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Analysis of the diversity of water kefir microbiota by culture-dependent and -independent approaches

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List of abbreviations

Ac.	*Acetobacter*
ARDRA	amplified ribosomal DNA restriction analysis
AE	apricot extract
ATP	adenosintriphosphate
B.	*Bifidobacterium*
BC	Before Christ
BLAST	basic local alignment search tool
Bp	base pair
C.	*Clostridium*
CE	cranberry extract
Cfu	colony forming unit
Cy5	hydrophilic sulphoindocyanine dye
DDBJ	DNA Data Base of Japan
DGGE	denaturating gradient gel electrophoresis
DNA	desoxyribonucleic acid
DSM	Deutsche Sammlung von Mikroorganismen
E.	*Escherichia*
EDTA	ethylenediaminetetraacetic acid
EPS	exopolysaccharide
FISH	fluorescent in situ hybridization
FLUOS	5(6)-carboxyfluorescein-N-hydroxysuccinimde ester
FTIR	Fourier transform infrared spectroscopy
G.	*Gluconobacter*
G	gram
GM	*Gluconobacter* Medium

h	hour
HGC probe	probe for bacteria with high GC content in DNA
IPTG	isopropyl ß-D-1-thiogalactopyranoside
ITS	internal transcribed spacer
l	liter
LAB	lactic acid bacteria
Lb.	*Lactobacillus*
Lc.	*Leuconostoc*
Lcc.	*Lactococcus*
M	mega (10^6), molar
MALDI-TOF MS	matrix-assisted laser desorption/ ionization time of flight mass spectrometry
min	minute
NCBI	National center for Biotechnology Information
NGS	next generation sequencing
nd	not detectable
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RAPD	random amplified polymorphic DNA analysis
RDP	Ribocomal Database Project
RISA	ribosomal intergenic spacer analysis
rRNA	ribosomal ribonucleic acid
s	second
S.	*Saccharomyces*
TE	Tris, EDTA

TEMED	tetramethylethylendiamin
TMW	Technische Mikrobiologie Weihenstephan
TRFLP	terminal restriction fragment length polymorphism
Tris	tris (hydroxy) aminomethan
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
V	volt
v/ v	volume/ volume
w/ v	weight/ volume
Wk	water kefir
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
µ	micro
Z.	*Zygotorulaspora*

1 Introduction

1.1 Water kefir

1.1.1 Origin of water kefir and traditional preparation

Water kefir is a homemade fermented beverage based on a sucrose solution with different dried and fresh fruits. The origin of water kefir remains unclear. There are some descriptions of similar grains called “gingerbeer plants”, that English soldiers brought back from the Crimean war in 1855 (Ward, 1892) or “Tibi grains” (Lutz, 1899), that are known to originate from a Mexican cactus (*Optunia*) where they were taken off the leaves. The natives already used them for preparing a beverage. Also other names are collected by Kebler: e.g. “California bees”, “African bees”, “Ale nuts”, “Balm of Gilead” and “Japanese Beer Seeds” (Kebler, 1921). Pidoux called them “Sugary kefir grains” in order to differentiate them from the grains used for fermenting milk (Pidoux, 1989; Pidoux et al., 1990). The different speculations of the origin of “water kefir” indicates that they could not be attributed to one specific source of this special microbiota (Lutz, 1899).

In the traditional household process of kefir preparation, the kefir grains are put into a solution containing 8 % sucrose, different dried fruits and some slices of lemon. Typically dried figs are used but plums, apricot, cranberry and raisins could also be added. Fermentation induced by water kefir grains for one or two days at room temperature results in a cloudy, carbonated and straw coloured drink, which is acidic, poor in sugar and slightly alcoholic. The water kefir grains are removed by filtration and could be used for the next fermentation with fresh medium, the supernatant is qualified for consumption (see Figure 1). The consumption of water kefir is

anticipated with health beneficial effects but this has not scientifically been allocated yet.

Figure 1: Components for the preparation of water kefir.
On the left side sugar, figs, water kefir grains, lemon slices and water could be seen and on the right side the straw coloured beverage after two days of fermentation is shown.

1.1.2 Microbiota and appearance of water kefir

Generally, the microbiota of water kefir have been described to contain different lactic acid bacteria, acetic acid bacteria and yeasts in a stable symbiotic system (Franzetti et al., 1998; Galli et al., 1995; Horisberger, 1969; Lutz, 1899; Neve and Heller, 2002; Pidoux, 1989; Ward, 1892). The water kefir grains are used to initiate the fermentation. The grains show irregular dimensions ranging from few millimeters to centimeters, the consistency is inelastic and fragile (Franzetti et al., 1998). The appearance is whitish to translucent with a cauliflower/crystal-like shape. The grains increase in size during the fermentation and divide so that they multiply in large quantities. The cleavage of the grains could be caused due to bubbles of carbon dioxide (Reiß, 1990). Under identical growth conditions regular fermentation appears over a long time period (Moinas, 1980).

In the literature describing the composition of the microbiota of water kefir, the isolation and reliable identification of species has been rare (Waldherr et al., 2010). There are only few data available where the water kefir consortia were analyzed and different microorganisms were identified, but the occurrence of single species and

their percentages in the consortia were not determined. Bacteria and yeast species isolated from the water kefir microbiota before this work are shown in Table 1. The molecular background for the formation of a stable consortium is unknown and the comprehensive composition of the microbiota is not scientifically defined yet.

