03		< 0.06		< 0.06	
24	0.75	0.12	1.41	0.12	< 0.01		0.07	0.01	0.79	0.02	0.84	0.04	2.83	1.03	2.84	0.06
48	0.93	0.16	1.98	0.48	< 0.01		0.11	0.02	1.27	0.03	1.00	0.12	5.75	0.18	5.95	0.96
72	1.17	0.31	2.99	0.12	< 0.01		0.18	0.03	1.53	0.11	1.24	0.03	9.28	0.45	10.96	0.43

* **c**alculated **M**ixed-**C**ulture fermentation ** **M**ixed-**C**ulture fermentation

Sugar consumption and metabolite production of different co-cultivation systems in comparison to the pooled single culture values, declared as “calculated co-culture”, of the individual organisms. All values are referred to a specific cell concentration (OD 1).

3.5.3.3 Proteome analysis of mixed- and pooled single-cultures

Further interactions between water kefir isolates were determined on the proteomic level with comparative 2D gel electrophoresis to investigate, whether protein regulation is altered in mixed-culture compared to pooled single-cultures of the individual organisms. Therefore, whole cell protein of a mixed-culture was isolated and matched with the pooled cell protein of individual single-cultures after 24 and 48 h of cultivation.

In mixed-culture cell protein of *Lb. hordei* resp. *Lb. nagelii* with *Z. florentina* more proteins were up-regulated compared with their respective pooled single-culture protein. 24 h and 48 h as well as the different lactobacilli resulted in the same protein 2D gel pattern. Up-regulated proteins were isolated and identified by LC-MS/MS.

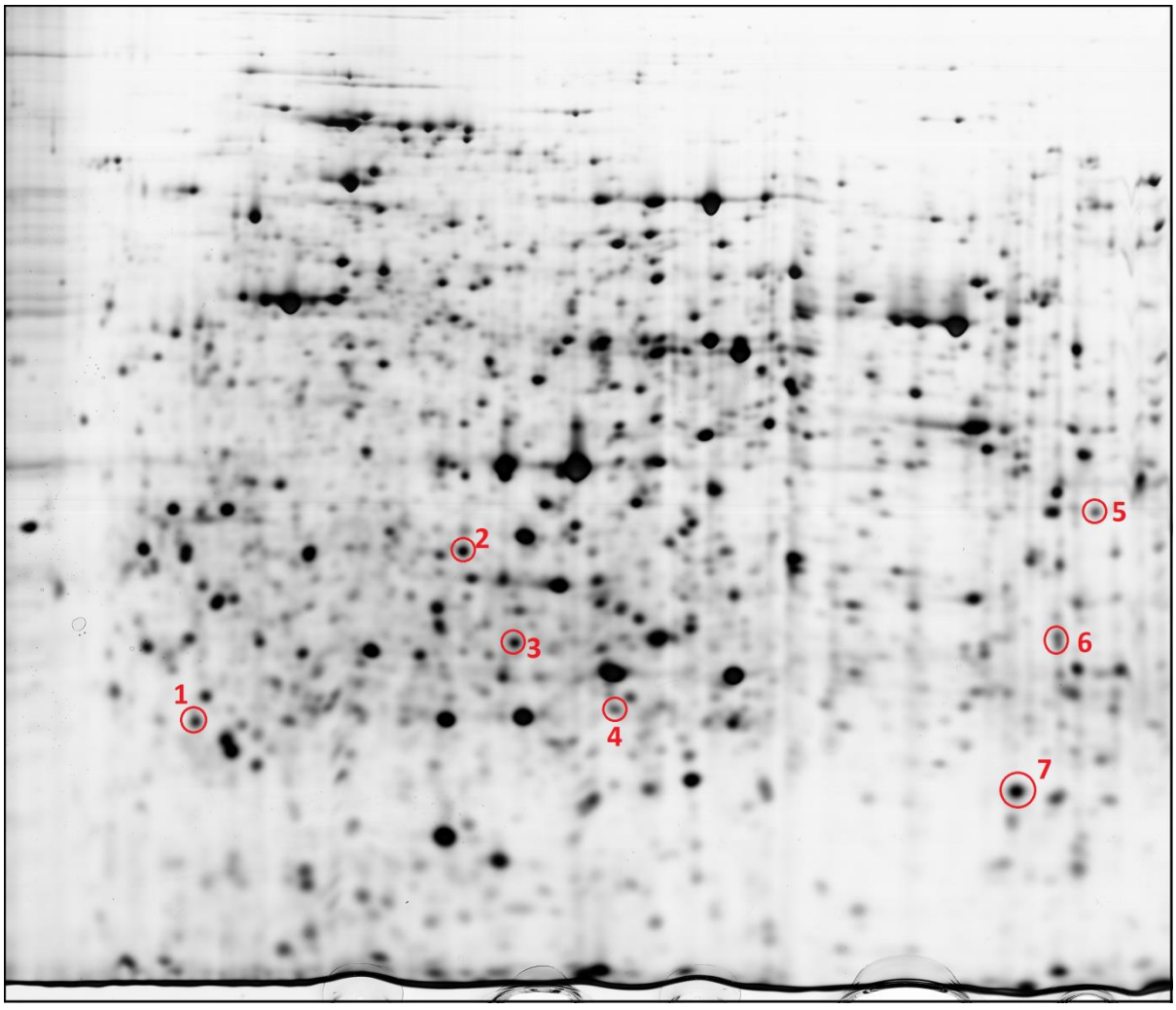


Fig. 28: 2D-gel electrophoretic analysis of mixed-cultured *Lb. nagelii* and *Z. florentina* after 48 h of fermentation in WKM

Marked proteins were only up-regulated in the mixed-culture.

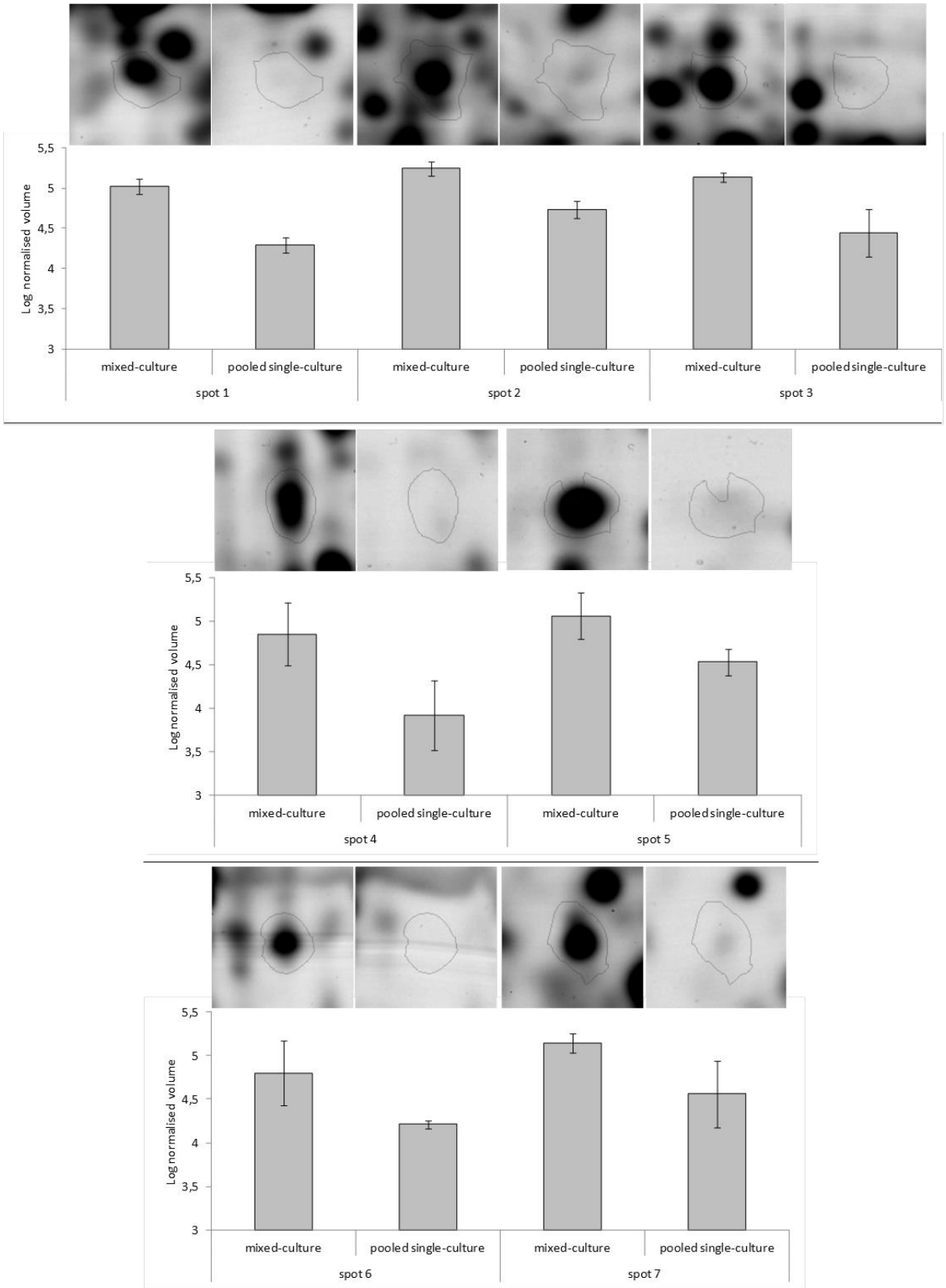


Fig. 29: Differential expression analysis of mixed-culture up-regulated proteins depicted as logarithmic normalized spot volume of mixed-culture and pooled single culture

Table 28: Up-regulated proteins in mixed culture of lactobacilli and *Z. florentina*

spot	Mw [kDa]	iP	function	Best hit UniProt Accession nr.	producer
1	21	4,60	Heat shock protein	Q707X3	yeast
2	29	4.98	Glutamine synthetase	F7QXU8	LAB
3	24	5.29	Enolase	F7QSE4	yeast
4	21	5.48	L-lactate dehydrogenase	G2SRC0	LAB
5	30	6.55	Phosphoglycerate kinase	Q1WTB5	LAB
6	24	6.48	Phosphoribosylaminoimidazole -succinocarboxamide synthase	E7FNF4	LAB
7	16	6.38	Glyceraldehyde-3-phosphate dehydrogenase	C5E0E4	yeast

Mixed-culture cell protein of *B. psychraerophilum* and *Z. florentina* in comparison to cell pool of their individual single-cultures showed only one over-expressed protein in mixed-culture cell protein. This protein could be found at the same position like spot 7 in the protein 2D gel pattern and was also identified as glyceraldehyde-3-phosphate dehydrogenase.

3.5.3.4 Aggregation of mixed water kefir isolates

Microscopic pictures (Fig. 30) of a mixed-culture of water kefir yeasts and lactobacilli display small communities therefore their mixed-aggregation was compared to their auto-aggregation.

Mixed-aggregation of both lactobacilli tested with *Z. florentina* displayed 3 % stronger aggregation than their individual mean auto-aggregation. Trend of mixed-aggregation of *B. psychraerophilum* and *Z. florentina* seemed to be similar, but the difference to their individual auto-aggregation is not significant.

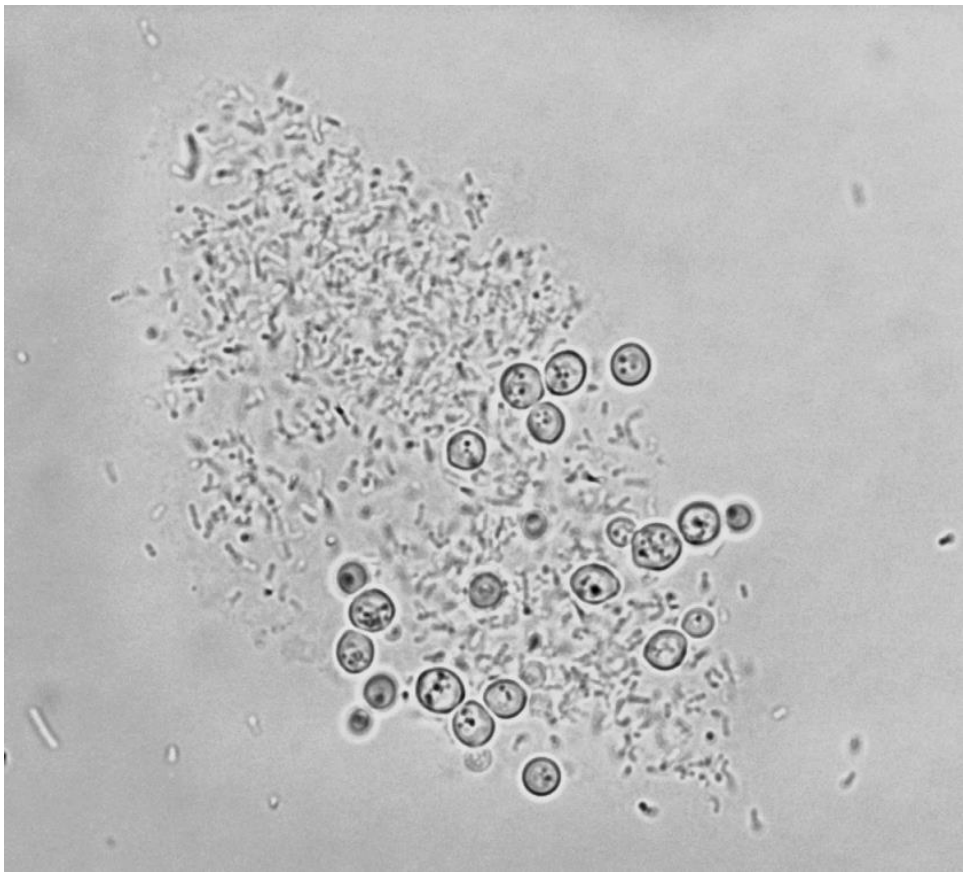


Fig. 30: Microscopic picture of *Lb. nagelii* in mixed-culture with *Z. florentina* after 24 h of fermentation

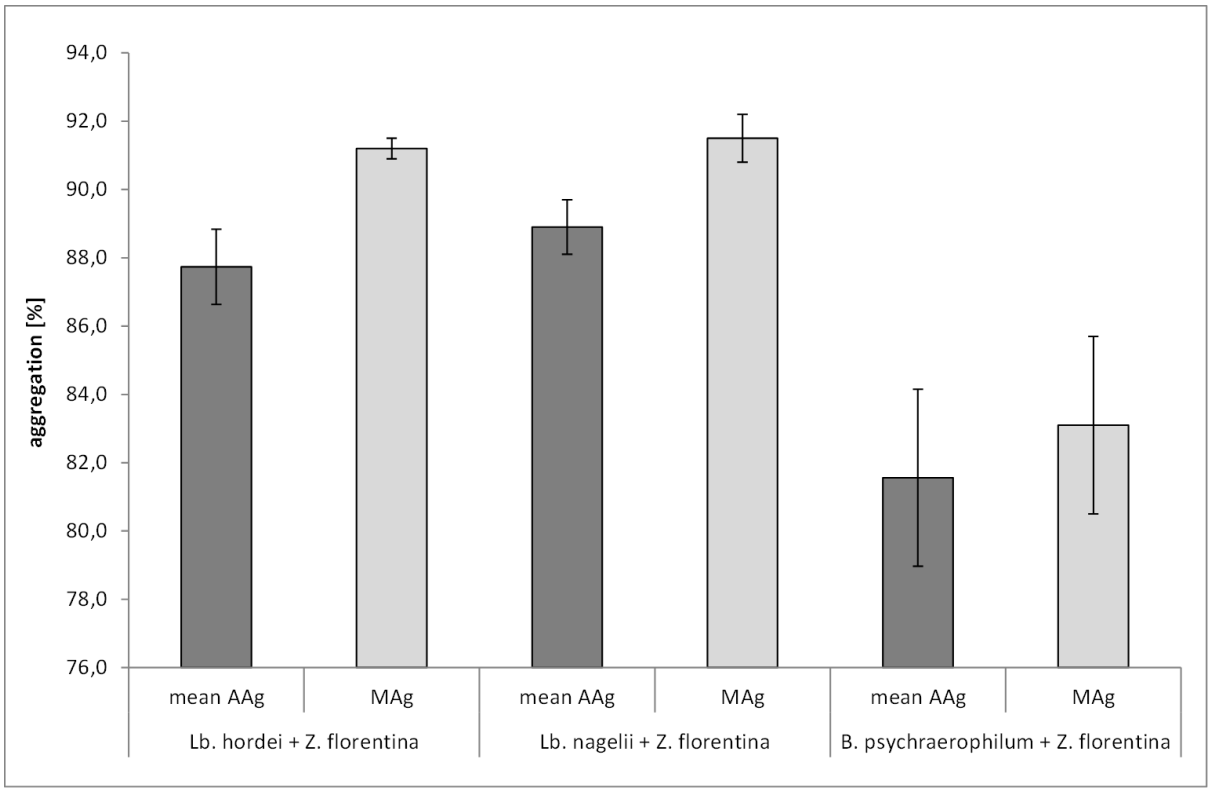


Fig. 31: Comparison of mean auto-aggregation (mean AAg) of the single isolates with their respective mixed-aggregation (MAg)

Since mannan containing surface proteins of yeasts are known as an important adhesion factor for aggregation of yeast and bacteria (Katakura, Sano, Hashimoto, Ninomiya, & Shioya, 2010), the influence of supplemented mannose to the aggregation buffer was determined.

Addition of 1 % mannose to PBS did not clearly change the mixed-aggregation. Though 5 % mannose decreased mixed-aggregation in the system with *Lb. hordei* (Fig. 32 A) about 40 %, in the system with *Lb. nagelii* the mixed-aggregation declined about 30 % and only 10 % in the system with *B. psychraerophilum*.

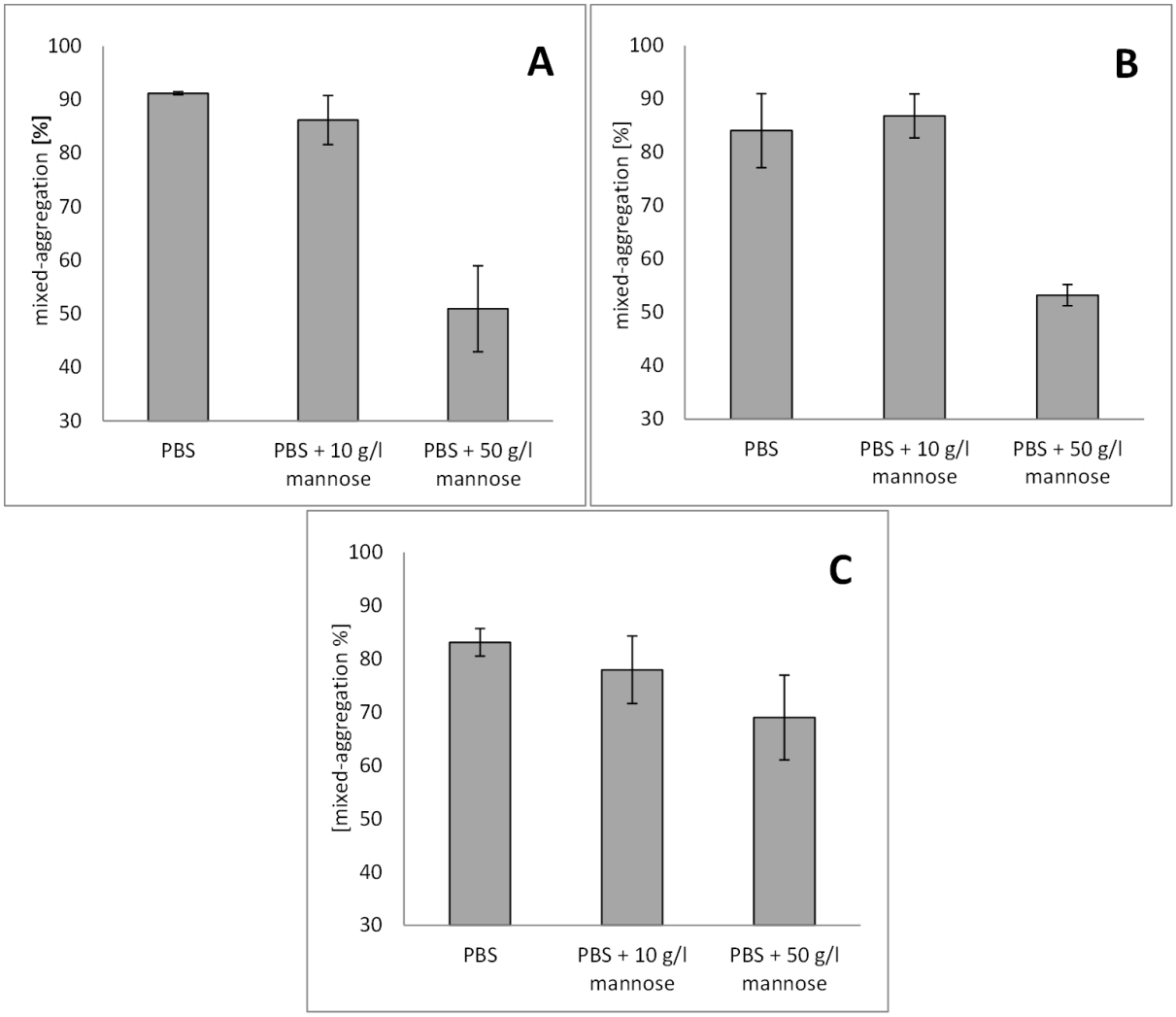


Fig. 32: Influence of mannose to the mixed-aggregation

Mixed-aggregations of *Lb. hordei* (A), *Lb. nagelii* (B) and *B. psychraerophilum* (C) mixed with Z. florentina and the influence of mannose in two different concentration (10 g/l and 50 g/l) were determined.

3.6 Metabolic synergism between main representatives of water kefir isolates

Co-cultivation of yeasts and lactobacilli in water kefir medium significantly increased cell yields of all interaction partners. The metabolic interaction of water kefir isolates was determined in a simplified chemically defined medium (SCDM) as described by Hebert et al. (2000) and in modified WKM.

3.6.1 Identification of essential nutrients for water kefir yeasts and lactobacilli in a simplified chemically defined medium

First, essential nutrients for water kefir isolates used in this work were investigated. Growth of *Lb. hordei* (TMW 1.1822), *Lb. nagelii* (TMW 1.1825), *Z. florentina* (TMW 3.220) and *S. cerevisiae* (TMW 3.221) was tested in full medium (SCDM) in comparison to media where one nutrient was omitted. *Z. florentina* and *S. cerevisiae* did not show an auxotrophy for any of the substances tested. Lag phase of *Z. florentina* was prolonged but after 24 h exponential phases started in every medium. Lactobacilli tested showed an auxotrophy for some amino acids (Table 29), *Lb. hordei* revealed an additional auxotrophy for pyridoxal, *Lb. nagelii* for L-arginine. The addition of ornithine instead of arginine was not sufficient for the growth of *Lb. nagelii*.

Table 29. Essential nutrients for *Lb. hordei* and *Lb. nagelii*, respectively

substance	*Lb. hordei* (TMW 1.1822)	*Lb. nagelii* (TMW 1.1825)
pyridoxal	X[A]	
L-arginine		X
L-leucine	X	X
L-isoleucine	X	X
L-methionine	X	X
L-phenylalanine	X	X
L-trytophan	X	X
L-tyrosine	X	X
L-valine	X	X

[A]X represents the auxotrophy for the substance.

3.6.2 Impact of co-cultivated yeasts to lactobacilli in SCDM

To determine if water kefir yeasts can provide essential nutrients for lactobacilli, three chemically defined media were prepared, first a medium without pyridoxal for interaction experiments with *Lb. hordei*, second a medium without L-arginine for *Lb. nagelii* and third a medium without the amino acids, which are essential for both lactobacilli. In co-cultivation both lactobacilli were able to grow in media without their essential nutrients within 48 h of fermentation in comparison to single cultivation in these media, the starting OD_{590} 0.1 did not change (Fig. 33). The influence of *Z. florentina* was higher than that of *S. cerevisiae*, the OD_{590} after 48 h of co-cultivation of *Lb. hordei* and *Z. florentina* in pyridoxal free medium is with 1.25 twofold higher than in co-cultivation with *S. cerevisiae* (OD_{590} 0.63). Also the stimulation of *Lb. nagelii* in arginine free medium was 1.5 fold higher in the cultivation system with *Z. florentina* (OD_{590} 0.68) than in the system with *S. cerevisiae* (OD_{590} 0.41). *Z. florentina* was not able to grow in SCDM-7AS thus in this medium no interaction could be shown. Experiments where only one of the seven amino acids was omitted each medium showed that also *Z. florentina* is able to support both lactobacilli with these amino acids (Table 30).

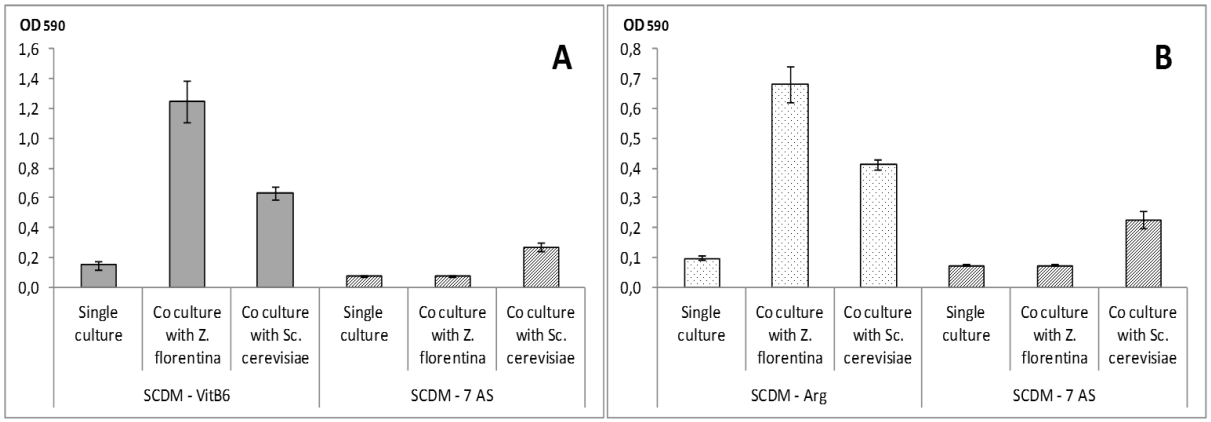


Fig. 33: Co-cultivation of water kefir isolated yeasts and lactobacilli in modified SCDM (Stadie et al., 2013)

OD_{590} after 48 h cultivation of *Lb. hordei* (A) and *Lb. nagelii* (B) grown in SCDM-VitB6 (grey bars), SCDM-Arg (spotted bars) and SCDM3-7AS (striped bars), respectively, single cultivated or in co-culture with *Z. florentina* and *S. cerevisiae*, respectively

Table 30: OD_{590} of *Lb. hordei* and *Lb. nagelii* in co-cultivation with *Z. florentina*, start OD_{590} 0.1

As positive control lactobacilli were singly cultivated in SCDM containing all nutrients. For negative controls lactobacilli were singly cultivated in each SCDM without an essential amino acid. *Z. florentina* grew to OD_{590} 0.75 in every medium. (Stadie et al., 2013)

medium	Growth of lactobacilli in **co-cultivation** in the model system with *Z. florentina*		Growth of lactobacilli singly cultivated	
	Lb. hordei [OD_{590}]	***Lb. nagelii*** [OD_{590}]	***Lb. hordei*** [OD_{590}]	***Lb. nagelii*** [OD_{590}]
SCDM – Val	0.45 ± 0.03	0.34 ± 0.03	0.09 ± 0.02	0.12 ± 0.03
SCDM – Tyr	0.35 ± 0.02	0.43 ± 0.01	0.11 ± 0.03	0.12 ± 0.03
SCDM – Phe	0.34 ± 0.03	0.52 ± 0.01	0.09 ± 0.03	0.11 ± 0.04
SCDM – Ile	0.55 ± 0.05	0.37 ± 0.01	0.08 ± 0.03	0.12 ± 0.02
SCDM – Leu	0.45 ± 0.03	0.31 ± 0.01	0.09 ± 0.03	0.11 ± 0.03
SCDM – Trp	0.38 ± 0.03	0.28 ± 0.03	0.10 ± 0.02	0.11 ± 0.01
SCDM – Met	0.46 ± 0.03	0.38 ± 0.00	0.10 ± 0.02	0.09 ± 0.02
SCDM	n.d.*	n.d.	0.35 ± 0.04	0.28 ± 0.05

* n.d. = not determined

3.6.3 Modification of SCDM

Z. florentina promoted *Lb. nagelii* with arginine in co-culture in the model system on arginine free medium. As no arginine in a yeast-fermented medium was detectable, the yeast does not produce arginine as single substance, but may produce arginine-containing compounds that could be used by the *Lactobacillus*. To find out under which circumstances the yeast released arginine sources, growth of *Lb. nagelii* in different pre-fermented arginine free medium was determined. Table 31 displays results of growth tests of *Lb. nagelii* in modified SCDM-Arg. *Z. florentina* did not produce any arginine available components for *Lb. nagelii* in pure culture. Therefore, sterile filtered supernatant of a 24 h fermented mixed-culture with *Z. florentina* and alive or dead cells of *Lb. nagelii*, respectively, were chosen for further growth experiments. By dead *Lb. nagelii* cells yeast might be induced to produce arginine, but without any living consumer inside of the fermentation vessel it should remain detectable. Mixed-culture incubation in comparison to co-cultivation of *Z. florentina* and *Lb. nagelii* in the cell-separating transwell system showed a twofold higher growth rate thus, the influence

of cell wall parts of the yeast on the *Lactobacillus* was determined. Since cell debris did not contain the stimulation factors for *Lb. nagelii*, yeast cells extract was added to arginine free medium. Addition of yeast cell extract yielded in stimulation of the *Lactobacillus* similar to SCDM with all essential nutrients.

Table 31. Growth of *Lb. nagelii* under different conditions (Stadie et al., 2013)

Since *Z. florentina* was able to support *Lb. nagelii* in co-cultivation in the model system on SCDM – Arg, it was to find out under which circumstances *Z. florentina* released arginine sources. Growth of *Lb. nagelii* in different modified media was compared with its growth behavior in SCDM with all nutrients and SCDM – Arg. Single culture growth experiments were executed in microplates for 72 h of fermentation

Growth conditions	Impact on growth of *Lb. nagelii*
Single cultivation in SCDM with all essential nutrients	growth
Single cultivation in SCDM – Arg	no growth
Co-cultivation of *Lb. nagelii* with *Z. florentina* in SCDM – Arg in the model system	growth
Single cultivation of *Lb. nagelii* in SCDM – Arg added with pre-fermented SCDM – Arg. Pre-fermentation with *Z. florentina* in single-cultivation	no growth
Single cultivation of *Lb. nagelii* in SCDM – Arg added with pre-fermented SCDM – Arg. Pre-fermentation with *Z. florentina* and *Lb. nagelii* in mixed-culture	no growth
Single cultivation of *Lb. nagelii* in SCDM – Arg added with pre-fermented SCDM – Arg. Pre-fermentation with *Z. florentina* and dead cells of *Lb. nagelii* in mixed-culture	no growth
Single cultivation of *Lb. nagelii* in SCDM – Arg added with cell debris of *Z. florentina*	no growth
Single cultivation of *Lb. nagelii* in SCDM – Arg added with cell extract of *Z. florentina*	growth

3.6.4 Modification of WKM

As water kefir lactobacilli did not grow well in WKM hence, the influence of essential nutrients in WKM in different concentrations was determined. During 20 h of fermentation growth of both lactobacilli was higher in the modified medium than in pure WKM. After 40 h optical density (590 nm) of both lactobacilli rose only in the supplemented medium with fivefold higher concentration of essential nutrients, the OD_{590} in the lower concentration stagnated (Fig. 34).

To determine if water kefir yeasts produce essential nutrients for lactobacilli in single cultivation in WKM the pre-fermented medium was used for growth experiments. Cultivation of *Lb. hordei* resp. *Lb. nagelii* in 24 h pre-fermented WKM by *Z. florentina* and *S. cerevisiae*, respectively, deteriorates growth of the lactobacilli.

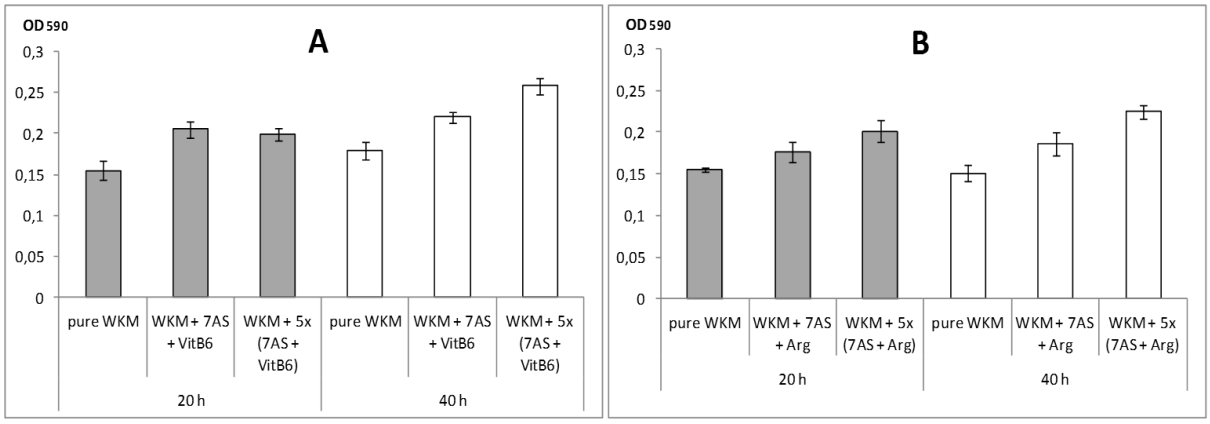


Fig. 34: Growth of Lb. hordei (A) and Lb. nagelii (B) in pure WKM and modified WKM with essential nutrients in different concentrations after 20 h (grey bars) and 40 h (white bars) of fermentation (Stadie et al., 2013)

3.6.5 pH optimum of yeast isolates from water kefir

To determine the influence of the physiochemical environment on yeasts the growth in media with different starting pHs was examined. *Z. florentina* shows its pH optimum for reproduction after 72 h incubation at a starting pH 4 (Fig. 35), *S. cerevisiae* is not influenced by acidification of the medium.

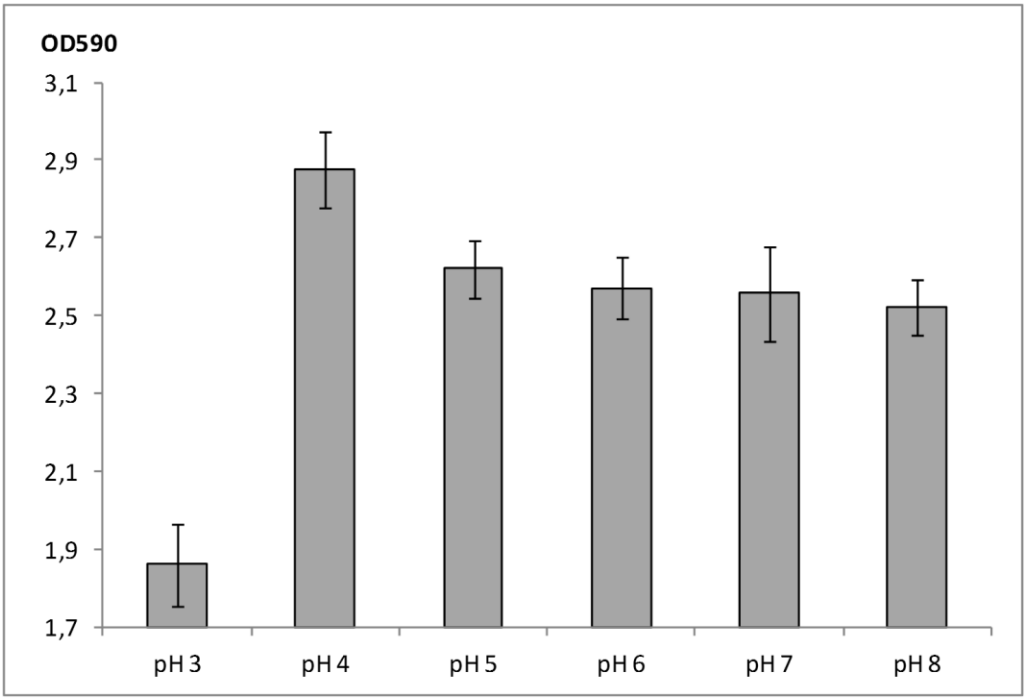


Fig. 35: Starting pH optimum for *Z. florentina* (Stadie et al., 2013)

OD_{590} was measured after 72 h of incubation

Since lactic acid bacteria are able to produce lactic and acetic acid, the impact of these substances on growth of water kefir yeasts was measured. Acidification with lactic acid showed the same influence for both yeasts as described above. To determine if the acidification or the addition of lactic acid is the reason for the improvement, growth of *Z. florentina* in WKM added with lactic acid at a constant pH was examined. The addition of lactic acid did not reveal a difference in growth in comparison to pure WKM.