Table 1: Organisms isolated from water kefir (Waldherr et al., 2010)

species	literature
Bacteria	
Lb. brevis	Moinas et al., 1980
Lb. hilgardii	Pidoux, 1989; Waldherr et al., 2010
Lb. casei subsp. *casei*	Pidoux, 1989; Galli et al., 1995
Lb. casei subsp. *rhamnosus*	Pidoux 1989
Lb. casei subsp. *pseudoplantarum*	Galli et al., 1995
Lb. plantarum	Pidoux, 1989
Lb. buchneri	Galli et al., 1995
Lb. fructiovorans	Galli et al., 1995
Lb. collinoides	Galli et al., 1995
Lcc. lactis supbsp. *lactis*	Moinas et al., 1980; Pidoux, 1989; Waldherr et al., 2010
Lcc. lactis supbsp. *cremoris*	Pidoux, 1989
Lc. mesenteroides subsp. *mesenteroides*	Galli et al., 1995; Waldherr et al., 2010
Lc. mesenteroides subsp. *dextranicum*	Pidoux, 1989
Enterobacter hormachei	Waldherr et al., 2010
Gb. frateuri	Waldherr et al., 2010
Yeasts	
Saccharomyces bayanus	Waldherr et al., 2010
Saccharomyces cerevisiae	Moinas et al., 1980; Galli et al., 1995; Franzetti et al., 1998
Saccharomyces pretoriensis	Galli et al., 1995
Zygosaccharomyces florentinus	Pidoux, 1989; Neve and Heller, 2001, Galli et al., 1995
Hanseniaspora valbyensis	Pidoux, 1989; Galli et al., 1995; Neve and Heller, 2001
Hanseniaspora vinae	Pidoux, 1989; Galli et al., 1995
Hanseniaspora yalbensis	Franzetti et al., 1998
Kloeckera apiculata	Pidoux, 1989; Franzetti et al., 1998
Candida lambica	Pidoux, 1989
Candida valida	Pidoux, 1989

1.1.3 Composition of water kefir grains

The unique structure of the water kefir grain shows a gelatinous consistency and they are described to contain dextran, an α 1-6 linked glucose polymer (Galli et al., 1995, Horisberger, 1969, Pidoux, 1989). *Lactobacillus* (*Lb*.) *hilgardii* was described by Pidoux et al., 1988 as an important organism for the stability of the water kefir grain by producing the polysaccharide dextran. Dextran is a sugar consisting of different amounts of the subunit glucose. Horisberger described the dextran produced by “tibi grains” as a single insoluble polysaccharide containing only D-glucose (Horisberger, 1969). The strain *Lb. hilgardii* isolated from water kefir by Waldherr was described as a granule-forming bacterium by producing large amounts of dextran (Waldherr et al., 2010).

1.1.4 Comparison to milk kefir

Water kefir is not studied in detail like other food fermentations. The microbiota of milk kefir is an example for a better characterized community and could be compared to the symbiosis of water kefir. Milk kefir is a beverage of fermented milk, which results in a carbonated slightly alcoholic and sour taste with a creamy consistency (Kok-Tas et al., 2013; Lopitz-Otsoa et al., 2006). A lot of investigations have been made to analyze milk kefir reviewed by Lopitz-Otsoa et al., 2006. The origin of milk kefir is also not defined but believed that it was found in the Caucasian mountains. Milk kefir is also known under different names like kephir, kiaphur, kefer, knapon, kepi and kippi (Farnworth, 2005). Milk kefir also is a natural starter culture for producing kefir in widespread countries like Argentina, Taiwan, Portugal, Turkey and France. Like water kefir it is improbable that the milk kefir originates from one single original starter culture as microbial investigations of milk kefirs taken from several sample sides showed differences in their microbiota (Farnworth, 2005).

The milk kefir grains share some characteristics with the water kefir grains as they also show gelatinous grain structure which consist of a various mixture of microorganisms mostly lactobacilli, lactococci and yeasts casually acetic acid bacteria and bifidobacteria (Dobson et al., 2011). Under traditional treatment of the milk kefir the starter grain could be used again after approximately 24h of fermentation in milk. The milk is then removed and appropriate for consumption (Lopitz-Otsoa et al., 2006). The microbiota of milk kefir grains is also very stable when incubated under suitable conditions. The polysaccharide produced from microorganisms of the milk kefir grains are called kefiran which is a water-soluble polysaccharide consisting of branched glucogalactan with equal amounts of the monomers D-glucose and D-galactose produced by *Lb. kefiranofaciens* (Kok-Tas et al., 2013). The milk kefir grains consist of a polysaccharide-protein matrix where kefiran is the main component. The viscosity of kefiran decreases with higher concentrations showing a pseudoplastic behavior (Piermaria et al., 2008). Milk kefir has been a source for isolation and description of new species like *Lb. kefiri*, *Lb. kefirgranum*, *Lb. parakefir*, *Candida kefyr* and *Saccharomyces turicensis* (Lopitz-Otsoa et al., 2006). Milk kefir could be produced commercially but only by using starter cultures with specific groups of microorganisms (lactic acid bacteria, acetic acid bacteria and yeasts) as the kefir grains show differences in their composition during the fermentation procedure resulting in deficient organoleptic characteristics (Lopitz-Otsoa et al., 2006). The consumption of traditional and industrial kefir shows a possible beneficial effect on health, especially kefiran might show therapeutic immunostimulatory, antimutagenic, antiallergic and antiulcer activity (Kok-Tas et al., 2013). Kefiran could also modulate the gut immune system and epithelial cells are protected against *Bacillus cereus* exocellular factors (Piermaria et al., 2008).

The milk kefir is an example for a symbiotic coexistence of bacteria and yeast dependent on each other with beneficial effects.

1.2 Role of fermented food in human nutrition

The history of the usage of fermented food is very old (Tamang and Kasipathy, 2010). The food processing technology regarding the production of food fermentation is one of the oldest known to human kind (Caplice and Fitzgerald, 1999). The oldest record of food fermentation goes back to 6000 BC in the Fertile Crescent (Blandino et al., 2003). The knowledge of food preparations was transferred from generation to generation producing small amounts of the traditional product for consumption. Traditionally, the way for obtaining fermented food in former times was done by indigenous knowledge but without understanding the meaning of microbial mechanisms. With the industrialization and growth of towns or cities the requirements of foods raised. The microbial understanding of communities immersed from 1850s onwards (Caplice and Fitzgerald, 1999). The products originating from fermented foods show a huge variety around the world. Different substrates are metabolized by different microorganisms resulting in unique foods with typical characteristics (Caplice and Fitzgerald, 1999). Fermented foods include a huge variability of substrates ranging from vegetables, cereals, milks, legumes, meat and fish products and grains (Tamang and Kasipathy, 2010; van Hijum et al., 2013). The food fermentation could occur as natural (spontaneous) or controlled fermentation by using starter cultures (monoculture or multiculture microorganisms) (Tamang and Kasipathy, 2010). With respect to the increasing consumption of fermented food and industrial preparation the need of understanding the interaction and identifying the microorganisms responsible for the characteristics is an irreplaceable requirement.