The acidification with acetic acid decreased growth of both yeasts. At pH 3 growth of yeasts tested was completely inhibited.

4 DISCUSSION

Water kefir grains harbor an association of different lactic acid bacteria, acetic acid bacteria, yeasts and bifido-bacteria (Gulitz et al., 2013, 2011). The consortium ferments a sugar solution with an extract of dried figs producing grains and a fizzy, cloudy beverage. This work could demonstrate that independent of the origin of the consortium and the related different distribution of microbes the resulting beverages were similar in their content of major metabolic compounds. On the other hand, growth behavior of single water kefir isolates in water kefir medium differed. Yeasts were able to grow well, whereas lactobacilli were limited in growth due to the lack of nitrogen sources. Additionally, Gulitz et al. (2013) could even find bifodobacteria which are uncultivable, yet (Gulitz et al., 2013). The presence of uncultivable organisms pinpoints an essential interaction in the consortium water kefir. It may reside in grain formation, because many water kefir isolates were able to produce EPS, but no insoluble EPS could be found to be produced by a single bacterium under the conditions used. As water kefir isolates were not able to ferment EPS produced by single water kefir organisms, grain EPS is not used as storage compound, but rather to ensure a stable order inside the consortium. Once destroyed, this grain order can never be rebuilt. The synergism between the numerically predominant representatives of water kefir yeasts interacting with lactobacilli and *B. psychraerophilum*, respectively, was studied in a co-culture model system. Enhanced formation of metabolites and glycolytic proteins could demonstrate the increasing metabolic activity of single organisms during co-cultivation. Based on the limited nitrogen content in WKM, water kefir isolates have to support each other in this ecological niche. It was shown that co-cultivated yeasts are induced to supply amino acids and vitamins by lactobacilli, whereas lactobacilli optimize the physical environment for yeasts while acidifying the milieu. It appears that lactobacilli “domesticate” yeasts to enable a better living. In order to produce an industrial beverage based on water kefir, such interacting organisms should be chosen as starter cultures to ensure best support in growth and fermentation. Therefore, the use of yeasts as starter cultures appears to be essential, but in less concentration than bacteria, otherwise they will overgrow the fermentation broth.

4.1 Water kefir supernatant analysis

Comparison of two water kefir consortia with different origin but similar fermentation conditions showed equal findings for metabolic composition. The pH of both water kefir supernatants decreased within 48 h from 6.5 to 3.5 because of produced organic acids.

4.1.1 Sugars, organic acids and ethanol

Within 48 h sucrose is completely inverted by invertases of containing yeasts. During this time about 70 % of the emerging glucose was consumed in both consortia whereas from emerging fructose only 12 % were consumed. High amounts of sucrose were also consumed for grain formation, this point is discussed later on in detail (4.2). Increasing ethanol concentrations were produced by alcoholic fermentation of yeasts and as a metabolite of heterofermentative LAB. Distinct differences between the supernatant analysis of the two water kefir fermentations could be displayed for mannitol production. Where WkI could only produce about 1 g/l, the mannitol concentration in the supernatant of WkW increased to 8 g/l. On the one hand mannitol results as a yeast metabolite on the other hand in the presence of fructose as an electron acceptor and mannitol-2-dehydrogenase, heterofermentative LAB are able to produce acetate and mannitol instead of ethanol (Groleau et al., 1995; Saha & Racine, 2011). Mannitol production of heterofermentative LAB could be increased with a fructose:glucose ratio of 2:1 (Saha & Racine 2011) for example for *Leuconostoc* sp. (Von Weymarn et al., 2002). After 48 h of fermentation this ratio was reached and fructose depletion started. Gulitz et al. (2013) could demonstrate that grains of WkW included with 29.1 % much more *Leuconostocaceae* than WkI, therefore the high amounts of mannitol are the result of the metabolic performance of these organisms.
A high amount of acetate was produced by heterofermentative LAB and acetic acid bacteria from the consortia. The increased acetate production in the supernatant of WkW after 110 h can also be explained by the use of fructose as electron acceptor, stagnating ethanol amounts confirms this assertion.

4.1.2 Volatile compounds

Measurement of volatile compounds in water kefir supernatant also displayed equal results for both consortia tested (Fig. 9). During fermentation several fruity esters, medium chain alcohols and branched-chain fatty acids were produced (Table 32). Most of the volatiles

detected could be also found during wine, cheese and sourdough fermentation. Fruity esters and odorous alcohols are welcome during fermentation whereas branched chain fatty acids and acetoin could be seen as off flavors because of their buttery rancid odor (Carrau et al., 2008).

Table 32: Volatile compounds of 48 h fermented water kefir supernatant

compound	feature	producing organisms	source
Ethyl acetate	fruity, pear, acescence	yeast, LAB	Carrau et al. 2008; Engels et al. 1997; Longo et al. 1992; Mauricio et al. 2003; Damiani et al. 1996
Isobutanol	sweet, vinous	yeast	Carrau et al. 2008; Longo et al. 1992; Damiani et al., 1996
Isoamyl acetate	banana, pear	yeast	Carrau et al. 2008; Mauricio et al. 2003
Caproic acid ethyl ester	fruity	yeast, LAB	Carrau et al. 2008; Engels et al. 1997; Liu et al. 2003; Costello et al. 2013
Acetoin	buttery	yeast, LAB	Goyal 1999; Romano et al. 1996
Caprylic acid ethyl ester	flory, fruity, vinous	yeast, LAB	Carrau et al. 2008; Costello et al. 2013
Benzaldehyd	bitter almond	LAB	Engels et al. 1997; Sinz 2011; Damiani et al., 1996
Isobutyric acid	rancid, buttery, cheesy	yeast, *Clostridiaceae*	Allison 1978; Carrau et al. 2008; Thierry et al. 2002
Isovaleric acid	sweet, cheesy, rancid	yeast, *Clostridiaceae*	Allison 1978; Carrau et al. 2008; Thierry et al. 2002
β-phenethyl acetate	rose, honey, tobacco	yeast	Carrau et al. 2008
2-phenylethanol	rose	yeast	Carrau et al. 2008; Etschmann et al. 2002, 2003

S. cerevisiae in the early phase of wine fermentation, weakly fermenting yeasts and LAB anaerobically produce acetoine by condensation of two pyruvate molecules (catalysis with acetyl lactate synthase) and subsequent decarboxylation (Goyal 1999; reviewed by Romano

et al. 1996). Since water kefir fermentation is normally stopped after 48 to 72 h no following reduction of acetoin to 2,3-butandiol could be detected in water kefir supernatant. Other volatile compounds were metabolic products of amino acid degradation. For example benzaldehyde is produced during catabolism of free phenylalanine or phenylalanine containing peptides by lactic acid bacteria in raw sausage fermentation (Sinz, 2011). Transamination, decarboxylation and reduction of phenylalanine (Ehrlich pathway) by *S. cerevisiae* leads to 2-phenylethanol production (reviewed by Etschmann et al. 2002). Water kefir contains yeasts as well as LAB therefore both metabolites could be found in the supernatant. Wine yeast strains of *S. cerevisiae* produce in media with low nitrogen content more unpleasant volatile compounds like isobutyric and isovaleric acid out of valine and leucine, respectively (Allison, 1978; Carrau et al., 2008; Thierry et al., 2002). In WKM with its low amino acid concentration these rancid odorants could also be detected but it was not possible to determine the quantity with the method used. Other volatile metabolites, namely caproic and caprylic acid ethyl ester could be synthesized during a reaction of fatty acids and alcohols (Engels et al., 1997; Liu et al., 2003).

4.1.3 Amino acids

For WKM an extract of dried figs was prepared. Protein concentration of fresh figs is about 1.3 g/100g (Scherz & Senser, 1999). During drying process the fruits lose about 75 % water resulting in a protein concentration of 5.2 g/100g dried fruits. One liter WKM contained an extract of 48 g dried figs, therefore 2.5 g protein per liter WKM. During extraction only soluble proteins, peptides and aminoacids were eluted therefore the protein concentration decreased. For example while preparing grape juice protein concentration decreases to one third (from 0.68 g protein/100 g grapes to 0.21 g protein/100 g juice) (Scherz & Senser, 1999), thus the protein concentration of WKM should be about 0.83 g/l. Measured concentration of free amino acids was about 8 mg/l, however the analytic method used did not include any peptides and proteins. Anyway Sinz (2011) could show for *Lb. sakei* and Saguir et al. (2008) for *Lb. plantarum* that these lactobacilli could assimilate di-peptides much better than free amino acids. Since all amino acids had been consumed after 72 h of fermentation and addition of amino acids to WKM improved growth of lactobacilli (3.6.4) it could be shown, that limiting growth factor in WKM was the nitrogen concentration.

4.1.4 Change of parameters for water kefir fermentation

Since water kefir fermentation is based on a biological system, fermentation differed from time to time. For this experiment water kefir grains of the same batch were divided and used for fermentation under different conditions. In this way equal requirements in grain activity could be ensured. In the systems with fig extract under different temperatures it could be displayed that invertase activity was increased at higher temperatures because sucrose concentration mainly decreased at 37°C. Glucose concentration only rose at 37°C, this showed that invertase activity was higher than the consumption of the consortium under this condition. Additional, yeasts as consumers diminished because *Z. florentina* as dominant yeast in water kefir (Gulitz et al., 2011) did not grow well and even lyse at this temperature (Fig. 19 A). Under the other conditions it could be shown that all glucose from inversion of sucrose was immediately consumed because of the stagnating glucose concentration during 72 h of fermentation, whereas fructose concentrations increased in response to sucrose depletion. Until the end of this experiment (72 h) no fructose under any condition was consumed. Fructose concentrations that were used for mannitol and EPS production were only less and could be included in measurement errors of sucrose determination (StD 0.73 g/l). Sucrose reduction did not correlate with EPS production because in the highest sucrose consuming system, namely at 37°C humid grain mass only increased about 3 % per day of fermentation. The highest increase of humid grain mass could be detected under standard conditions (30 % per day), the increase at 12 °C and with apricot extract was similar (10 % per day) and with cranberry extract almost any growth could be determined (1 % per day) (Gulitz, 2013). Acetate concentration increased most at 21°C. Since mannitol concentration did not raise appropriately to acetate production at 21°C, amounts of acetic acid did not emerge due to heterofermentative fermentation, therefore other bacteria of the consortium caused the increase of acetate, for example acetic acid bacteria. It has been demonstrated, that at 21°C most AAB were present for the production of acetic acid (Gulitz, 2013). Highest amounts of lactate could be detected at 37°C. The main carbohydrate and nitrogen consumer *Z. florentina* did not grow at 37 °C therefore more nitrogen sources remain for LAB that were predominant at this temperature (more than 90 %, (Gulitz, 2013)). Addition of other dried fruits instead of figs let to a reduction of the metabolic activity and with cranberries even to an inhibition of growth and metabolism of the water kefir system. Reiß (1990) could also describe the best biological value of figs for the mass increase of Tibi grains (synonym for water kefir). In the system with cranberry extract no sugar consumption and metabolite

production could be detected. Cranberries contain amounts of benzoic acid, but at pH 6 of the prepared medium for water kefir fermentation this preservative is not active. Other substances of cranberries, namely phenolic compounds, are known as strong antioxidants and antimicrobials. Inhibition of growth of Gram-positive and –negative bacteria is caused by several mechanisms, like inhibition of extracellular enzymes, destruction of membranes and direct and indirect (deprivation of substrates) actions on microbial metabolism (Cesoniene et al., 2009; Puupponen-Pimiä et al., 2005).

Taken together it could be shown that changing conditions for water kefir fermentation led to concentration changes in the metabolite spectrum. Therefore, playing with fermentation parameters could supply varieties in flavor for production of water kefir supernatant as a beverage. Conversely, for grain formation usual household conditions are necessary for most productive increase of grain mass.

4.2 EPS production in water kefir

During water kefir fermentation the consortium produced amounts of EPS in forms of grains (insoluble) and solubilized in the supernatant. Supernatant EPS and grains were determined along their sugar monomer components and the grains were additionally analyzed for their linkage types by NMR. Single water kefir isolates were determined for their ability to produce EPS to find their influence on grain formation.

4.2.1 EPS production of the whole consortium

Analysis of water kefir supernatant after 72 h of fermentation displayed a low EPS concentration (< 1g/l). Gently hydrolysis displayed fructose and glucose monomers but additional peaks with lower retention times as it was typical for oligosaccharides. Gently hydrolyzed glucans showed similar fractionation patterns but without a fructose peak. Stronger hydrolysis could only emerge glucose. Fructose did not exist anymore because of its heat and acid sensitivity. Analysis of different hydrolysis varieties displayed that water kefir supernatant EPS contained glucan as well as fructan but only in low concentrations (< 1 g/l).
Conversely mass increase of grains was about 100 % after 72 h of fermentation, corresponding to a formation of 3.4 g/l. Analysis of grain EPS displayed, that water kefir grains consisted of glucose polymers. Water kefir grains include many different EPS

producers, for example *Lc. mesenteroides*, *Lc. citreum*, *Lb. hilgardii* and *Lb. hordei* as glucan producing bacteria and *Gluconobactieriaceae* as a fructan producers (Gulitz et al., 2011; Jakob et al., 2013; Waldherr et al., 2010). Presented results could show, that produced fructans were soluble and not incorporated into the grains, whereas most of produced glucans were involved in grain formation. Comparison of NMR analysis of grain EPS with other bacterial EPS could reveal the grain EPS as a dextran with mainly α-1,6-linear-linkages (Table 33; Seymour et al. 1976; Shukla et al. 2011). Dextranase treatment of grains confirmed this assumption (3.2.2). The cloudy remaining solution after dextranase treatment, on the one hand, could be caused by microorganisms that were included in the grains and, on the other hand, by insoluble parts of the dextran which were not split by dextranase and therefore displayed another linkage type. Additional anomeric resonances at 5.33 ppm in the ^{1}H-NMR spectrum of the grain EPS displayed 10% α-1,3-branching, whereas the peak at 4.99 ppm belonged to the H-1 of α-1,6-glucosyl residues of the main chain (Seymour et al., 1976; Shukla et al., 2011). Dextrans with a high percentage of α-1,3-branching are characterized as insoluble dextrans (Côté & Skory, 2012; Shukla et al., 2011). Horisberger (1969) could demonstrate that the dextran of Tibi grains (synonym for water kefir) consists of α-1,3 and α-1,6-linked glucose units in equal proportions. The insoluble dextran of *Leuconostoc mesenteroides* NRRL B-1149 is known to produce a dextran with 52 % α-1,6 and 40 % α-1,3-linkages (Shukla et al., 2011). For water kefir grain EPS only 10% branching was determined, maybe because it was diluted in D_2O although it was very difficult to solubilize grain EPS in water and so the insoluble parts were not included during the measurement. For further investigations it should be better to prepare the sample in NaOD, because water kefir grains were soluble in 1 M NaOH. The insoluble dextran of *Leuconostoc mesenteroides* B-1149 used in the study of Shukla et al. (2011) showed additional shift values of C-1 and C-6 in comparison to the linear dextran (Table 33). For water kefir grain EPS only the resonances for the 6 main carbon signals comparable with linear dextran could be measured indicating that no carbon atoms involved in branching were detectable. Dextrans with high branching are described to be insoluble but other polysaccharides, for example cellulose are also known to be insoluble without branching. Cellulose is a high molecular polysaccharide (degree of polymerization until 14 000) with β-1,4-glycosidic linkages. Because of its high molecular weight and its highly ordered structure based on intermolecular hydrogen bonds and hydrophobic interactions beside the linear glycosidic linkages cellulose is insoluble in water (Belitz et al., 2001). On the one side water kefir grain EPS could be highly branched, but the characteristic NMR spectra could not be measured, yet, because of

the complicated handling with the insoluble EPS. On the other hand it also seems to be possible that the dextran is insoluble because of its high molecular linearity comparable with cellulose, since no additional ^{13}C NMR resonances could be detected.

Table 33: Comparison of ^{13}C NMR spectral data of water kefir grain EPS with an insoluble dextran containing 40 % α-1,3-branching and with linear dextran

	α-1,6 linear dextran B-640*	Insoluble dextran from *Lc. mesenteroides* B1149*	Water kefir grain EPS
C-1	98.67	101.38, 100.94, 97.94	97.65
C-2	74.36	73.57	73.31
C-3	72.37	71.58	71.30
C-4	71.14	70.46	70.11
C-5	70.52	69.65	69.46
C-6	66.56	65.75, 60.64	65.50

* Data for comparison determined by Shukla et al., 2011

Water kefir preparation with demineralized water led to the deterioration of grain formation. Under standard conditions humid grain mass increased about 30 % per day, whereas in the medium prepared with dH_2O only about 7 %. This may be explained as some minerals and trace elements are essential for microorganisms (Fiil & Branton, 1969). For example manganese is used by LAB as cofactor for enzymes and also to handle oxidative stress. *Lb. plantarum* is additionally able to accumulate manganese via a specific active transport system (Archibald & Duong, 1984; Archibald, 1986). At the first cultivation step grains in dH_2O grew as well as under standard conditions because microorganisms could use their accumulated minerals for growth but from the second cultivation step on the storage was exhausted and the consortium could only use the low mineral concentration of the fig extract. The addition of calcium ions did not increase production of grain mass therefore calcium concentrations in water kefir seemed to be enough, or calcium as single mineral is not enough. Therefore, a balanced mineral composition of the medium is essential for water kefir fermentation.