Nowadays, it is assumed that 5-40 % of the total daily food consumption is coped by fermented food and beverages and the importance of fermented food consumed globally is rising (Tamang and Kasipathy, 2010).

The microbial stability has to be proven for ensuring food safety and typical organoleptic characteristic which is nearly impossible when the mechanisms of the fermentation are rarely understood. Therefore the interest of the characterization of the microbial composition of a fermented food is increasing constantly. Often fermented foods are providing health benefits by enhanced nutritional content like bioavailability of minerals and production of antioxidants, improving digestibility and could reduce toxicity (Bokulich and Mills, 2012). The diversity of microbiota in fermented foods and their functional microorganisms also provide several novel properties like enzyme and alcohol producing bacteria and yeasts which can be used industrially.

1.2.1 Fermented beverages

A lot of investigations examined alcoholic fermentation, such as wine and beer. The predominant and most important species in these fermentations is the yeast *Saccharomyces cerevisiae.* At this juncture the fermentation process is an alcoholic fermentation resulting in the formation of ethanol. Alcohol fermentation is not the only procedure taking place in beverage fermentations. Milk and cereals are fermented by lactic acid fermentation due to lactic acid bacteria. The metabolic characteristics of this group can be divided into homofermentative and heterofermentative species whereas homofermentative bacteria produce lactic acid as major end product. Species representing the homofermentative pathway are members of the genera *Pediococcus*, *Streptococcus*, *Lactococcus* and *Lactobacillus*. Heterofermentative bacteria produce same amount of lactate, ethanol and CO_2. Species representing

this group belong to the genera *Weissella*, *Leuconostoc* and *Lactobacillus* (Blandino et al., 2003). The interaction between the different organisms is not analyzed in depth but the technology of beer and wine making is well documented in literature. The mechanisms of other fermented beverages are not completely understood and the interest of analyzing their microbiota, due to their probably health benefits and for development of possible starter culture is increasing fast. One example is water kefir.

There are different molecular procedures to investigate the microbiota ranging from culture-dependent to culture-independent methods both with advantages and disadvantages.

1.3 Application of culture-dependent and- independent analyses of microbiota

1.3.1 Advantages and disadvantages of culture-dependent analyses

The conventional techniques of a microbiota analyses relies on culture-dependent methods where bacteria, yeasts or fungi are cultured with or in nutrient media (Gong and Yang, 2012). The identification of microorganisms is done regarding their morphology, biochemical, physiological and genetic characteristics after growing on suitable media (Jany and Barbier, 2008). One of the most important advantage of culture-dependent analysis is the ability to obtain pure cultures and to characterize these species in detail. Especially in the field of food microbiology it is necessary to correctly profile bacterial or yeast species like in fermented foods for obtaining the food with wanted texture, flavor and possible health benefit properties (Kesmen et al., 2012).

The difficulty in culturing microorganisms from food environments is that only a small percentage of food-associated microorganisms can be cultured under standard

laboratory procedures (Giraffa and Neviani, 2001). It is very difficult to simulate the natural environment or habitat of the microorganisms within a microbiota under laboratory conditions. Not only the nutritional requirements are often unknown but also the interaction and symbiosis of microorganisms is nearly impossible to simulate (Gong and Yang, 2012). The culture-dependent analyses of complex microbiota often results in a misleading interpretation of the diversity and composition of the microbes (van Hijum et al., 2013). Enrichment cultures used for growth of microorganisms within microbiota could influence the community structure by inserting new selective conditions (Giraffa and Neviani, 2001). Species appearing in low cell numbers in the microbiota could be missed by culturing procedures due to the competiveness of numerically more abundant species (Hugenholtz et al., 1998). Predominant species might overgrow less abundant species which could also have an important impact in the stability of the community. The culture-dependent analyses are very time-consuming resulting from long culture periods and complex culture techniques like isolating single pure cultures (Gong and Yang, 2012).

1.3.2 High through-put sequencing as a tool for characterizing microbiota in food fermentations

Molecular techniques for analyzing microbiota like detection, identification and characterization of microorganisms within these environmental habitats exhibit an outstanding tool (Giraffa and Neviani, 2001). Therefore technologies that do not require cultivation have proven useful to identify non-culturable microorganisms. Technologies without the need of culturing microorganisms established over the last decades (van Hijum et al., 2013). There are several culture-independent molecular methods applied analyzing microbial food community each with its own limits like Denaturating/Thermal Gradient Gel Electrophoresis (DGGE/TGGE), terminal restriction fragment length polymorphism (TRFLP), fluorescence in situ hybridization

(FISH), quantitative PCR (QPCR) and next generation sequencing (NGS) (van Hijum et al., 2013). The technology of next generation sequencing was firstly introduced to the market in 2005 (Morozova and Marra, 2008). This relatively new technique provides high speed and high-throughput sequencing without the need of any culture step or cloning of DNA fragments (Ansorge, 2009).