4.2.2 Influence of water kefir isolates on grain formation

Water kefir isolates were analyzed for their ability to produce EPS to learn more about the key players for grain formation. 17 of 37 isolates were able to produce EPS and even 6 strains

produced very much EPS on sucrose containing agar plates. All EPS found were soluble in water in different concentrations. From 5 *Lb. hordei* isolates 4 strains were identified as strong EPS producers. Three strong and one weak EPS producer of 5 *Lb. nagelii* isolates could be found and from 6 *Leuconostocaceae* only one strain was unable to produce EPS. *Lb. hordei* and *Lb. nagelii* were found as main representative lactobacilli in water kefir and grains of WkW also contained 29.1 % *Leuconostocaceae* (Gulitz et al., 2013, 2011). Since 13 of 16 strains of the organisms mentioned were able to produce EPS and are additionally predominant in water kefir grains, these organisms seemed to play an important role in grain formation. Additional EPS producing organisms were *Lb. hilgardii*, *Lb. satsumensis* and *G. albidus. G. albidus* could be quitted as being part in grain formation because this strain produced a fructan, which could only be found in the supernatant. *Lb. satsumensis* was only less found in water kefir, *Lb. hilgardii* was hardly found in WkA and WkF whereas WkW and WkI displayed a higher concentration of this species (Gulitz et al., 2013). Since WkA and WkF were also able to produce grains *Lb. hilgardii* did not seemed to be that necessary for grain formation. On the other side Pidoux (1989) and Waldherr et al. (2010) described *Lb. hilgardii* as the important species for grain formation. Pidoux was able to produce grains with *Lb. hilgardii* in pure culture on a specific medium containing yeast extract. In this work almost 50 % of the water kefir isolates were able to produce EPS in different concentrations and hardly any *Lb. hilgardii* was found. Therefore, it could be assumed that a metabolic core producing EPS is more important for water kefir grain formation than the single species designation.

For strong EPS-producing isolates about 10 to 20 g/l EPS could be determined, whereas very strong EPS-producing strains released a concentration about 30 g/l after 48 h of fermentation in MRS-Sac. *Lc. mesenteroides* (TMW 2.1073) and *G. albidus* (TMW 2.1191) were identified as very strong EPS producing strains on sucrose containing agar plates whereas their EPS formation in liquid medium was less (about 10 g/l) and rather be comparable with strong EPS producers. In liquid medium cells were more distributed than as colony forming unit on the agar plate. Biofilm formation of several species is known to be quorum sensing controlled at high cell densities (Parsek & Greenberg, 2005). *Pseudomonas aeruginosa* produces biofilms involving quorum sensing (Davies et al., 1998). It is likely that *Lc. mesenteroides* as well as *G. albidus* produce one of these signaling molecules at high cell densities that are not reached in liquid medium at the same level as on agar plates.

Very strong EPS producing organisms were additionally determined for their EPS production in WKM. It could be shown that only *Leuconostocaceae* were able to produce EPS in appreciable amounts anymore, but in much lower concentrations than in MRS-Sac. *Lb. hilgardii* and *G. albidus* could hardly produce any EPS, because revealed concentrations after 48 h of fermentation were < 1 g/l. Nitrogen concentrations in WKM were much lower than in MRS, therefore, organisms could only grow and produce less EPS. As mentioned before *Leuconostocaceae* could be found in high amounts in WkW and they were additionally better able to produce EPS in the water kefir system. *Leuconostoc sp*. are known to spoil lemonades, a medium with high sugar and low nitrogen concentrations (Müller & Reuter, 1968). Therefore, it can be assumed that *Leuconostocaceae* are well adapted to such ecological niches. In future it could be interesting to determine the EPS production of *Lb. hordei* and *Lb. nagelii* in WKM because several strains were identified as EPS producers on MRS. Good EPS production in WKM could confirm their influence of grain formation.

Growth experiments of water kefir isolates in WKM supplemented with different EPS could demonstrate that EPS did not increase growth of the isolates. Experiments in media with EPS as single carbon source displayed that water kefir isolates were not able to ferment EPS. It is more likely that water kefir organisms produce EPS to handle osmotic stress while reducing sugar concentration and not as energy source because most EPS producers are not able to catabolize their EPS (Cerning, 1990).

Some bifidobacteria are able to ferment fructooligosaccharide (Korakli et al., 2002). During this work the influence of glucooligosaccharides was tested, with the result that water kefir isolated *B. psychraerophilum* was not able to use these EPS as carbon source. Water kefir acetic acid bacteria were identified as fructan producers, therefore more interaction experiments between *Bifidobacteriaceae* and acetic acid bacteria might be interesting in future.

4.2.3 Glucansucrases catalyzing production of water insoluble α-D-glucan

EPS obtained from water kefir isolates were soluble in water in different concentrations. Since water kefir grains were insoluble in water backgrounds of the ability of single isolates and the whole consortium to produce water insoluble EPS were determined. DNA of different *Leuconostoc* isolates and water kefir grain DNA were analyzed along their genes for glucansucrases catalyzing the production of water insoluble α-D-glucans. Côté and Skory (2012) described a glucansucrase (YP_819212) isolated from *Leuconostoc mesenteroides* (NRRL B-1118) catalyzing the production of an insoluble α-D-glucan. For 3 of 6 water kefir isolates and for DNA of WkW the gene of exactly this glucansucrase was found. Further enzymes catalyzing α-1,3-linkages could not be determined. *Lc. mesenteroides* strains TMW 2.1075, TMW 2.1076 and TMW 2.1193 were theoretically able to produce a water insoluble glucan, but the isolated EPS was soluble. The triggers inducing these strains to express this protein are still unknown.

The background of water kefir grain formation remains unclear, because single water kefir isolates produced soluble dextrans and even solubilized grains in 1 M NaOH after neutralization did not precipitate but a clear solution remained. After precipitation with ethanol and subsequent freeze drying water kefir grain EPS was poorly soluble but no flocculation under any circumstances could be observed. Maybe yet uncultivable species play an important role in grain formation.

4.3 Interaction of water kefir isolates

4.3.1 Experiments for reconstruction of water kefir grains

Mixture of isolated organisms could not produce any grains because of missing uncultivable organisms. Besides the cultivable species with state of the art methods Gulitz et al. (2013) could demonstrate that water kefir grains contain a number of *Bifidobacteriaceae*, that could not be cultivated, yet. Disruption of water kefir grains and using the supernatant did not lead to grain formation. While destroying the grains the physical configuration of single water kefir organisms was disorganized and during fermentation with the supernatant the organisms were far apart from each other and not able to reorganize. On the one hand it can be assumed that water kefir organisms need a strict organization that is only given inside the grains.

Confining the organisms in alginate beads seemed to force water kefir bacteria and yeasts in wrong positions. Leroi and Pidoux (1993a) could also demonstrate that embedding water kefir isolates in alginate beads led to decreased metabolic activity. On the other hand it could also be possible that some organisms adhere close to grain EPS, like it can be also observed in biofilms (Cerning, 1990; De Vuyst & Degeest, 1999). Thus, these particles were too large to pass the membrane of the mixing bag and so these organisms were missing for grain formation. Pidoux (1989) could demonstrate new grain formation with *Lb. hilgardii* in pure culture on a specific medium containing yeast extract but they did not present following studies on sucrose medium comparable with natural growth of water kefir grains as announced during the study. Also for other food fermentations an artificial construction of a new consortium seemed to be hardly achievable. A reconstruction of milk kefir grains was not described, yet. For sourdough organisms cultivation in laboratory media is difficult because the organisms of the consortium have been selected during many propagation and are finely balanced concerning nutrient requirements and growth conditions (reviewed by De Vuyst & Neysens, 2005).

4.3.2 Interaction of single isolates in co-cultivation without cell-cell contact

Interaction between different water kefir isolates was determined concerning their growth and metabolites by comparison of co-cultivation in a model system and their respective pure culture. Inoculation with a bacteria : yeast ratio of 10% was similar to the average viable cell counts per gram water kefir grains (Gulitz et al., 2011).

Each co-cultivation of yeasts and lactobacilli tested showed an increase of cell yield for both interaction partners in comparison to pure cultures of single water kefir isolates from the beginning of fermentation, delineating the interaction of these water kefir isolates as mutualism (Stadie et al., 2013). The effect of interdependency of lactobacilli and yeast has also been observed in sourdough (Damiani et al., 1996; Gobbetti et al., 1994a, 1994b), in milk kefir (Cheirsilp, Shimizu, et al., 2003; Cheirsilp, Shoji, et al., 2003), fermented milks (Gadaga et al., 2001; Shao-Quan Liu & Tsao, 2009) and in sugary kefir grains (synonym for water kefir; (Leroi & Pidoux, 1993a)). These studies just present a stimulation of co-cultured lactobacilli, yeasts were either unaffected or even decreased in growth for 65% (Leroi & Pidoux, 1993a). A support of yeast, as could be demonstrated in this work, has not been described, before (Stadie et al., 2013). Merely, Leroi and Pidoux (1993b) could determine a small increase of yeast cell yield in mixed-cultures employing a bacteria : yeast ratio of 5%.

Also, their study remains descriptive leaving the metabolic background unsolved. In our experiments we started with a bacteria : yeast cell ratio of 10%, thus an increase of yeast cell rate seems to enhance the interaction in water kefir.

Co-cultivation of *B. psychraerophilum* with *Z. florentina* and *S. cerevisiae*, respectively, increased growth of both yeasts, whereas the *Bifidobacterium* remained unaffected, that delineates their interaction as commensalism. Enhancement of yeast growth was lower than that in co-cultivation with lactobacilli. *Bifidobacteriaceae* acidify the medium during fermentation by production of lactic and acetic acid and growth of *Z. florentina* was increased in media at lower pH-values (for more details see 3.6.5). Since *B. psychraerophilum* was able to grow pure cultured in WKM better than lactobacilli tested, yeasts and the *Bifidobacterium* compete for nitrogen nutrients that were quantitatively limited in WKM. Although lactobacilli and yeasts should compete for nitrogen sources as well, just the opposite could be observed while both interaction partners were promoted. This counts for the fact that yeasts and lactobacilli mutually support each other during water kefir fermentation. Still, their interaction remains opportunistic as they can also grow alone.

Similar observations could be made for sourdough fermentation. *S. cerevisiae* rapidly consumes glucose and maltose and would therefore inhibit maltose fermenting *Lb. sanfransiscensis*. But excluding *S. cerevisiae* from the microbial community of sourdough leads to repression of maltose fermentation and to depletion of sucrose concentrations (De Vuyst and Neysens, 2005).

Metabolic activity in co-cultivation was enhanced for all interaction systems tested. In every co-cultivation experiment sucrose depletion was increased whereas theoretically produced glucose and fructose from inversion did not appear and were therefore consumed. Higher metabolic activity could also be detected for lactate production especially in the co-cultivation systems with yeasts and lactobacilli. Glycolysis of some bacteria, e.g. streptococci, could be stimulated by environmental conditions, delineating a host-symbiont interaction (Kleinberg et al., 1973).

Growth of yeasts in co-cultivation with *B. psychraerophilum* was increased and therefore more cells compete for same nutrient concentrations as in pure culture. Yeasts do not produce any acetate or lactate thus the concentrations of these metabolites were due to the *Bifidobacterium*. During fermentation of 2 mol glucose *Bifidobacteriaceae* usually produce 3 mol acetate and 2 mol lactate (Korakli et al., 2002; Pokusaeva et al., 2011). Even for calculated co-culture in the system with *B. psychraerophilum* and both yeasts in WKM acetic

and lactic acid amounts were similar. For *B. bifidum* an enhanced lactic acid production could be detected during fermentation in the presence of honey and equal amounts of lactate and acetate could be determined as well (Chick et al., 2001). Therefore, with the natural source of figs *B. psychraerophilum* seemed to be stimulated for lactate production. Metabolism of *B. psychraerophilum* in co-cultivation with *S. cerevisiae* was inhibited because of reduced lactic and acetic acid production. In co-cultivation with *Z. florentina* acetate production was decreased whereas lactate concentration rose. The succinate concentrations produced by *Z. florentina* rose as well, thus a stronger acidification of the milieu could be possible, but was not determined. The lower the pH the less is the acetate production for *B. bifidum* (Mlobeli et al., 1998). Acidification of the milieu, co-cultivation with *Z. florentina* in comparison with natural source medium (fig concentrate) seemed to induce *B. psychraerophilum* to change its metabolic pathway.

Fig. 36 displays a summary of interaction determined in co-culture in the model system. Mutualism between lactobacilli and yeasts is reflected in growth as well as in metabolic activity of all interaction partners. Depicted interaction between yeasts and *B. psychraerophilum* reveals commensalism because yeasts were increased in growth and metabolic activity whereas *B. psychraerophilum* is unaffected in growth. Co-cultivation of *S. cerevisiae* and *B. psychraerophilum* exhibits decreased metabolic activity for the *Bifidobacterium*, whereas co-cultivation with *Z. florentina* seemed to change metabolism of *B. psychraerophilum* towards enhanced lactate and decreased acetate production. Between lactobacilli and *B. psychraerophilum* no interaction could be detected.

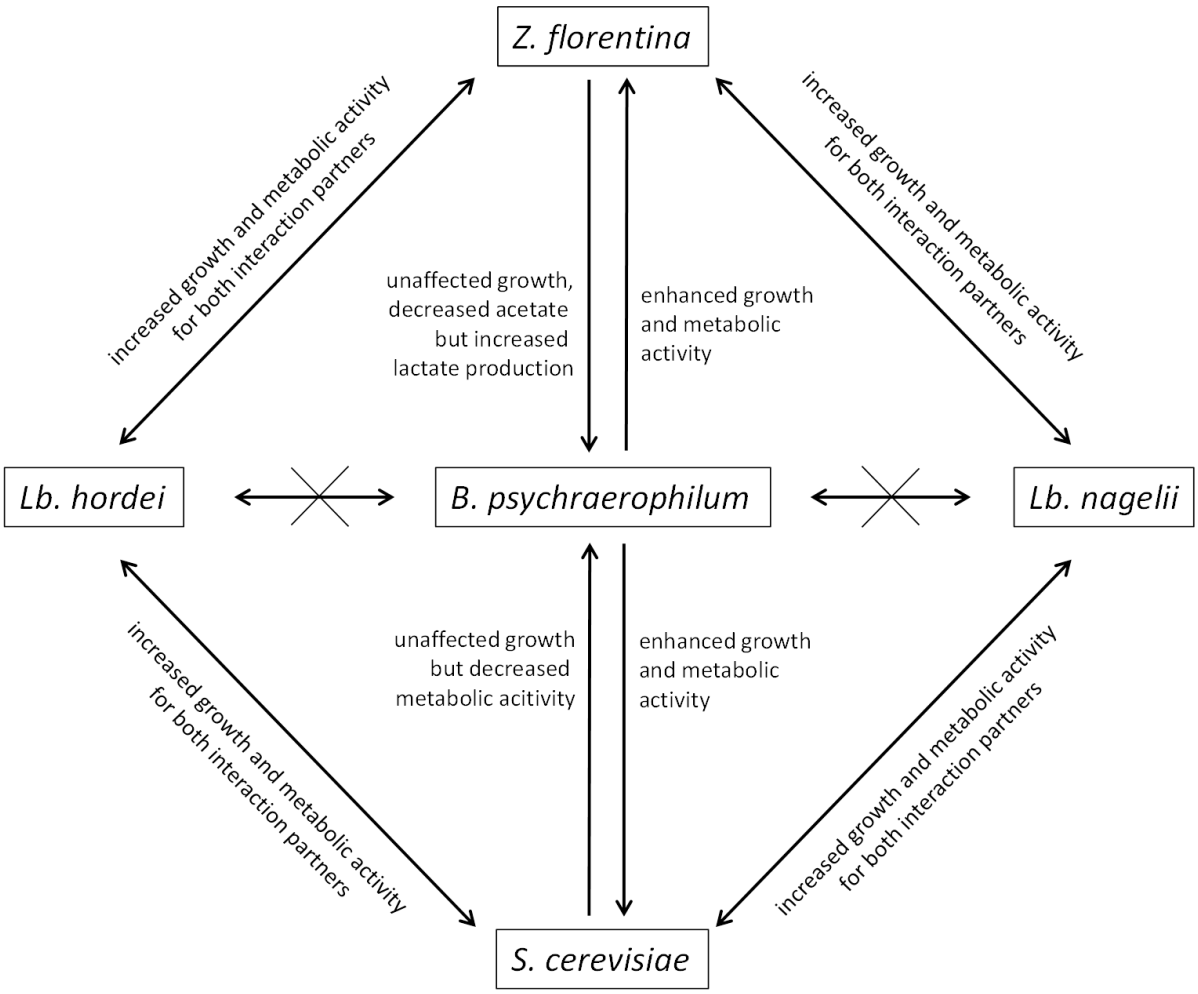


Fig. 36: Summarized results of interaction experiments of different water kefir isolates co-cultivated in the model system

Influence of organism A ⟶ on growth and metabolism of organism B.

4.3.3 Interaction of isolates in mixed-culture with cell contact

During water kefir fermentation the organisms live in a close community, thus interaction between different isolates with cell-cell contact was determined.

Comparison of growth of the mixed- and calculated-mixed culture did not differ for interaction experiments with lactobacilli, whereas mixed-culture of *B. psychraerophilum* and *Z. florentina* decreased growth in comparison to calculated-mixed culture. As mentioned above *B. psychraerophilum* as well as *Z. florentina* were able to grow pure cultured in WKM, and therefore, in mixed-culture they compete for limited nitrogen nutrients. The experimental setup of calculated mixed-culture provided twice as much nutrients as in mixed-culture, because for calculated-mixed culture both organisms were pure cultured in each 45 ml WKM, whereas during mixed-culture both organisms had to compete for 45 ml WKM. This nutrient

competition was also reflected in the metabolite spectrum. Concentrations of lactate, acetate and succinate were reduced in comparison to calculated mixed-culture.

Distinct differences in the metabolite spectrum could be displayed for the lactate production in mixed-culture of *Lb. nagellii* and *Z. florentina,* lactate concentration rose from 1.17 g/l in calculated mixed-culture to 2.99 g/l in mixed-culture. *Z. florentina* is not able to produce lactate, thus the *Lactobacillus* was more effective in lactate production as pure cultured.

4.3.4 Comparison of co-culture and mixed-culture experiments

During co-cultivation in the model system the interaction of water kefir isolates was obvious while all interaction partner benefited from co-cultivation, except growth of *B. psychraerophilum* remained unchanged. The support between water kefir organisms could not be detected during mixed-culture experiments. This fact could be due to the differences in the experimental setup. In the Transwell® system 5 ml fermentation broth displayed a surface of 4.5 cm^2. Therefore, oxygen concentration was higher in percentage to the mixed-culture experimental setup, where 45 ml fermentation broth displayed a surface of 6.2 cm^2. Thus, the yeast was able to breathe and produce more cell mass for supporting the lactobacilli in the model system than mixed-cultured. During water kefir fermentation grains increase in size and caused by CO_2 production the grains are transported to the vessel surface. The vessel is only slightly covered to ensure a gas exchange and limited oxygenation. After dividing, grains fall down to the bottom of the vessel, thus, a steady oxygen exchange during fermentation is ensured. Contemplating oxygen exchange during fermentation the model system is likely closer to household water kefir fermentation than the mixed-culture setup used.

Cheirsilp, Shoji, et al. (2003) explained that the observed interaction between milk kefir organisms *Lactobacillus kefiranofaciens* and *Saccharomyces cerevisiae* were based on the physical contact between them. During this work it could be shown that interactions of water kefir isolates do not obligatory depend on cell contact. Although mixed-culture experiments in the simplified chemically defined medium without arginine could increase interaction between *Lb. nagelii* and *Z. florentina* in comparison to experiments in the model system (see 3.6.3). Therefore, it can be concluded that cell-cell contact of water kefir isolates tested is not obligatory but auxiliary.

4.3.5 Up-regulated proteins in mixed-culture

Whole cell protein in mixed-culture was compared to pooled whole cell protein of individual single-cultures with 2D gel electrophoresis. Seven proteins up-regulated in mixed-culture could be found in the system of *Lb. hordei* and *Lb. nagelii*, respectively, with *Z. florentina*, whereas no additional proteins could be identified that were only up-regulated in their individual single-cultures.

Glyceraldehyd-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), enolase (ENO) and L-lactate dehydrogenase (LDH) are glycolytic enzymes and therefore important for energy production out of carbohydrates. Fig. 37 displays their function in the glycolytic pathway. Glycolytic enzymes are usually located in the cytosol. However, for several glycolytic enzymes non-glycolytic functions are known. Proteins that have more than one function are termed moonlighting proteins (Brandina et al., 2006; Commichau et al., 2009; Entelis et al., 2006; Hughes et al., 2002; Katakura et al., 2010; reviewed by Henderson and Martin, 2011). For *S. cerevisiae* macromolecular complexes out of glycolytic enzymes, especially enolase and GAPDH could be found on the surface of mitochondria. In this association these enzymes are responsible for channeling pyruvate into the mitochondria, namely into the site of consumption (Brandina et al., 2006; Entelis et al., 2006). Additionally, enolase, PGK and GAPDH were observed in interaction with replication proteins in *B. subtilis* (Commichau et al., 2009; Jannière et al., 2007). Enhanced expression of the glycolytic enzymes with their usual function in glycolysis as well as in order to channeling of pyruvate and interaction with replication proteins proves increased metabolic activity of interacting lactobacilli and yeast.

Comparison of whole cell proteins obtained in mixed-culture with pooled whole cell proteins of individual single-cultures of *B. psychraerophilum* and *Z. florentina* displayed one more time with only one up-regulated protein the exiguous interaction between these water kefir organisms. It is noteworthy, that the experimental setup was planktonic and not immobilized as in their natural environment in water kefir grains. Therefore, it remains unclear if yeasts and *Bifidobacteriaceae* interact in water kefir grains.

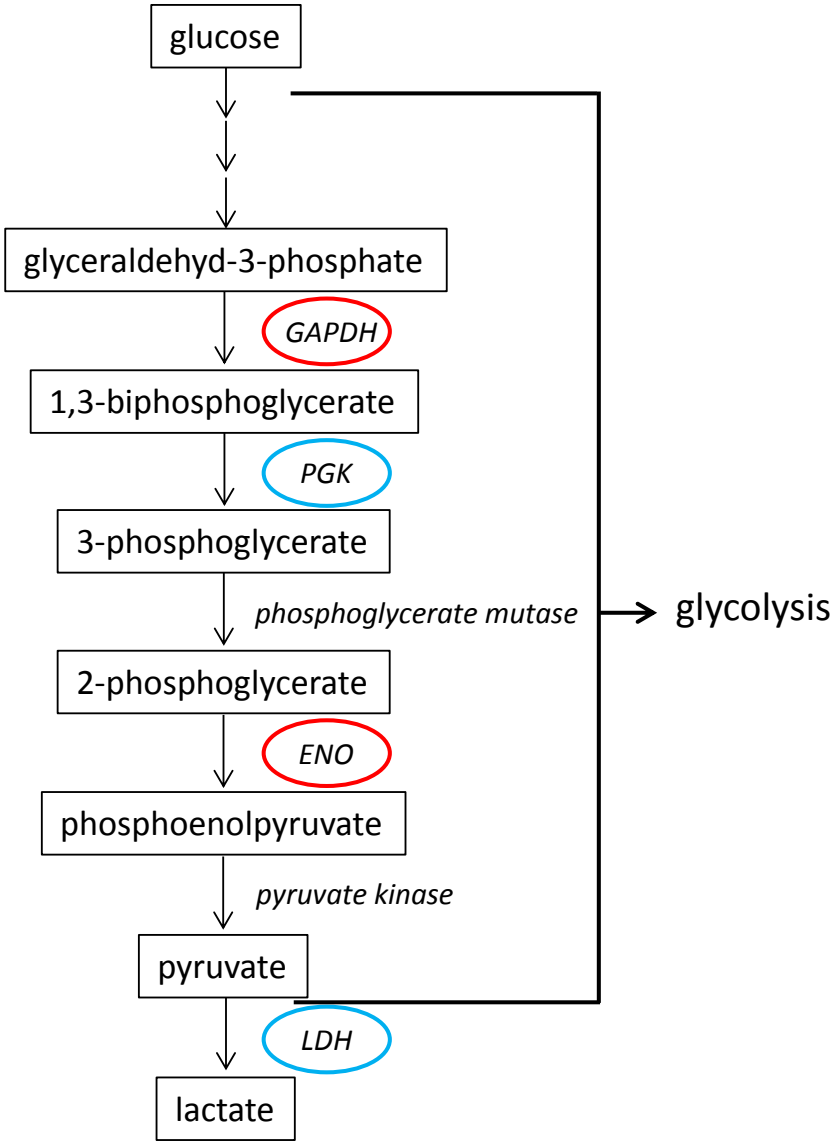


Fig. 37: Schematic representation of the glycolytic pathway and the final lactic acid fermentation

Red circled enzymes represent proteins that were up-regulated from the yeast and blue circled enzymes were up-regulated from both lactobacilli tested in mixed-culture of *Z. florentina* and lactobacilli. Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; ENO, enolase; LDH, lactate dehydrogenase

Another up-regulated protein in mixed-culture of lactobacilli and *Z. florentina* could be identified as a heat shock protein produced by the yeast. Since a molecular weight of 21 kDa could be determined, this protein belongs to the family of small heat shock proteins (sHsps). Hsps are expressed under stress, not only heat stress but also a variety of other stresses, e.g. acid, heavy metal ions or anoxia. Production of Hsps is already induced under moderate stresses to protect the cell from more severe stress conditions (Lindquist & Craig, 1988). In co- and mixed-cultivation with lactobacilli and even during water kefir fermentation *Z. florentina* has to cope with acid stress. During water kefir fermentation several organic

acids are produced and decrease the pH of the fermentation broth. To come along with this acid stress *Z. florentina* induced production of heat shock proteins.

Additionally, glutamine synthetase (EC 6.3.1.2) up-regulated by lactobacilli could be found. This enzyme catalyzes the production of glutamine out of glutamate and ammonia. It is an ATP and therefore energy consuming reaction.

ATP + L-glutamate + NH_3 → ADP + phosphate + L-glutamine

This reaction plays an important role in nitrogen metabolism of micro-organisms. Glutamine can be used as nitrogen donor in the biosynthesis of proteins and nucleic acids (Gancedo & Holzer, 1968; Rhodes, Rendon, & Stewart, 1975). Yeast extract contains glutamate, thus, partial autolysis of yeast cells in mixed-culture as also described for arginine sustenance supply glutamate for glutamine production of lactobacilli.
Phosphoribosylaminoimidazole-succinocarboxamide synthase (SAICAR synthase; EC 6.3.2.6), also up-regulated by lactobacilli, is an important enzyme for *de novo* purine nucleotide synthesis (Herve-Jimenez et al., 2009).

GAPDH, PGK, ENO, LDH, glutamine synthase and SAICAR synthase, namely 6 of 7 over-expressed proteins in mixed-culture are basic enzymes for metabolic activity and replication. Interaction experiments could demonstrate enhanced metabolic activity in terms of increased sugar consumption and metabolite production, especially for lactate. Co-cultivation in the model system particularly displayed enhanced replication because cell density of yeasts as well as lactobacilli increased.

4.3.6 Aggregation of mixed water kefir isolates

Several glycolytic enzymes, that are usually located inside the cytosol, could be found on cell surfaces of different bacteria and yeasts bound to the cell wall (reviewed by Henderson and Martin, 2011). Katakura et al. (2010) could determine PGK and GAPDH and several other enzymes, located on the surface of *Lactococcus lactis* as cell-wall-associated proteins. Furthermore they could exhibit another protein, namely DnaK as adhesion factor to yeast mannan. DnaK usually is a chaperon of the Hsp70 family but could be revealed as moonlighting protein to adhere LAB to yeasts. During mixed culture experiments of water

kefir isolates associations of yeasts and lactobacilli could be examined under microscope (Fig. 30). Since GAPDH as well as PGK and another heat shock protein could be identified as up-regulated proteins in mixed-culture, the mixed-aggregation of water kefir isolates was examined. Cells of *Lb. nagelii* and *Lb. hordei*, respectively, together with cells of *Z. florentina* in PBS buffer exhibited 3 % stronger aggregation than their individual auto-aggregation. Mixed-aggregation of *B. psychraerophilum* and *Z. florentina* did not display a significant difference to their respective auto-aggregation. This illustrates another time the unincisive interaction between these water kefir isolates as already described for growth and metabolic activity. Addition of mannose to the buffer could inhibit mixed-aggregation of lactobacilli and yeasts for about 35 %. Thus, it could be shown that aggregation of water kefir organisms is due to proteins that aggregate with mannose containing components similar to the description of Katakura et al. (2010). They could display that DnaK on the cell surface of *Lacotcoccus lactis* binds to a mannoprotein, namely invertase, of *S. cerevisiae.*

It is noteworthy, that the organisms tested have not been cultivated together but mixed in equal cell densities in buffer. Singly cultured cells did not reveal up-regulated proteins as described before. Therefore, this experiment could determine basic interaction in aggregation of water kefir organisms. To confirm the assumption that glycolytic enzymes are placed on the surface of water kefir organisms to aggregate with each other and be the basis for grain formation more detailed aggregation experiments should follow. Aggregation experiments of co- or mixed-cultured cells should reveal stronger aggregation than already described if the assumption could be confirmed.

4.4 Metabolic synergism between main representatives of water kefir isolates

With a content of 31.3 and 22.7 % of water kefir bacteria, *Lb. hordei* and *Lb. nagelii*, respectively, are the predominant bacteria in water kefir ascertained with state of the art methods. As main representatives of yeasts *Z. florentina* and *S. cerevisiae* were described (Gulitz et al., 2011). Thus, these apparent key players in water kefir were used for more detailed interaction experiments to reveal to metabolic synergism between water kefir isolates.

Co-cultivation of yeasts and lactobacilli in water kefir medium significantly increased cell yields of all interaction partners. Growth of *Z. florentina* was increased in pH lowered YPG at pH 4 in comparison to higher starting pHs (Fig. 35) (Stadie et al., 2013). Lactobacilli produce lactic and acetic acid during fermentation and therefore, they optimize the milieu for *Z. florentina*. The growth of food fermenting yeasts is known to be improved at decreasing pH. For the brewing yeast *S. carlsbergensis* Vosti and Joslyn (1954) described growth optimum at pH 3.83. The metabolic interaction for *Z. florentina* relies on the adjustment of the physiochemical environment. Interaction of milk kefir isolated *L. kefiranofaciens* and *S. cerevisiae* relies on the consumption of lactate by *S. cerevisiae* and the following intensified production of capsuled kefiran by the *Lactobacillus* (Cheirsilp, Shimizu, et al., 2003). Water kefir isolated *Z. florentina* and *S. cerevisiae* were not able to perform gluconeogenesis, thus this point is not applicable for water kefir fermentation.

Growth of both yeasts is decreased in WKM with acetic acid; at pH 3 growths were inhibited. Acetic acid in acidic solutions is largely undissociated and as an uncharged particle it can diffuse freely into the cells. Intracellular pH of *S. cerevisiae* is usually between 5.5 and 6.8, depending on the growth phase (Imai & Ohno, 1995). If the external pH is lower than the internal, acetic acid can dissociate inside the cell and decrease internal pH. Under expense of metabolic energy (ATP) and decrease of growth, cells can regulate the intracellular pH by pumping out the protons (Abbas, 2006; Warth, 1988). The lower the pH, the more undissociated acetic acid exists in the medium, therefore cell yield of both yeasts declines in pH lowered media. The inhibition at pH 3 can be explained by the acetic acid concentration in this medium (347 mM), 167 mM suffice for complete repression of *S. cerevisiae* (Thomas et al., 2002).

Pre-fermented media with yeasts could stimulate *Lb. hilgardii* in growth and lactic acid production, whereas free amino acids and vitamins did not show an effect (Leroi & Pidoux, 1993b). We could not confirm the support by pre-fermentation with our water kefir species tested, conversely, addition of vitamins and free amino acids played an important role, because *Lb. hordei* and *Lb. nagelii* showed auxotrophies for some amino acids and vitamin B_6, respectively. Addition of essential nutrients to WKM in different concentrations showed the influence and lack of these substances in WKM. After 20 h of fermentation the content of essential nutrients in pure and lower modified WKM were already consumed, because the optical density of both lactobacilli stagnated in these media. Only in the medium with higher concentrations of essential nutrients cells were able to replicate furthermore.

WKM contains glucose out of the fig concentrate as well as sucrose that is inverted to fructose and glucose, and yeasts are known to secrete vitamin B_6 during fermentation in the presence of glucose (Abbas, 2006). Thus, a part of trophic interaction between *Lb. hordei* and both yeasts is revealed as delivery of vitamin B_6 by *Z. florentina* and *S. cerevisiae* (Stadie et al., 2013).

In co-cultivation with yeasts *Lb. nagelii* was able to grow in simplified chemically defined medium without addition of essential arginine. The support of *Z. florentina* showed 1.5 fold higher cell yield than the interaction with *S. cerevisiae*. With 52.5% of water kefir isolated yeasts *Z. florentina* is the predominant yeast in water kefir grains, thus it seems to play a more important role in mutualism therefore further interactions were only analyzed with this yeast. Addition of yeast fermentation broth, yeast cell debris, co-cultivation fermentation broth and pre-fermented medium with yeast and dead cells of *Lb. nagelii* did not support growth of *Lb. nagelii* in arginine free medium (Table 31). This accounts for the fact that *Z. florentina* only excretes amino acids essential for *Lb. nagelii* in co- or in mixed-culture, but not if they are single cultivated (Stadie et al., 2013). The effect that yeasts release essential nutrients for lactobacilli was observed in sourdough by Challinor and Rose and Gobbetti et al (Challinor & Rose, 1954; Gobbetti et al., 1994a). On the one hand release of amino acids by yeasts can be explained by the change in membrane permeability in presence of glucose (Lewis & Stephanopoulos, 1967) or by autolysis of yeast cells (Vosti & Joslyn, 1954). This work could demonstrate for the first time that the stimulation must be caused by the *Lactobacillus*, because support only occurs in co-cultivation. The addition of yeast cell extract to arginine free medium resulted in growth of *Lb. nagelii* similar to medium with arginine. Therefore it is suggested that the co-culture of these two organisms partially affects autolysis in yeasts or triggers other mechanisms of (selective) nutrient release. Autolysis of yeasts can be induced e.g. by various proteins, peptides and amino acids leading to a change in membrane permeability (Babayan & Bezrukov, 1985), and thus *Lb. nagelii* may produce such molecules signaling the yeast to autolyze. Such symbiotic association is called cooperative metabolism.

Fig. 38 displays an overview about the revealed metabolic interaction of main representative cultivable water kefir isolates. *Z. florentina* is depicted closer to the lactobacilli and the arrows are shown more intensive because of the higher influence of this yeast in comparison to *S. cerevisiae*. In co-cultivation of *S. cerevisae* with lactobacilli the yeast is improved in growth but the reason has still to be ascertained. During this work metabolic interactions of

single water kefir isolates in a model system with planktonic cultures could be explained. Interaction experiments with organisms embedded in grains, closer to their natural association, are technically limited and have not been possible, yet. This may be due to the fact, that the water kefir consortium contains partly unculturable types of bifidobacteria (Gulitz et al., 2013). Interactions in water kefir are likely more complex than the interaction of only two organisms demonstrated here, and therefore, the role of other species, namely acetic acid bacteria, (yet unculturable) bifidobacteria and *Leuconostocaceae* remain to be elucidated.