To overcome misinterpretation of a microbial community resulting from growth bias, molecular techniques have been established over the past decades. In the field of food fermentation next generating high through-put sequencing such as 454 FLX Titanium sequencing are state of the art techniques for monitoring the microbial flora and/or changes within a microbial community (Bokulich and Mills, 2012; van Hijum et al., 2013). The 454 high through-put sequencing is a sequencing by synthesis technique and chemiluminescence is measured when inorganic pyrophosphate is released. For detailed information of the procedure see Morozova and Marra, 2008. Universal primers are used to amplify the DNA template from microbial communities; normally the 16S rRNA genes in bacteria and ITS genes in yeast and fungi are amplified. The length of sequence in 454 Pyrosequencing could be up to 700 bp (van Hijum et al., 2013) providing greater taxonomic information than shorter sequences (Bokulich and Mills, 2012). The length of sequences is an important factor for the analysis of a community. The identification of organisms on species level is only possible when sequence length is long enough and a good quality of the sequences is given. The term pyrosequencing implies different technologies for analyzing a community, including genome/metagenome, transcriptome and epigenome characterization.

The 16S rRNA amplicon sequencing is a part of the metagenomic high through-put technology whereas metagenomic analysis studies the collective set of mixed

microbial communities (Petrosino et al., 2009). With the amplicon sequencing organisms are identified on either family or species level but no information about their metabolic activity are obtained.

Several fermented foods were analyzed by high-throughput sequencing such as pearl millet fermentation (Humblot and Guyot, 2009), fermented fish/rice called narezushi (Kiyohara et al., 2012; Koyanagi et al., 2012), nukadoko (rice bran) (Sakamoto et al., 2011), Chinese liquor fermentations (Li et al., 2011) and cheese fermentation like Polish Oscypek cheese (Alegria et al., 2012). A lot of investigations have been done regarding Korean food fermentation like fermented seafood (Roh et al., 2010), soybean pastes like meju (Kim et al., 2011) and doenjang (Nam et al., 2012a), rice beer called Makgeolli (Jung et al., 2012), kochujang (red pepper, rice, soybean mix) (Nam et al., 2012b) and fermented raddish/cabbage called kimchi (Park et al., 2012) reviewed recently by Bokulich and Mills, 2012; van Hijum et al., 2013.

High-throughput sequencing offers a new view of different environmental microbiota and changes of a community could be measured when altering growth conditions. When detecting new species in a community the step of cultivation is needed for characterizing these microorganisms, but needed growth parameters could be applied in laboratory scale more easily.

1.4 Preservation of food, beverages and starter cultures used for production

The preservation of food and beverage is very important for stability and microbial safety of these products around the world. For ensuring the food safety combined preservation factors are used, called hurdles (Leistner, 2000). Applied hurdles are the control of temperature (low/high), water activity, acidity (pH), redox potential, addition

of preservatives like nitrite, sorbate or bacteriocines and the usage of competitive microorganisms such as lactic acid bacteria (Leistner, 2000). The organoleptic characteristics of each end-product have to be guaranteed. Therefore it is necessary to evaluate suitable mix of hurdles for each product. Nowadays the focus of hurdle techniques is to generate a multitarget preservation with applied gentle hurdles. These hurdles are mixed in a way that they show synergistic effects for gaining food and beverages with wanted characteristics (Leistner, 2000). The globalization of food market and the introduction of novel food and beverages are linked to new demands for preservation techniques. The demands of the consumers are changing towards products with natural characteristics and minimally processed food like preservation by microorganisms or other biopreservation techniques.

1.4.1 Biopreservation

One of the oldest biopreservation is the technology of fermentation (Ross et al., 2002). Over the past few years the role of microorganisms in preservation of food and beverage raised. The process of preservation due to microorganisms is dependent o their biological activity and the formation of metabolites which could suppress unwanted microbiota in foods and beverages (Ross et al., 2002). Nowadays yeasts are widely used in the production of alcoholic beverages like beer, wine and spirits. Lactic acid bacteria are important microorganisms for the production of different fermentation processes like dairy, meat and vegetable fermentation. Genera belonging to LAB are *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Leuconostoc* and *Pediococcus* (Ross et al., 2002). The raw material of each food or beverage exhibits substrates for different mixture of microorganisms normally a defined mix of starter culture (Ross et al., 2002). LAB play an important role in the food industry not only because of contributing in flavor, texture and in enhancing the nutritional value in foods and beverages, they also prevent spoilage

and extent shelf life of these products (Topisirovic et al., 2006). The production of antimicrobial substances as metabolites could be important in several fermentations, but LAB are also known to produce antimicrobial proteinaceous substances, called bacteriocins. Substances showing antimicrobial effects toward pathogenic organisms are several types of acids like organic and fatty acids, alcohol production and carbon dioxide (Ohlsson and Bengtsson, 2002; Ross et al., 2002). The antimicrobial effects often are attended by lowering the pH, or negatively affecting the cell membrane potential, preventing active transport and influencing different metabolic functions (Ross et al., 2002).

The interest in using substances which can be marketed as natural is rising and therefore the interest of microorganisms isolated from natural niches with potential of new variants of antimicrobial, antifungal or antioxidantal effects is also increasing (Ohlsson and Bengtsson, 2002; Topisirovic et al., 2006).

1.4.2 Stress tolerance of microorganisms used as starter cultures regarding freeze-drying technology

The procedure of freezing and freeze-drying are standard methods for the long-term storage of bacteria used as starter cultures. The difficulty of bacterial storage is to reduce damages resulting from freeze-drying technology and rehydration with a maximal cell survival.