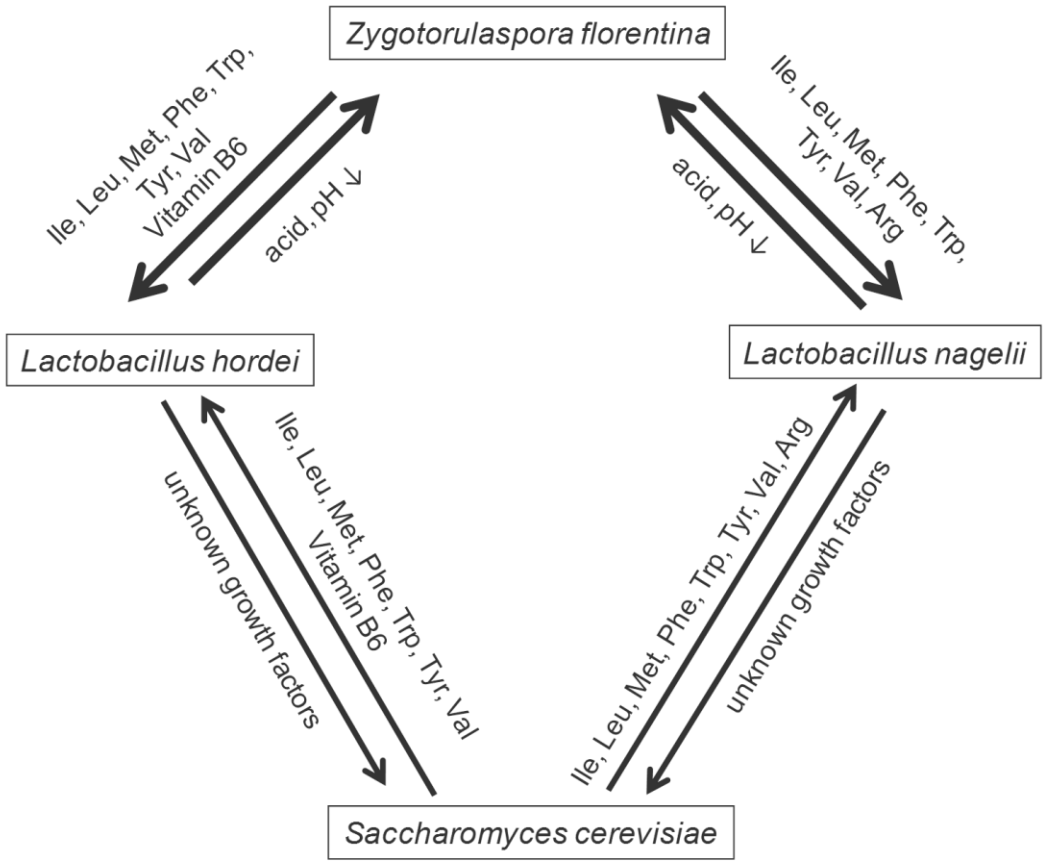


Fig. 38: Interaction overview of main representative cultivable water kefir isolates (Stadie et al., 2013)

WKM is a high sugar and low amino acid containing medium therefore, in this ecological niche only well adapted microorganisms are able to grow. *Lb. nagelii* was first isolated from slightly fermented grape juice that is a demanding habitat similar to water kefir, even the existence of yeasts implies that *Lb. nagelii* is well adapted in such ecological niches and lives in mutualism with yeasts.

If we perceive water kefir as an organism rather than a mere association it forces itself that induction of autolysis of yeast cells on the one hand plays an important role for nutrient

exchange and on the other hand for species regulation in the consortium, preventing organisms overgrow by others. Such social behaviors are known in other complex multicellular communities, namely biofilms, in terms of programmed cell death and lysis, while the control mechanisms are not fully understood (Rice & Bayles, 2008; Sadykov & Bayles, 2012). So it is appears likely that the water kefir consortium uses related mechanisms to regulate community composition and growth (Stadie et al., 2013).

SUMMARY

Water kefir grains are an association of different lactic acid bacteria, acetic acid bacteria, yeasts and bifido bacteria. The consortium is embedded in transparent, crushed-ice-shaped grains which mainly consist of an insoluble dextran. For the preparation of a homemade beverage grains were cultivated in a sucrose solution (8%) in mineral water supplemented with dried fruits. Dried figs ensure best growth of water kefir grains. Fermentation at room temperature for two or three days results in a fizzy, cloudy beverage that is low acid, somewhat sweet and slightly alcoholic. During water kefir fermentation sucrose is inverted to glucose and fructose by invertases of containing yeasts, whereas subsequently mainly glucose and less fructose are fermented. The bacteria of the consortium produce lactic and acetic acid as well as little amounts of ethanol. Water kefir yeasts produce amounts of ethanol, succinate and mannitol during fermentation. Additionally many volatile compounds are built, which can be also found during wine fermentation. Fruity esters and odorous alcohols are welcome whereas branched chain fatty acids and acetoin could be seen as off flavors because of their buttery rancid odor. Water kefir organisms compete for nitrogen compounds that are the limiting growth factors for the consortium. Comparison of pH, sugar consumption and metabolite production of two water kefir consortia with different origin revealed equal fermentation patterns. Change of parameters for water kefir fermentation could display that fermentation conditions have more impact on the concentrations of the metabolite spectrum and the resulting taste of the beverage than consortia with a different origin. However, for a stable consortium with grain formation usual household conditions are necessary. In order to produce an industrial beverage based on water kefir, interacting organisms should be chosen as starter cultures to ensure best support in growth and fermentation. Therefore, the use of yeasts as starter cultures appears to be essential, but in less concentration than bacteria, otherwise they will overgrow the fermentation broth.

Beside the fermentation of sugar for energy production 17 of 37 water kefir isolates were able to produce EPS. Merely one strain, namely *G. albidus,* could be identified as a fructan producer, the other strains released a glucan composed of glucose monomers. Since water kefir grain EPS consists of glucose monomers the identified glucan producing strains are likely important for water kefir grain formation. Pidoux (1989) and Waldherr et al. (2010)

described *Lb. hilgardii* as the most important species for grain production. The water kefir consortia that were used during this work contained only small amounts of *Lb. hilgardii* strains (Gulitz et al., 2013, 2011). Therefore, it could be assumed that other genera can also provide this function and a metabolic core producing EPS is more important for water kefir grain formation than the single species designation. Water kefir grains are insoluble in water. Isolated EPS from single strains were soluble in different concentrations. Genes encoding a glucansucrase catalyzing the production of water insoluble α-D-glucan could be identified for three strains of *Lc. mesenteroides* isolated from water kefir and in DNA of WkW. The induction for the production of insoluble EPS as it could be found during water kefir grain formation remains unclear. NMR analysis could reveal the grain EPS as a dextran with mainly α(1→6) linear linkages and 10 % α(1→3) branching.

Further investigations during this work addressed the interaction of main representative water kefir organisms in planktonic cultures. With its high sugar content and low amino acid concentration water kefir medium represents a demanding habitat. In this ecological niche only well adapted microorganisms which are fit to the consortium are able to grow and symbiotically provide essential nutrients. The synergism between main representatives of water kefir yeasts interacting with lactobacilli and *B. psychraerophilum*, respectively, was studied in a co-culture model system. Co-cultivation of yeasts and lactobacilli in water kefir medium significantly increased cell yield of all interaction partners, delineating the interaction of these water kefir isolates as mutualism, whereas the impact of *Z. florentina* is greater than that of *S. cerevisiae*. Commensalic interaction between *B. psychraerophilum* with *Z. florentina* and *S. cerevisiae* could be revealed since growth of both yeasts was increased whereas the *Bifidobacterium* remained unaffected. Enhanced metabolic activity for interacting lactobacilli and yeasts could be illustrated through increased metabolite production, e.g. lactate, succinate and mannitol, intensified sugar depletion and over-expression of glycolytic enzymes. The support of *Zygotorulaspora (Z.) florentina* is due to acidification of the medium by lactobacilli, whereas lactobacilli are improved in growth by the disposal of essential nutrients produced by yeasts. The trophic interaction between *Lactobacillus (Lb.) hordei* and yeasts is constituted by the release of amino acids and Vitamin B_6 from yeasts, whereas *Lb. nagelii* is supported in growth by their production of amino acids. The interaction of *Z. florentina* and *Lb. nagelii* was further examined to reveal that co-cultivation induced the yeast to release arginine, which was essential for *Lb. nagelii*. During interaction experiments concerning growth, metabolism and aggregation of mixed

cells clarified special synergism between *Z. florentina* and predominant water kefir lactobacilli (*Lb. hordei* and *Lb. nagelii*) since all reactions are particularly pronounced between these organisms.

ZUSAMMENFASSUNG

Wasserkefir Granula bestehen aus einer Gemeinschaft von Hefen und verschiedenen Bakterien, darunter Milchsäure-, Essigsäure- und Bifiobakterien. Das Konsortium dieser Mikroorganismen ist in transparente, crushed-ice-ähnliche Granulen eingebettet, die hauptsächlich aus unlöslichem Dextran bestehen. Im Hausgebrauch wird Wasserkefir für die Herstellung eines spritzigen, leicht sauren und wenig alkoholischen Getränks verwendet, dafür werden die Granulen in einer Saccharoselösung (8%) in Mineralwasser mit getrockneten Früchten für zwei bis drei Tage bei Raumtemperatur kultivert. Getrocknete Feigen liefern hierbei die höchste Zunahme an Granula-Masse. Während der Fermentation von Wasserkefir wird der Gehalt an Saccharose durch die Invertaseaktivität der Hefen reduziert und zu Glucose und Fructose gespalten. Die entstehende Glucose wird zugleich abgebaut, während Fructose nur in geringen Mengen verstoffwechselt wird. Die im Konsortium befindlichen Bakterien produzieren neben Milch- und Essigsäure auch geringe Mengen an Ethanol. Der größere Anteil an gebildetem Ethanol kann den Hefen zugeschrieben werden, welche zusätzlich für die Bildung von Bernsteinsäure und Mannitol verantwortlich sind. Weiterhin entstehen bei der Fermentation von Wasserkefir einige flüchtige Aromakomponenten, die vergleichbar mit denen bei der Weinbereitung sind. Während fruchtige Ester und wohlriechende Alkohole wünschenswert sind, gelten verzweigtkettige Fettsäuren und Acetoin aufgrund ihres buttrigen, ranzigen Geschmacks eher als Fehlaromen. Der geringe Stickstoffgehalt im Wasserkefirmedium ist ein limitierender Wachstumsfaktor für das Konsortium. Wasserkefirorganismen konkurrieren um die vorhandenen Stickstoffverbindungen, die aus den zugegebenen Feigen resultieren. Der pH-Verlauf, die Zuckerverwertung und die Metabolitproduktion von zwei Konsortien unterschiedlicher Herkunft erwiesen sich als ähnlich. Im Gegensatz dazu konnten durch geänderte Fermentationsbedingungen hinsichtlich Kultivierungstemperatur und Art der zugesetzten Früchte Unterschiede im Geschmack, infolge von Konzentrationenänderungen der gebildeten Metabolite, festgestellt werden. Für ein stabiles, sich reproduzierendes Konsortium mit Granulabildung wurde gezeigt, dass die Fermentation unter den für den Hausgebrauch üblichen Bedingungen notwendig ist. Im Hinblick auf eine industrielle Nutzung von Wasserkefirorganismen zur Produktion eines Getränks auf Basis von Wasserkefir, sollten Isolate verwendet werden, für die eine positive Interaktion beschrieben

ist, dies sichert ein optimales Wachstum und eine aktive Fermentation. Hefen erscheinen hierbei essentiell für den Einsatz als Starterkulturen, sollten aber in geringeren Konzentrationen als Bakterien eingesetzt werden, da sie ansonsten den Fermentationsansatz zu schnell überwachsen können.

Von den 37 untersuchten Wasserkefirisolaten verwerten 17 die im Medium enthaltene Saccharose nicht nur zur Energieproduktion sondern auch zur Bildung von EPS. Lediglich einer dieser Stämme produzierte ein Fructan, die anderen konnten als Produzenten von Glucan identifiziert werden. Granula-EPS besteht ebenso aus Glucoseeinheiten, daher ist es wahrscheinlich, dass diese Glucan-produzierenden Isolate einen wichtigen Einfluss auf die Granulabildung haben. Pidoux (1989) und Waldherr et al. (2010) identifizierten *Lb. hilgardii* als die wichtigste Spezies der Granulaproduktion in Wasserkefir. Wasserkefirkonsortien, die in dieser Arbeit verwendet wurden, enthielten nur einen geringen Anteil an *Lb. hilgardii*, demnach ist anzunehmen, dass eine metabolische Einheit zur Granulaproduktion wichtiger erscheint als der Einfluss einzelner Spezies. Während die Wasserkefir-Granula unlöslich in Wasser sind, konnte gezeigt werden, dass die isolierten EPS der einzelnen Isolate wasserlöslich in Abhängigkeit der eingesetzten Konzentrationen sind. Für drei *Lc. mesenteroides* Stämme wurde das Gen für die Bildung einer Glucansucrase, welche die Produktion eines unlöslichen α-D-Glucans katalysiert, nachgewiesen. Dieses Gen wurde ebenfalls in der DNA von WkW gefunden. Was die Stämme bzw. das Konsortium letztendlich zur Produktion eines unlöslichen EPS veranlasst ist weiterhin unklar. NMR Analysen von Granula EPS zeigten die Struktur eines Dextrans mit dem größten Anteil an linearen α(1→6)-Bindungen und 10% α(1→3)-Verknüpfungen.

Weitere Untersuchungen behandelten die Interaktion zwischen den Hauptvertretern der Wasserkefirorganismen in planktonischer Kultur. Das Wasserkefirmedium ist aufgrund seines hohen Zucker- und niedrigen Stickstoffgehalts ein anspruchsvoller Lebensraum für Mikroorganismen. In dieser ökologischen Nische können nur gut angepasste Organismen bzw. Organismen, die sich gut in das Konsortium eingliedern, wachsen und sich symbiotisch mit essentiellen Nährstoffen unterstützen. In einem Modell System wurde der Synergismus zwischen verschiedenen Hefen und Laktobazillen, die in großer Anzahl im Wasserkefir gefunden werden konnten, untersucht. Weiterhin wurde deren Wechselwirkung mit *B. psychraerophilum* analysiert. Die Co-Kultivierung von Hefen und Laktobazillen zeigte einen Wachstumsanstieg aller beteiligten Mikroorganismen. Eine mutualistische Beziehung dieser Wasserkefirisolate konnte demnach aufdeckt werden, wobei der Einfluss von

Z. florentina stärker ist als der von *S. cerevisiae*. Kommensalische Wechselwirkungen konnten dagegen zwischen *B. psychraerophilum* und den Hefen aus Wasserkefir aufgedeckt werden, da die Hefen im Wachstum unterstützt wurden wobei das *Bifidobacterium* unbeeinflusst blieb. Die Mehrproduktion von Milchsäure, Bernsteinsäure und Mannitol, eine gesteigerte Zuckerverwertung sowie die Überexpression von glykolitschen Enzymen belegen eine erhöhte metabolische Aktivität der Mikroorganismen in Co-Kultivierung. *Z. florentina* wurde durch die Absenkung des pH-Wertes des Mediums anhand der Säurebildung von Laktobazillen gefördert. Im Gegenzug werden die Laktobazillen mit essentiellen Nährstoffen, welche von Hefen produziert werden, versorgt. Trophische Interaktionen zwischen *Lb. hordei* und Hefen aus Wasserkefir bestehen aus der Freisetzung von Aminosäuren und Vitamin B_6 durch die Hefen. *Lb. nagelii* wird ebenfalls durch von Hefen abgegebene Aminosäuren unterstützt. Eine genauere Untersuchung der Interaktionen zwischen *Z. florentina* und *Lb. nagelii* konnte zeigen, dass die Hefe in Co-Kultivierung zur Freisetzung von, für *Lb. nagelii* essentielles Arginin, veranlasst wird. Innerhalb der Co-Kultivierungsversuche wird deutlich, dass ein besonderer Synergismus zwischen den Hauptvertretern der Laktobazillen und *Z. florentina* vorherrscht, da sowohl das verstärkte Wachstum, der angeregte Metabolismus als auch die Aggregation der vermischten Zellen zwischen diesen Akteuren besonders ausgeprägt sind. Diese Ergebnisse zeigen eine deutliche Anpassung der Wasserkefirorganismen. Die Wasserkefirorganismen sind demnach für das Leben im Konsortium besonders aufeinander abgestimmt.

REFERENCES

Abbas, C. A. (2006). Production of antioxidants, aromas, colours, flavours, and vitamins by yeasts. In A. Querol & G. H. Fleet (Eds.), *Yeasts in Food and Beverages* (Vol. 184, pp. 285–334). Berlin: Springer.

Allison, M. J. (1978). Production of branched-chain volatile fatty acids by certain anaerobic bacteria. *Applied and Environmental Microbiology*, *35*(5).

Archibald, F. (1986). Manganese: Its aquisition by and function in lactic acid bacteria. *Critical Reviews in Microbiology*, *13*(1).

Archibald, F. S., & Duong, M. N. (1984). Manganese acquisition by Lactobacillus plantarum. *Journal of bacteriology*, *158*(1), 1–8.

Babayan, T. L., & Bezrukov, M. G. (1985). Autolysis in yeasts. *Acta Biotechnologica*, *5*(2), 129–136.

Ballongue, J. (1993). Bifidobacteria and probiotic action. In S. Salminen & A. Wright (Eds.), *Lactic acid bacteria* (pp. 357–428). New York: Dekker, Marc.

Barnett, J. a, & Entian, K.-D. (2005). A history of research on yeasts 9: regulation of sugar metabolism. *Yeast (Chichester, England)*, *22*(11), 835–894.

Bartóak, T., Szalai, G., Lőrincz, Z. S., Bőurcsök, G., & Sági, F. (1994). High-Speed RP-HPLC/FL analysis of amino acids after automated two-step derivatization with o-phthaldialdehyde/3-mercaptopropionic acid and 9-fluorenylmethyl chloroformate. *Journal of Liquid Chromatography*, *17*(20), 4391–4403.

Belitz, H.-D., Grosch, W., & Schieberle, P. (2001). Kohlenhydrate. *Lehrbuch der Lebensmittelchemie* (5th ed.). Berlin: Springer-Verlag.

Blum, H., Beier, H., & Gross, H. J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis*, *8*(2), 93–99.

Brandina, I., Graham, J., Lemaitre-Guillier, C., Entelis, N., Krasheninnikov, I., Sweetlove, L., Tarassov, I., et al. (2006). Enolase takes part in a macromolecular complex associated to mitochondria in yeast. *Biochimica et Biophysica Acta*, *1757*(9-10), 1217–1228.

Carrau, F. M., Medina, K., Farina, L., Boido, E., Henschke, P. a, & Dellacassa, E. (2008). Production of fermentation aroma compounds by Saccharomyces cerevisiae wine yeasts: effects of yeast assimilable nitrogen on two model strains. *FEMS yeast research*, *8*(7), 1196–1207.

Cerning, J. (1990). Exocellular polysaccharides produced by lactic acid bacteria. *FEMS Microbiology Reviews*, *87*, 113–130.

Cesoniene, L., Jasutiene, I., & Sarkinas, I. (2009). Phenolics and anthocyanins in berries of European cranberry and their antimicrobial activity. *Medicina (Kaunas)*, *45*(12), 992–999.

Challinor, S., & Rose, A. (1954). Interrelationships between a yeast and a bacterium when growing together in defined medium. *Nature (London)*, *174*, 877–878.

Cheirsilp, B., Shimizu, H., & Shioya, S. (2003). Enhanced kefiran production by mixed culture of Lactobacillus kefiranofaciens and Saccharomyces cerevisiae. *Journal of biotechnology*, *100*(1), 43–53.

Cheirsilp, B., Shoji, H., Shimizu, H., & Shioya, S. (2003). Interactions between Lactobacillus kefiranofaciens and Saccharomyces cerevisiae in mixed culture for kefiran production. *Journal of bioscience and bioengineering*, *96*(3), 279–84.

Chick, H., Shin, H. S., & Ustunol, Z. (2001). Growth and acid production by lactic acid bacteria and bifidobacteria grown in skim milk containing honey. *Journal of Food Science*, *66*(3), 478–481.

Collado, M. C., Hernandez, M., & Sanz, Y. (2005). Production of Bacteriocin-like inhibitory compounds by human fecal Bifidobacterium strains. *Journal of Food Protection*, *68*(5), 1034–1040.

Commichau, F. M., Rothe, F. M., Herzberg, C., Wagner, E., Hellwig, D., Lehnik-Habrink, M., Hammer, E., et al. (2009). Novel activities of glycolytic enzymes in Bacillus subtilis: interactions with essential proteins involved in mRNA processing. *Molecular & Cellular Proteomics*, *8*(6), 1350–1360.

Costello, P. J., Siebert, T. E., Solomon, M. R., & Bartowsky, E. J. (2013). Synthesis of fruity ethyl esters by acyl coenzyme A: alcohol acyltransferase and reverse esterase activities in Oenococcus oeni and Lactobacillus plantarum. *Journal of Applied Microbiology*, *114*(3), 797–806.

Côté, G. L., & Skory, C. D. (2012). Cloning, expression, and characterization of an insoluble glucan-producing glucansucrase from Leuconostoc mesenteroides NRRL B-1118. *Applied microbiology and biotechnology*, *93*(6), 2387–94.

Courtin, P., & Rul, F. (2003). Interactions between microorganisms in a simple ecosystem: yogurt bacteria as a study model. *Le Lait*, *84*, 125–134.

Damiani, P., Gobbetti, M., & Cossignani, L. (1996). The sourdough microflora. Characterization of hetero-and homofermentative lactic acid bacteria, yeasts and their interactions on the basis of the volatile compounds. *LWT-Food Science and Technology*, *70*, 63–70.

Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W., & Greenberg, E. P. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science*, *280*(5361), 295–298.

De Bary, H. A. (1879). *Die Erscheinung der Symbiose* (Vol. 8). Strasbourg: Karl J. Trubner.

De Klerk, J. L. (2010). *Succinic acid production by wine yeasts*. Stellenbosch University.

De Vries, W., & Stouthamer, A. H. (1967). Pathway of glucose fermentation in relation to the taxonomy of Bifidobacteria. *Journal of Bacteriology*, *93*(2), 574–576.

De Vries, W., & Stouthamer, A. H. (1968). Fermentation of glucose , lactose , galactose , mannitol and xylose by Bifidobacteria. *Journal of Bacteriology*, *96*(2), 472–478.

De Vuyst, L, & Degeest, B. (1999). Heteropolysaccharides from lactic acid bacteria. *FEMS microbiology reviews*, *23*(2), 153–177.

De Vuyst, Luc, & Neysens, P. (2005). The sourdough microflora: biodiversity and metabolic interactions. *Trends in Food Science & Technology*, *16*(1-3), 43–56.

Dickinson, J. R., & Kruckeberg, A. L. (2006). Carbohydrate Metabolism. In A Querol & G. H. Fleet (Eds.), *Yeasts in Food and Beverages* (pp. 215–242). Berlin.

Dobson, A., O'Sullivan, O., Cotter, P. D., Ross, P., & Hill, C. (2011). High-throughput sequence-based analysis of the bacterial composition of kefir and an associated kefir grain. *FEMS microbiology letters*, *320*(1), 56–62. doi:10.1111/j.1574-6968.2011.02290.x

Doenecke, D., Koolman, J., Fuchs, G., & Gerok, W. (2005). *Karlsons Biochemie und Pathobiochemie* (15th ed., pp. 245–250). Stuttgart: Georg Thieme Verlag.

Engels, W. J. M., Dekker, R., Jongb, C. De, Neeter, R., & Visser, S. (1997). A comparative study of volatile compounds in the water-soluble fraction of various types of ripened cheese. *International dairy journal*, *6946*(97), 255–263.

Entelis, N., Brandina, I., Kamenski, P., Krasheninnikov, I. a, Martin, R. P., & Tarassov, I. (2006). A glycolytic enzyme, enolase, is recruited as a cofactor of tRNA targeting toward mitochondria in Saccharomyces cerevisiae. *Genes & Development*, *20*(12), 1609–1620.

Etschmann, M., Bluemke, W., Sell, D., & Schrader, J. (2002). Biotechnological production of 2-phenylethanol. *Applied Microbiology and Biotechnology*, *59*(1), 1–8.

Etschmann, M. M. W., Sell, D., & Schrader, J. (2003). Screening of yeasts for the production of the aroma compound 2-phenylethanol in a molasses-based medium. *Biotechnology Letters*, *25*(7), 531–536.

Farnworth, E. (2005). Kefir - a complex probiotic. *Food Science and Technology*, *2*, 1–17.

Feldmann, H. (2005). Yeast metabolism. *Yeast molecular biology*. München: Adolf-Butenandt-Institut.

Fiil, A., & Branton, D. (1969). Changes in the plasma membrane of Escherichia coli during magnesium starvation. *Journal of Bacteriology*, *98*(3).

Franzetti, L., Galli, A., Pagani, M. A., & De Noni, L. (1998). Microbiological and chemical investigations on “Sugar Kefir” drink. *Annali di Microbiologia ed Enzimologia*, *48*, 67–80.

Fredrickson, A. G. (1977). Behavior of mixed cultures of microorganisms. *Annual reviews of Microbiology*, *31*, 63–87.

Frey-Klett, P., Burlinson, P., Deveau, A., Barret, M., Tarkka, M., & Sarniguet, A. (2011). Bacterial-fungal interactions: hyphens between agricultural, clinical, environmental, and food microbiologists. *Microbiology and molecular biology reviews : MMBR*, *75*(4), 583–609.

Gadaga, T. H., Mutukumira, a N., & Narvhus, J. a. (2001). The growth and interaction of yeasts and lactic acid bacteria isolated from Zimbabwean naturally fermented milk in UHT milk. *International journal of food microbiology*, *68*(1-2), 21–32.

Gancedo, C., & Holzer, H. (1968). Enzymatic inactivation of glutamine synthetase in Enterobacteriaceae. *European Journal of Biochemistry*, *4*(2), 190–192.

Gancedo, C., & Serrano, R. (1989). Energy-Yielding Metablism. In A. Rose & J. Harrison (Eds.), *The yeasts volume 3* (2nd ed., pp. 205–259). London.

Gobbetti, M., Corsetti, A., & Rossi, J. (1994a). The sourdough microflora. Interactions between lactic acid bacteria and yeasts: metabolism of amino acids. *World Journal of Microbiology and Biotechnology*, *10*, 275–279.

Gobbetti, M., Corsetti, A., & Rossi, J. (1994b). The sourdough microflora. Interactions between lactic acid bacteria and yeasts: metabolism of carbohydrates. *Applied Microbiology and Biotechnology*, 456–460.

Görtz, H. D. (1988). *Formen des Zusammenlebens* (pp. 1–4). Darmstadt: Wissenschaftliche Buchgesellschaft.

Goyal, R. K. (1999). Biochemistry of Fermentation. In V. K. Joshi & A. Pandey (Eds.), *Biotechnology: Food Fermentation Volume 1* (Vol. 99). New Delhi.

Groleau, D., Chevalier, P., & Hing Yuen, T. (1995). Production of polyols and ethanol by the osmophilic yeast Zygosaccharomyces rouxii. *Biotechnology letters*, *17*(3), 315–320.

Gulitz, A. (2013). *Analysis of the diversity of water kefir microbiota by culture-dependent and -independent approaches*. Technische Universität München.

Gulitz, A., Stadie, J., Ehrmann, M. A., Ludwig, W., & Vogel, R. F. (2013). Comparative phylobiomic analysis of the bacterial community of water kefir by 16S rRNA gene amplicon sequencing and ARDRA analysis. *Journal of Applied Microbiology*, 1–10.

Gulitz, A., Stadie, J., Wenning, M., Ehrmann, M. A., & Vogel, R. F. (2011). The microbial diversity of water kefir. *International Journal of Food Microbiology*, *151*(3), 284–8.

Gullo, M., & Giudici, P. (2008). Acetic acid bacteria in traditional balsamic vinegar: Phenotypic traits relevant for starter cultures selection. *International Journal of Food Microbiology*, *125*(1), 46–53. doi:10.1016/j.ijfoodmicro.2007.11.076

Hebert, E. M., Raya, R. R., & De Giori, G. S. (2000). Nutritional requirements and nitrogen-dependent regulation of proteinase activity of Lactobacillus helveticus CRL 1062. *Applied and Environmental Microbiology*, *66*(12), 5316–21.

Henderson, B., & Martin, A. (2011). Bacterial virulence in the moonlight: multitasking bacterial moonlighting proteins are virulence determinants in infectious disease. *Infection and Immunitymmunity*, *79*(9), 3476–3491.

Herve-Jimenez, L., Guillouard, I., Guedon, E., Boudebbouze, S., Hols, P., Monnet, V., Maguin, E., et al. (2009). Postgenomic analysis of streptococcus thermophilus cocultivated in milk with Lactobacillus delbrueckii subsp. bulgaricus: involvement of nitrogen, purine, and iron metabolism. *Applied and environmental microbiology*, *75*(7), 2062–2073.

Horisberger, M. (1969). Structure of the dextran of the tibi grain. *Carbohydrate Research*, *10*, 379–385.

Hughes, M. J. G., Moore, J. C., Lane, J. D., Wilson, R., Pribul, P. K., Younes, Z. N., Dobson, R. J., et al. (2002). Identification of major outer surface proteins of Streptococcus agalactiae. *Infection and Immunity*, *70*(3), 1254–1259.

Imai, T., & Ohno, T. (1995). Measurement of yeast intracellular pH by image processing and the change it undergoes during growth phase. *Journal of Biotechnology*, *38*(2), 165–72.

Jakob, F., Pfaff, A., Novoa-Carballal, R., Rübsam, H., Becker, T., & Vogel, R. F. (2013). Structural analysis of fructans produced by acetic acid bacteria reveals a relation to hydrocolloid function. *Carbohydrate Polymers*, *92*(2), 1234–1242.

Jakob, F., Steger, S., & Vogel, R. F. (2012). Influence of novel fructans produced by selected acetic acid bacteria on the volume and texture of wheat breads. *European Food Research and Technology*, *234*(3), 493–499. doi:10.1007/s00217-011-1658-7

Jannière, L., Canceill, D., Suski, C., Kanga, S., Dalmais, B., Lestini, R., Monnier, A.-F., et al. (2007). Genetic evidence for a link between glycolysis and DNA replication. *PloS ONE*, *2*(5), e447.

Jay, J. M. (1992a). Fermented Foods and Related Products of Fermentation. *Modern Food Microbiology* (Vol. 45, pp. 371–409). New York: Van Nostrand Reinhold.

Jay, J. M. (1992b). History of Microorganisms in Food. *Modern Food Microbiology* (4th ed., pp. 3–10). New York: Van Nostrand Reinhold.

Jolly, L., Vincent, S. F., Duboc, P., & Neeser, J.-R. (2002). Exploiting exopolysaccharides from lactic acid bacteria. *Antonie van Leeuwenhoek*, *82*(1-4), 367–374.

Kaditzky, S. B. (2008). *Sucrose metabolism in lactobacilli and bifidobacteria*. Technische Universität München.

Katakura, Y., Sano, R., Hashimoto, T., Ninomiya, K., & Shioya, S. (2010). Lactic acid bacteria display on the cell surface cytosolic proteins that recognize yeast mannan. *Applied Microbiology and Cell Physiology*, *86*(1), 319–326.

Kebler, L. (1921). California bees. *Journal of the Amercian Pharmaceutical Association*, *10*, 939–943.

Kleinberg, I., Craw, D., & Komiyama, K. (1973). Effect of salivary supernatant on the glycolytic activity of the bacteria in salivary sediment. *Archs oral Biology*, *18*, 787–798.

Korakli, M, Gänzle, M. G., & Vogel, R. F. (2002). Metabolism by bifidobacteria and lactic acid bacteria of polysaccharides from wheat and rye, and exopolysaccharides produced by Lactobacillus sanfranciscensis. *Journal of Applied Microbiology*, *92*(5), 958–65.

Korakli, Maher, & Vogel, R. F. (2006). Structure/function relationship of homopolysaccharide producing glycansucrases and therapeutic potential of their synthesised glycans. *Applied microbiology and biotechnology*, *71*(6), 790–803.

Kos, B., Susković, J., Vuković, S., Simpraga, M., Frece, J., & Matosić, S. (2003). Adhesion and aggregation ability of probiotic strain Lactobacillus acidophilus M92. *Journal of Applied Microbiology*, *94*(6), 981–7.

Krämer, J. (2007). *Lebensmittel Mikrobiologie*. *Lebensmittelmikrobiologie* (5th ed.). Stuttgart: Verlag Eugen Ulmer.

Kurtzman, C. (2003). Phylogenetic circumscription of , and other members of the Saccharomycetaceae, and the proposal of the new genera Lachancea, Nakaseomyces, Naumovia, Vanderwaltozyma and Zygotorulaspora. *FEMS Yeast Research*, *4*(3), 233–245.

La Rivière, J. W. M., & Kooiman, P. (1967). Kefiran, a novel polysaccharide produce ub the kefir grain by Lactobacillus brevis. *Archives of Microbiology*, *59*, 269–278.

Lee, J.-K., Song, J.-Y., & Kim, S.-Y. (2003). Controlling substrate concentration in fed-batch candida magnoliae culture increases mannitol production. *Biotechnology Progress*, *19*(3), 768–775.

Leroi, F., & Pidoux, M. (1993a). Detection of interactions between yeasts and lactic acid bacteria isolated from sugary kefir grains. *Journal of Applied Microbiology*, *74*(1), 48–53.

Leroi, F., & Pidoux, M. (1993b). Characterization of interactions between Lactobacillus hilgardii and Saccharomyces florentinus isolated from sugary kefir grains. *Journal of Applied Microbiology*, *74*(1), 54–60.

Lewis, M. J., & Stephanopoulos, D. (1967). Glucose-induced release of amino acids from Saccharomyces carlsbergensis by action on the cytoplasmic membrane. *Journal of Bacteriology*, *93*, 976–984.

Lindquist, S., & Craig, E. a. (1988). The heat-shock proteins. *Annual review of genetics*, *22*, 631–677.

Liu, Shao-Quan, & Tsao, M. (2009). Enhancement of survival of probiotic and non-probiotic lactic acid bacteria by yeasts in fermented milk under non-refrigerated conditions. *International Journal of Food Microbiology*, *135*(1), 34–38.

Liu, S-Q, Holland, R., & Crow, V. L. (2003). Ester synthesis in an aqueous environment by Streptococcus thermophilus and other dairy lactic acid bacteria. *Applied microbiology and biotechnology*, *63*(1), 81–8.

Longo, E., Velazquez, J., Sieiro, C., Cansado, J., Calo, P., & Villa, T. (1992). Production of higher alcohols , ethyl acetate , acetaldehyde and other compounds by 14 Saccharomyces cerevisiae wine strains isolated from the same region (Salnes , N . W . Spain). *World Journal of Microbiology and Biotechnology*, *8*, 539–541.

Lopitz-Otsoa, F., Rementeria, A., Elguezabal, N., & Garaizar, J. (2006). Kefir: A symbiotic yeasts-bacteria community with alleged healthy capabilities. *Revista Iberoamericana de Micologia*, *23*, 67–74.

Lüttge, U., Kluge, M., & Bauer, G. (2005). Ernährungsphysiologische Besonderheiten: Symbiose, Parasitismus, Carnivore. *Botanik* (5th ed., pp. 495–516). Weinheim: WILEY-VCH Verlag GmbH.

Lutz, M. L. (1899). Recherches biologiques sur la constitution du Tibi. *Bulletin de la Societe Mycologique de France*, *15*, 68–72.

Macfarlane, G. T., & Englyst, H. N. (1986). Starch utilization by the human large intestinal microflora. *Journal of Applied Microbiology*, *60*(3), 195–201. doi:10.1111/j.1365-2672.1986.tb01073.x

Mauricio, J. C., Ortega, J. M., Plata, C., & Mill, C. (2003). Formation of ethyl acetate and isoamyl acetate by various species of wine yeasts. *Food Microbiology*, *20*, 217–224.

Mlobeli, N. T., Gutierrez, N. a., & Maddox, I. S. (1998). Physiology and kinetics of Bifidobacterium bifidum during growth on different sugars. *Applied Microbiology and Biotechnology*, *50*, 125–128.

Moinas, M., Horisberger, M., & Bauer, H. (1980). The structural organization of the Tibi grain as revealed by light, scanning and transmission microscopy. *Archives of Microbiology*, *128*, 157–161.