The viability and functional activity of preserved starter cultures are dependent on the preservation technologies used, but starter cultures used industrially should maintain high levels of viability during freeze-drying and after rehydration (Carvalho et al., 2002a). Although this technology is commonly used, undesirable side-effects often occur and hamper the viability of these organisms (Carvalho et al., 2002b). During the freeze-drying procedure cells are exposed to extreme stress situations including

high concentrations of solutes, extremes in pH, low temperature, ice crystal formation, water removement from within the cell (Carvalho et al., 2002a). The loss of viability of dried cultures is a result of cell damage at several target sites, like cell membrane and cell wall damage, DNA denaturation and on membrane lipid oxidation (Carvalho et al., 2004; Zhao and Zhang, 2005). Cell damage during freezing is depending on the cooling rate. Slow cooling could result in extracellularly ice crystal formation due to osmotic water flow. The solute concentration outside the cell increases resulting from ice crystal formation and osmotic imbalance occurs. But if the cooling rate is too fast ice crystals could be formed intracellularly leading to lethal cell damage (Zhao and Zhang, 2005). The mechanisms of desiccation tolerance are not completely understood. Gram positive bacteria are more stress tolerant than gram negative bacteria. Investigations of Fredrickson et al. showed a correlation of Mn^{2+} accumulation and high Mn^{2+}/Fe^{2+} ratio in the cell and stress tolerance. Mn^{2+} might protect proteins from oxidative damage after desiccation, the intracellular Mn^{2+}/Fe^{2+} concentration is related to desiccation resistance although the mechanisms behind it are not understood completely (Fredrickson et al., 2008). For Gram negative bacteria on the other hand desiccation tolerance has to be improved by accumulation of intracellular protective agents such as non-reducing disaccharides like sucrose and trehalose.

Cells are protected by the replacement of water due to formation of hydrogen bonds with the sugars and the saccharides are involved in vitreous cytoplasmatic matrix formation. Not only intracellular but also extracellular protective agents could be used. Gram negative cells could also be trained for stress tolerance by multiple exposure and the physiology/metabolism prior to stress could be altered (Fredrickson et al., 2008).

For cell survival during and after freeze-drying many factors have to be considered like growth conditions, protective medium, initial cell concentration, freezing temperature and rehydration conditions. The rehydration of desiccated cells is an important factor and the survival of the cells is depending on the rehydration medium. Complex rehydration media might show higher survival rates of microorganisms due to the finding of nutrients for repairing damaged cells. The osmotic pressure of complex media is higher and therefore the effect of the osmotic shock in desiccated cells could be minimized (Morgan et al., 2006).

The optimal application freezing temperature, freeze-drying procedure and the rehydration process is highly depending on genera (Zhao and Zhang, 2005).

1.5 *Bifidbacteriaceae* in food

Bifidobacteriaceae are heterofermentative Gram-positive bacteria (Pokusaeva et al., 2011). The family of bifidobacteria have different habitats mostly in the human and/or animal gut (Biavati and Mattarelli, 2006). Normally they are not pathogenic with the exception of bifidobacteria isolated from dental caries and *Gardnerella vaginalis* which might cause urogenetal tract infections. Most of them normally show probiotic and or prebiotic characteristics.

Bifidobacteriaceae are very important in the field of treatment and prevention of diseases either in medicine as well as supplement or natural occurring in food (Arunachalam, 1999). In humans bifidobacteria are naturally found in the gastrointestinal gut showing beneficial health effects (Pokusaeva et al., 2011).

The health benefits of regularly intake of bifidobacteria are reported as suppression of putrefactive bacteria and intestinal putrefaction, prevention of constipation and

geriatric diseases including cancer, prevention and treatment of antibiotic-associated diarrhea and stimulation of immune response resulting in greater resistance to infection (Mitsuoka, 1990). The species of bifidobacteria often are difficult to isolate due to their special growth requirements and extreme habitats (Arunachalam, 1999). Their ability to survive and live in extreme habitats could be explained by their physiological capability to metabolize different oligosaccharides (Pokusaeva et al., 2011). Fermented products are claimed to have health promoting benefits like yoghurt and milk kefir, especially in the human digestive tract. For manufacturing health promoting fermented milk products five species are mostly used namely *B. adolescentis*, *B. bifidum*, *B. breve*, *B. infantis* and *B. longum* (Arunachalam, 1999).

Some species of *Bifidobacteriaceae* are able to form different vitamins like thiamin, folic acid, ascorbic acid, pyridoxine, nicotine etc. (Arunachalam, 1999).

The health promoting effect in human is due to production of antimicrobial substances suppressing pathogens by competition for nutrients and adhesion receptors and stimulating immunity (Kailasapathy and Chin, 2000). In the past few years the public awareness of probiotic food raised and so did the demands and so some strains have been introduced to the commercially market, especially as functional substances of dairy- based probiotic drinks (Pokusaeva et al., 2011). In the industry prebiotics have become a very important role, especially in the functional food market (Pokusaeva et al., 2011). Therefore it is interesting to find new natural habitats and/or new bifidobacteria species and characterize them in terms of a potential prebiotic or probiotic effect.

1.6 Objectives of the work

The aim of this thesis was to elucidate the composition and dynamic of the water kefir consortium to enable a stable and reproducible fermentation of water kefir with a constant end product showing desired characteristics. Therefore the microorganisms of the water kefir microbiota should be isolated and identified. The identification should be done by culture-dependent procedures like RAPD pattern comparison followed by 16S rRNA analysis. As a second approach for identifying bacteria in the water kefir community culture-independent analyses such as 16S rRNA gene amplicon sequencing, ARDRA and RISA should be applied. The isolates should be characterized regarding their possibility of EPS production. The composition of different water kefir microbiota grown under standardized conditions should be determined for indentifying the core microorganisms within this community. The alteration of the water kefir microbiota resulting from different growth conditions should be determined by convenient procedures. A possible conservation of the water kefir grains by freeze-drying technology should be analyzed with respect of industrial handling.

As the overall aim possible isolates of the water kefir should be identified, which could be used as starter cultures for a water kefir based beverage.