Müller, G., & Reuter, D. (1968). Zur Mikrobiologie des Zuckers. *Food / Nahrung*, *12*(1), 115–127.

Neve, H., & Heller, K. J. (2002). The microflora of water kefir: a glance by scanning electron microscopy. *Kieler Milchwirtschaftliche Forschungsberichte*, *54*, 1337–349.

Parsek, M. R., & Greenberg, E. P. (2005). Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends in Microbiology*, *13*(1), 27–33.

Pidoux, M. (1989). The microbial flora of sugary kefir grain (the gingerbeer plant): biosynthesis of the grain from Lactobacillus hilgardii producing a polysaccharide gel. *MIRCEN Journal*, *5*, 223–238.

Pidoux, M., Brillouet, J., & Quemener, B. (1988). Characterization of the polysaccharides from a Lactobacillus brevis and from sugary kefir grains. *Biotechnology letters*, *10*(6), 415–420.

Pokusaeva, K., Fitzgerald, G. F., & Van Sinderen, D. (2011). Carbohydrate metabolism in Bifidobacteria. *Genes & nutrition*, *6*(3), 285–306.

Puupponen-Pimiä, R., Nohynek, L., Alakomi, H.-L., & Oksman-Caldentey, K.-M. (2005). Bioactive berry compounds—novel tools against human pathogens. *Applied Microbiology and Biotechnology*, *67*(1), 8–18.

Rastall, R. a, Gibson, G. R., Gill, H. S., Guarner, F., Klaenhammer, T. R., Pot, B., Reid, G., et al. (2005). Modulation of the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human health: an overview of enabling science and potential applications. *FEMS microbiology ecology*, *52*(2), 145–152. doi:10.1016/j.femsec.2005.01.003

Reiß, J. (1990). Metabolic activity of Tibi grains. *Zeitschrift für Lebensmitteluntersuchung und Forschung*, *191*(6), 462–465.

Rhodes, D., Rendon, G. A., & Stewart, G. R. (1975). The control of glutamine synthetase level in Lemna minor L. *Planta*, *125*(3), 201–211.

Rice, K. C., & Bayles, K. W. (2008). Molecular control of bacterial death and lysis. *Microbiology and Molecular Biology reviews*, *72*(1), 85–109, table of contents.

Romano, P., Suzzi, G., Biologia, D., & Agro-forestali, D. B. (1996). Origin and Production of Acetoin during Wine Yeast Fermentation. *Applied and Environmental Microbiology*, *62*(2), 309–315.

Russel, R. R. B. (2009). Bacterial polysaccharides in dental plaque. In M. Ullrich (Ed.), *Bacterial Polysaccharides*. Norfolk: Caister Academic Press.

Sadykov, M. R., & Bayles, K. W. (2012). The control of death and lysis in staphylococcal biofilms: a coordination of physiological signals. *Current opinion in microbiology*, *15*(2), 211–5.

Saguir, F. M., Loto Campos, I. E., & Manca de Nadra, M. C. (2008). Utilization of amino acids and dipeptides by Lactobacillus plantarum from orange in nutritionally stressed conditions. *Journal of Applied Microbiology*, *104*(6), 1597–1604.

Saha, B. C., & Racine, F. M. (2011). Biotechnological production of mannitol and its applications. *Applied Microbiology and Biotechnology*, *89*(4), 879–91.

Scherz, H., & Senser, F. (1999). *Die Zusammensetzung der Lebensmittel*. (S. W. Souci, W. Fachmann, & H. Kraut, Eds.) (5th ed., Vol. 99). medpharm.

Seymour, F. R., Knapp, R. D., & Bishop, S. H. (1976). Determination of the structure of dextran by 13C-nuclear magnetic resonance spectroscopy. *Carbohydrate Research*, *51*, 179–194.

Shukla, R., Shukla, S., Bivolarski, V., Iliev, I., Ivanova, I., & Goyal, A. (2011). Structural characterization of insoluble dextranpProduced by Leuconostoc mesenteroides NRRL B-1149 in the presence of maltose. *Food Technology and Biotechnology*, *49*(3), 291–296.

Simpson, P. J., Ross, R. P., Fitzgerald, G. F., & Stanton, C. (2004). Bifidobacterium psychraerophilum sp. nov. and Aeriscardovia aeriphila gen. nov., sp. nov., isolated from a porcine caecum. *International Journal of Systematic and Evolutionary Microbiology*, *54*(2), 401–406.

Sinz, Q. (2011). *Aminosäure- und Peptidstoffwechsel von Lactobacillus sakei : Analytische und biochemische Untersuchungen zur Bildung von aromarelevanten Verbindungen*. Technische Universität München.

Stadelmann, E. (1957). Die Symbiose Tibi. *Bull Soc Fibourgeoise Sci Nat*, *47*, 16–19.

Stadie, J., Gulitz, A., Ehrmann, M. A., & Vogel, R. F. (2013). Metabolic activity and symbiotic interactions of lactic acid bacteria and yeasts isolated from water kefir. *Food Microbiology*, *35*(2), 92–98.

Stolz, P, Böcker, G., Hammes, W. P., & Vogel, R. F. (1995). Utilization of electron acceptors by lactobacilli isolated from sourdough. I. Lactobacillus sanfransiscensis. *Zeitschrift für Lebensmittel Untersuchung und Forschung*, *201*, 91–96.

Stolz, Peter, Vogel, R. F., & Hammes, W. P. (1995). Utilization of electron acceptors by lactobacilli isolated from sourdough. II. Lactobacillus pontis, L. reuteri, L. amylovorus and L. fermentum. *Zeitschrift für Lebensmittel-Untersuchung und Forschung*, *201*, 402–410.

Thierry, A., Maillard, M., & Yvon, M. (2002). Conversion of i-leucine to isovaleric acid by Propionibacterium freudenreichii TL 34 and ITGP23. *Applied and Environmental Microbiology*, *68*(2).

Thomas, K. C., Hynes, S. H., & Ingledew, W. M. (2002). Influence of medium buffering capacity on inhibition of Saccharomyces cerevisiae growth by acetic and lactic acids. *Applied and Environmental Microbiology*, *68*(4).

Van Geel-Schutten, G. H., Faber, E. J., Smit, E., Bonting, K., Smith, R., Brink, B. Ten, Kamerling, J. P., 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. *Applied and Environmental Microbiology*, *65*(7), 3008–3014.

Von Weymarn, N., Hujanen, M., & Leisola, M. (2002). Production of d-mannitol by heterofermentative lactic acid bacteria. *Process Biochemistry*, *37*(11), 1207–1213.

Vosti, D. C., & Joslyn, M. a. (1954). Autolysis of several pure culture yeasts. *Applied and Environmental Microbiology*, *1*, 79–84.

Waldherr, Florian W, Doll, V. M., Meißner, D., & Vogel, R. F. (2010). Identification and characterization of a glucan-producing enzyme from Lactobacillus hilgardii TMW 1.828 involved in granule formation of water kefir. *Food Microbiology*, *27*(5), 672–678.

Waldherr, Florian Wolfgang. (2009). *Comparative analysis of fructosyltransferases of lactobacilli*. TU München.

Ward, M. (1892). The ginger-beer plant, and the organisms composing it: a contribution to the study of fermentation yeasts and bacteria. *Philosophical Transactions of the Royal Society of London*, *183*, 125–197.

Warth, A. D. (1988). Effect of benzoic acid on growth yield of yeasts differing in their resistance to preservatives. *Applied and Environmental Microbiology*, *54*(8).

Werner, D. (1987). Die Rhizobium/Bradyrhizobium-Fabales-Symbiose. *Pflanzliche und mikrobielle Symbiosen* (pp. 31–112). Stuttgart: Georg Thieme Verlag.

Yokoi, H., Watanabe, T., Fujii, Y., Mukai, T., Toba, T., & Adachi, S. (1991). Some taxonomical characteristics of encapsulated Lactobacillus sp. KPB-167B isolated from kefir grains and characterization of its extracellular polysaccharide. *International Journal of Food Microbiology*, *13*(4), 257–264.

Zourari, A., Accolas, J., & Desmazeaud, M. (1992). Metabolism and biochemical characteristics of yogurt bacteria. A review. *Le lait*, 1–34.

APPENDIX

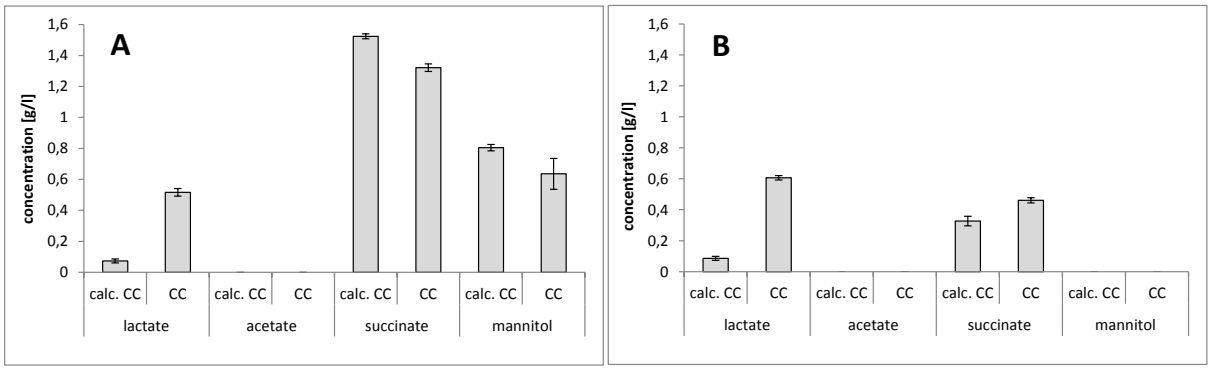


Fig. 39: Metabolite concentrations after 72 h fermentation of *Lb. hordei* in co-cultivation (CC) and as calculated co-culture (calc. CC) with *Z. florentina* (A) and *S. cerevisiae* (B), respectively

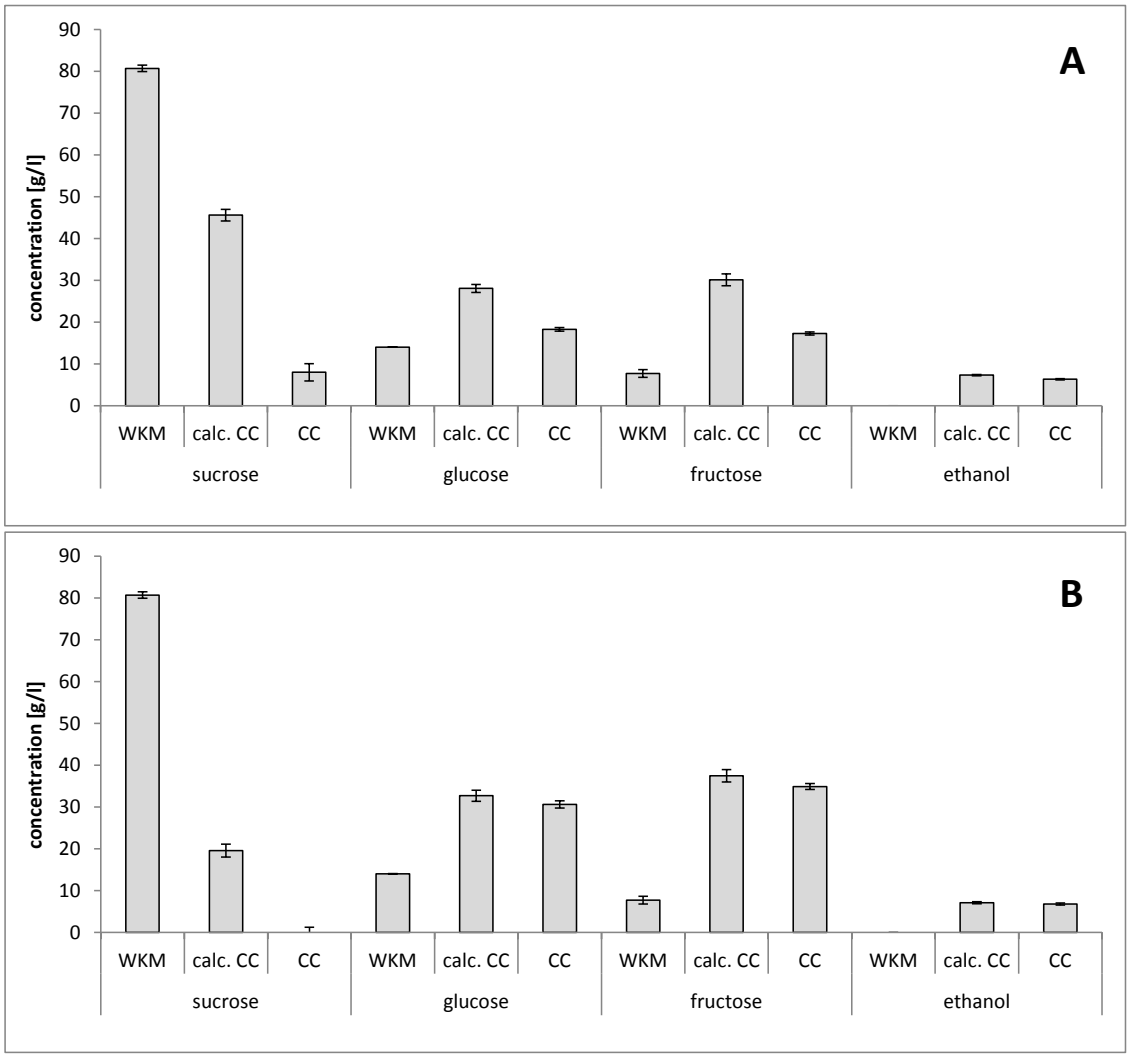


Fig. 40: Sugar and ethanol concentrations after 72 h fermentation of *Lb. hordei* in co-cultivation (CC) and as calculated co-culture (calc. CC) with *Z. florentina* (A) and *S. cerevisiae* (B), respectively

Table 34: Sugar and metabolite concentration during mixed- and calculated mixed-cultivation of *Lb. hordei* and *Z. florentina* in WKM

time [h]	sucrose [g/l per OD 1]				glucose [g/l per OD 1]				fructose [g/l per OD 1]				mannitol [g/l per OD 1]			
	cMC*		MC**		cMC		MC		cMC		MC		cMC		MC	
	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD
0	83.2	0.7	83.2	0.7	4.6	0.4	4.6	0.4	4.3	0.4	4.3	0.4	< 0.04		< 0.04	
24	53.4	0.7	49.2	1.2	14.9	0.1	16.5	0.7	18.6	1.0	19.4	0.5	0.31	0.01	0.24	0.01
48	24.5	3.0	21.3	2.1	24.1	0.3	25.8	0.6	32.2	1.5	30.8	0.6	0.41	0.02	0.38	0.03
72	< 0.07		< 0.07		31.8	0.5	30.7	0.5	41.3	1.4	37.7	0.7	0.54	0.01	0.48	0.02

time [h]	lactate [g/l per OD 1]				acetate [g/l per OD 1]				succinate [g/l per OD 1]				ethanol [g/l per OD 1]			
	cMC		MC		cMC		MC		cMC		MC		cMC		MC	
	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD
0	< 0.02		< 0.02		< 0.01		< 0.01		< 0.03		< 0.03		< 0.06		< 0.06	
24	1.17	0.02	1.25	0.06	< 0.01		< 0.01		0.76	0.02	0.73	0.04	2.73	1.03	2.47	0.15
48	1.75	0.28	1.85	0.02	< 0.01		< 0.01		1.11	0.03	0.96	0.03	5.04	0.18	5.73	0.04
72	2.17	0.14	2.34	0.03	< 0.01		< 0.01		1.35	0.11	1.15	0.06	8.15	0.45	9.28	0.11

* **c**alculated **M**ixed-**C**ulture fermentation ** **M**ixed-**C**ulture fermentation

Sugar consumption and metabolite production of different co-cultivation systems in comparison to the pooled single culture values, declared as “calculated co-culture”, of the individual organisms. All values are referred to a specific cell concentration (OD 1).

Table 35: Sugar and metabolite concentration during mixed- and calculated mixed-cultivation of *B. psychraerophilum* and *Z. florentina* in WKM

time [h]	sucrose [g/l per OD 1]				glucose [g/l per OD 1]				fructose [g/l per OD 1]				mannitol [g/l per OD 1]			
	cMC*		MC**		cMC		MC		cMC		MC		cMC		MC	
	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD
0	83.6	0.9	83.6	0.9	5.3	0.6	5.3	0.6	2.8	0.0	2.8	0.0	< 0.04		< 0.04	
24	40.9	1.9	46.4	0.3	16.6	3.4	16.6	0.6	14.3	0.8	8.9	0.4	1.37	0.04	1.57	0.09
48	12.8	1.8	12.0	0.3	31.2	1.5	28.8	1.7	29.0	1.7	19.0	1.1	1.29	0.07	1.59	0.09
72	< 0.07	2,7	< 0.07	1.5	38.4	0.7	34.2	0.3	38.3	4.5	26.6	1.2	1.22	0.25	1.39	0.09

time [h]	lactate [g/l per OD 1]				acetate [g/l per OD 1]				succinate [g/l per OD 1]				ethanol [g/l per OD 1]			
	cMC		MC		cMC		MC		cMC		MC		cMC		MC	
	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD	mean	StD
0	< 0.02		< 0.02		< 0.01		< 0.01		< 0.03		< 0.03		< 0.06		< 0.06	
24	0.49	0.00	0.25	0.02	0.43	0.00	0.19	0.02	0.78	0.00	0.73	0.04	3.57	0.07	3.92	0.03
48	0.71	0.03	0.45	0.03	0.66	0.04	0.30	0.02	1.06	0.04	0.96	0.03	7.58	0.46	8.99	0.67
72	1.03	0.07	0.55	0.03	0.87	0.06	0.35	0.02	1.25	0.02	1.15	0.06	11.19	0.08	13.58	0.38

* **c**alculated **M**ixed-**C**ulture fermentation ** **M**ixed-**C**ulture fermentation

Sugar consumption and metabolite production of different co-cultivation systems in comparison to the pooled single culture values, declared as “calculated co-culture”, of the individual organisms. All values are referred to a specific cell concentration (OD 1).