2 Material and Methods

2.1 Material

2.1.1 Equipment

Table 2: Overview of devices used

device	model	manufacturer
agarose gel chamber 25 x 20 cm	Easy Cast electrophoresis system	Owl Separation Systems, Portsmouth, NH, USA
agarose gel chamber 13.8 x 12 cm	Easy Cast electrophoresis system	
autoclaves	2540 ELV	Systec GmbH, Wettenberg, Germany
	Varioklav	H + P Labortechnik, Oberschleißheim, Germany
breeding/incubation	Certomat BS-1	Systec GmbH, Wettenberg, Germany
	Hereaus B5042E	Hereaus Instruments, Hanau, Germany
	Memmert INB series	Memmert GmbH & C. KG, Schwabach, Germany
	Memmert ICP 500	
	WiseCube® WIS-ML02	Witeg Labortechnik GmbH, Wertheim, Germany
centrifuges	Sigma 1 K 15	Sigma Labortechnik, Osterrode am Harz, Germany
	Sigma 6-16 K	
	Hermle Z382 K Hermle Z383 K Hermle Z216 MK	Hermle Labortechnik, Wehningen, Germany
	Mini Centrifuge MCF-1350	Laboratory Medical Supplies, Hongkong, China
freeze-drying	Freeze-drying zone 2.5^{plus}	Labconco, Kansas City, USA
cooled incubator	ICP 500	Memmert GmbH & Co. KG, Schwabach-Uigenau, Germany
incubation hood	Certomat H	B. Braun Biorech International, Melsungen, Germany
laminar flow sterile work bench	HERA safe	Heraeus Instruments, Hanau, Germany
MALDI-TOF-MS	microflex LT	Bruker Daltonics GmbH, Bremen
microscope	Axiolab	Carl Zeiss Micro Imaging GmbH, Germany

device	model	manufacturer
nanodrop	Nanodrop 1000	Peqlab Biotechnologie GmbH, Erlangen, Germany
PCR cycler	Primua 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	Pipeman	Gilson-Abomed, Langenfeld, Germany
power supplies	MPP 2 x 3000 Power Supply	MWG Biotech, AG, Ebersberg, Germany
	Electrophoresis Power Supply EPS 3000	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	Teche DRI-Blick DB3	Thermo-Dux Gesellschaft für Laborgerätebau mbH, Wertheim, Germany
ultra sonic water bath	Sonorex Super RK 103H	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 of chemical used

chemicals	purity	manufacturer
6 x DNA loading dye	-	Fermentas GmbH, St. Leon-Rot, Germany
acetic acid	99-100 % (glacial)	Merck, Darmstadt
acrylamide- Bis solution	(19:1); 30 % (w/v)	SERVA, Heidelberg, Germany
agar	european agar	Difco, BD Sciences, Heidelberg
ampicillin sodium salt	93.3 %	Gerbu Biotechnik GmbH, Gaiberg, Germany
ammonium chloride	≥99.5 % p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
ammonium persulfate (APS)	electrophoresis grade	SERVA, Heidelberg, Germany
anaerocult	-	Merck, Darmstadt, Germany
bromphenol blue	for electrophoresis	SIGMA-Aldrich, Steinheim, Germany
$CaCl*H_2O$	p.a.	Merck, Darmstadt, Germany
chloramphenicol	research grade	SERVA, Heidelberg, Germany
cycloheximide	98 %	SIGMA-Aldrich, Steinheim, Germany
EDTA	for molecular biology	SIGMA-Aldrich, Steinheim, Germany
ethanol, absolute	≥99.8 %	VWR, Prolabo, Foutenay-sous-Bois, France
$FeSO_4$	97 %	SIGMA-Aldrich, Steinheim, 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	p.a.	Merck, Darmstadt, Germany
kanamycin sulfate	98 %	SIGMA-Aldrich, Steinheim, Germany
KH_2PO_4	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$K_2HPO_4*3H_2O$	p.a.	Merck, Darmstadt, Germany
lysozyme	-	SERVA, Heidelberg, Germany
maltose	for microbiology	Gerbu Biotechnik GmbH, Gaiberg, Germany
mannitol	98 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

chemicals	purity	manufacturer
*mbo*I restriction enzyme	-	GATC, Fermentas GmbH, St. Leon-Rot, Germany
meat extract	for microbiology	Merck, Darmstadt, Germany
$MgSO_4*7H_2O$	p.a.	Merck, Darmstadt, Germany
$MnSO_4*4H_2O$	p.a.	Merck, Darmstadt, Germany
Na-acetate*$3H_2O$	≥99.5 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
NaCl	p.a.	Merck, Darmstadt, Germany
NaH_2PO_4	p.a.	Merck, Darmstadt, Germany
NaOH	50 %	J.T. Baker, Deventer, Netherlands
$(NH)_4H$-citrate	≥98 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
nucleobases	p.a.	SIGMA-Aldrich, Steinheim, Germany
neutralized peptone from soybeans	for microbiology	Oxoid, Hampshire, England
peptone from casein	for microbiology	Merck, Darmstadt, Germany
Primer	-	MWG-BiotechAG, Ebersberg, Germany
Raftilose	-	Orafti, Oraye, Belgium
ringer reagent	p.a.	Merck, Darmstadt, Germany
T4 DNA ligase	-	Fermentas GmbH, St. Leon-Rot, Germany
TEMED	p.a.	Merck, Darmstadt, Germany
Tris	ultra-pure	MP Biomedicals Solon, Ohio, USA
Tris-HCl	p.a.	Merck, Darmstadt, Germany
Tween 80	-	Mallinkrodt Baker B.v., Deventer, NL
X-Gal	research grade	Boehringer Ingelheim, Ingelheim, Germany
yeast extract	for microbiology	Merck, Darmstadt, Germany
$ZnSO_4*7H_2O$	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	apricots, cranberries, figs	Seeberger, Ulm, Germany
plastic vessel	2 l	-
sieve and spoon	autoclaved	-
mineral water	naturell	Residenzquelle, Bad Windsheim, Germany
sucrose	EG-Qualität-I	Tip, Düsseldorf, Germany
water kefir grains	-	different provider, Germany

2.1.4 Consumables

Table 5: Overview of consumables

material	type	manufacturer
anaerocult	A, A mini, C mini	Merck, Darmstadt, Germany
reaction tubes	200 µl, 1.5 ml, 2 ml	Eppendorf, Hamburg, Germany
sterile filter	Filtropur S 0.2 (0.2 µm)	Sarstedt, Nümbrecht, Germany
sterile ml tubes	15 ml, 50 ml	Sarstedt, Nümbrecht, Germany
UV cuvette	LCH 8.5 mm, from 220 mm	Sarstedt, Nümbrecht, Germany

2.1.5 Molecular biological kits

Table 6: Overview of molecular biological kits used

kit	type	manufacturer
E.Z.N.A. Bacterial DNA Kit	DNA isolation	Omega Bio-Tek Inc., Norcross, GA, USA
peq GOLD Gelextraction Kit	gel extraction	PEQLAB Biotechnologie GmbH, Erlangen, Germany
QIAquick PCR purification Kit	PCR purification	Quiagen GmbH, Hilden, Germany
Taq Core Kit	DNA polymerase	MP Biomedicals Solon, Ohio, USA

2.1.6 Bacterial and yeast strains

Table 7: Overview of bacterial strains

species	strain	source
Ac. fabarum	TMW 2.1192	water kefir W
Ac. fabarum	TMW 2.1197	water kefir F
Ac. fabarum	TMW 2.1198	water kefir F
Ac. orientalis	TMW 2.1196	water kefir W
B. psychraerophilum	TMW 2.1362	DSM 22366
B. psychraerophilum	TMW 2.1395	water kefir W
B. crudilactis	TMW 2.1369	LMG 21775
B. longum	TMW 2.448	DSM 20088^T
B. animalis subsp. *lactis*	TMW 2.462	DSM 10140^T
B. lactis	TMW 2.530	BB12
B. breve	TMW 2.447	DSM 20213^T
Lc. citreum	TMW 2.1194	water kefir W
Lc. mesenteroides	TMW 1.1961	water kefir A
Lc. mesenteroides	TMW 2.1073	water kefir A
Lc. mesenteroides	TMW 2.1076	water kefir F
Lc. mesenteroides	TMW 2.1193	water kefir W
Lc. mesenteroides	TMW 2.1195	water kefir W
Lc. mesenteroides	TMW 2.1199	water kefir A
Lc. mesenteroides	TMW 2.1343	water kefir A
Lb. casei	TMW 1.1814	water kefir A
Lb.casei	TMW 1.1957	water kefir A
Lb. hilgardii	TMW 1.1828	water kefir A
Lb. hilgardii	TMW 1.1910	water kefir A
Lb. hordei	TMW 1.1821	water kefir A
Lb. hordei	TMW 1.1822	water kefir F

species	strain	source
Lb. hordei	TMW 1.1907	water kefir W
Lb. hordei	TMW 1.1958	water kefir A
Lb. hordei	TMW 1.1959	water kefir A
Lb. hordei	TMW 1.1960	water kefir A
Lb. nagelii	TMW 1.1823	water kefir A
Lb. nagelii	TMW1.1825	water kefir W
Lb. nagelii	TMW 1.1826	water kefir F
Lb. nagelii	TMW 1.1827	water kefir F
Lb. nagelii	TMW 1.1908	water kefir A
Lb. nagelii	TMW 1.1909	water kefir A

Table 8: Overview of yeast species

species	strain	source
Hanseniaspora valbyensis	TMW 3.222	water kefir F
Hanseniaspora valbyensis	TMW 3.242	water kefir W
Hanseniaspora valbyensis	TMW 3.295	water kefir W
Pichia membranifaciens	TMW 3.241	water kefir W
Pichia membranifaciens	TMW 3.296	water kefir W
Saccharomyces cerevisiae	TMW 3.221	water kefir A
Zygotorulaspora florentina	TMW 3.220	water kefir A

2.2 Methods

2.2.1 Microbial methods

2.2.1.1 Media and growth conditions

2.2.1.1.1 Preparation of water kefir

The water kefirs were propagated in a medium containing 100 ml/l of fig extract and 80 g/l sucrose for at least two times to eliminate influences resulting from different cultivation procedures of the supplier before analysis. To guarantee a constant mineral content, we used mineral water (Residenz Quelle, naturell, Bad Windsheim, Germany). The fig extract was prepared by manually shaking 480 g dried figs cut into small pieces in 1 l of mineral water (for 20 min. This extract was centrifuged for 3 h at 17000 g and sterilized using a 0.20 µm membrane filter unit (Sarstedt, Germany). Dried figs that had not been treated with any preservative agents and non sulfurated (Seeberger, Germany) were obtained from a local supermarket.

The fermentation was performed at 21 °C for 72 h in a sterile 2 l plastic vessel with 40 g water kefir grains in a total volume of 500 ml. The supernatant was discarded with a sterile metallic sieve, and the kefir grains were strained, washed with tap water strained again and reused for the next start of fermentation with a new sterile 2 l plastic vessel. Lemon slices were not added to minimize potential contamination from handling the water kefir under aseptic conditions.

2.2.1.1.2 Cultivation and growth conditions of LAB

For the cultivation of lactobacilli modified MRS media was used (Table 9). All components were dissolved in 800 ml deionised water except of sugars. The pH was adjusted at 5.7. For agar plates 1.5 % agar was added. The sugar was dissolved in 200 ml deionised water and both solutions were autoclaved separately at 121 °C for

20 min to avoid Maillard products. Both solutions were mixed after autoclaving. After cooling the solution below 50 °C cycloheximide (150 µg/ml) was added to inhibit the growth of yeasts. The agar plates were incubated anaerobically at 30 °C for three days. Liquid cultures were incubated anaerobically in 15 ml Falcon tubes over night.

Table 9: Composition of mMRS medium used for lactobacilli

compound	concentration [g/l]
yeast extract	4
meat extract	2
peptone from casein	10
tween 80	1
K_2HPO_4*3 H_2O	2.5
Na-acetate*3 H_2O	5
$(NH_4)_2$ H citrate	2
$MgSO_4$*7 H_2O	0.2
$MnSO_4$*H_2O	0.038
glucose	20

2.2.1.1.3 Cultivation and growth conditions of acetic acid bacteria

For the cultivation of acetic acid bacteria GM media was used (Table 10). All components were dissolved in 800 ml deionised water except of sugar. The pH was adjusted at 6.0. For agar plates 1.5 % agar was added. Both solutions were mixed. To inhibit growth of yeasts 150 µg/ ml cycloheximide was added after cooling below 50 °C. The agar plates were incubated aerobically at 30 °C for three days. Liquid cultures were cultivated in 50 ml Erlenmeyer flask at 30 °C and 180 rpm over night.

Table 10: Composition of GM medium for cultivation of acetic acid bacteria

compound	concentration [g/l]
yeast extract	5
peptone from casein	3
mannitol	25

2.2.1.1.4 Cultivation and growth conditions of yeast strains

For the cultivation of yeasts YPG medium was used (Table 11). All components were dissolved in 800 ml deionised water except of sugar. The pH was adjusted at 6.5. For agar plates 1.5 % agarose was added and 0.01 g/l bromphenol blue for morphological differentiation. The glucose solution (200 ml deionized water) was autoclaved separately. After autoclaving (121 °C for 20 min) the solutions were mixed. For the inhibition of bacteria growth chloramphenicol (100 g/ml) was added after cooling below 50 °C. The agar plates were incubated aerobically at room temperature for 3 days. Liquid cultures were incubated in 50 ml Erlenmeyer flask under aerobic conditions over night at 180 rpm.

Table 11: Composition of YPG medium for yeast cultivation

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

2.2.1.1.5 Cultivation and growth conditions of *Bifidobacteriaceae*

For the cultivation of *Bifidobacteriaceae* modified Tryptone-Phytone (TP) medium was used (Table 12). All components were dissolved in 800 ml deionised water except of sugars. The pH was adjusted at 6.1. For agar plates 1.5 % agarose was added. For the inhibition of yeasts 400 µg/ml cycloheximide was added after cooling below 50 °C as well as 400 µg/ml kanamycin sulphate for the inhibition of lactic acid bacteria. The agar plates were incubated anaerobically at 30 or 37 °C for three to five days. Liquid cultures were incubated in falcon tubes at 37 °C for 24 h.

Table 12: Composition of TP medium for the cultivation of *Bifidobacteriaceae*

compound	concentration [g/l]
yeast extract	6
peptone from casein	10
neutralized soya peptone	10
NaCl	5
K_2HPO_4	2.5
glucose	2
raftilose	2

2.2.1.1.6 Cultivation and growth conditions of *E. coli*

For the cultivation of *E. coli* LB- medium (Luria- Bertani) medium was used (Table 13). All components were dissolved in 1 l deionised water. For agar plates 1.5 % agarose was added. The medium was autoclaved at 120 °C for 20 min. After cooling below 50 °C IPTG, X-Gal and ampicillin were added according to the manufacturer's instructions (AccepTor^{TM} Vector Kit, Novagen).

Table 13: Composition of LB medium for *E. coli* growth

compound	concentration [g/l]
yeast extract	5
peptone from casein	10
NaCl	5

2.2.2 Molecular biological methods

2.2.2.1 DNA isolation from single strains for identification of microorganisms with culture-dependent procedure

For the DNA isolation of bacteria from water kefir samples overnight cultures from single colonies were done in the appropriate medium. Therefore a serial dilution was prepared with 10 g of washed and strained water kefir grains. The grains were diluted with 90 ml of ¼ Ringer reagent and mechanically homogenized by a stomacher for

60 s. A serial dilution was prepared by mixing 1 ml of the grain suspension with 9 ml of ¼ Ringer reagent. The different serial dilutions were plated on the different media and incubated as described before. After the incubation the viable cell count was enumerated on a proper dilution and the single colonies were picked from each different plate. From every suitable serial dilution half of the colonies were picked and first grown on the appropriate plate for 3 days. For the growth of lactobacilli liquid MRS medium was used and for acetic acid bacteria liquid GM medium. The lactobacilli were grown in 2 ml reaction tubes at 30 °C over night. The acetic acid bacteria were grown in 50 ml Erlenmeyer flasks at 180 rpm and 30 °C over night. The overnight cultures were centrifuged at 5000 g for 10 min. The pellet was washed with 1 ml TE buffer (1 mM EDTA, 10 mM Tris, pH 8) and centrifuged again. The pellets were stored at -20 °C until further use. The DNA isolation was done with the Bacterial DNA Kit according to the instructions. The pellets were resuspended with 200 µl TE buffer containing lysozyme (10 mg/ml). The mixture was incubated at 37 °C for 1 h. The DNA was eluted with two times 50 µl of elution buffer. Quantification of the genomic DNA was done by agarose gel electrophoresis comparing band intensities with known DNA ladders.

2.2.2.2 RAPD PCR

For the RAPD PCR isolated genomic DNA was used as template. The primer used was the oligonucleotide primer M13V (5'-GTTTTCCCAGTCACGAC-3'). The PCR reaction (25 µl) contained 25 pmol primer, 0.2 mM each deoxyribonucleoside triphosphate, 3.5 mM $MgCl_2$, reaction buffer, 0.75 U *Taq* polymerase and 1 µl of DNA solution. Approximately the same amount of DNA (50-100 ng) was used. PCR was carried out by using a Primus 96^{plus} cycler. The amplification program was 94 °C for 45 s, 3 cycles of 94 °C for 3 min, 40 °C for 5 min, 72 °C for 5 min and 32 cycles 94 °C for 1 min, 60 °C for 2 min, 72 °C for 3 min. All PCR products were mixed with 5 µl 6 x

Loading dye (Fermentas) and then electrophoretically separated in a 1.3 % (w/ v) agarose gel. Registration of the PCR patterns, normalization of the densitometric traces, pattern storage, grouping of the strains using the Pearson product moment correlation coefficient and UPGMA cluster analysis were performed using BioNumcerics Version 6.50.

2.2.2.2.1 Identification of bacteria isolated with culture-dependent procedures

Isolated strains from water kefir samples showing different RAPD patterns were analyzed by comparative 16S rDNA sequencing. The 16S rDNA was amplified with the universal primer 616V (5'-AGAGTTTGATYMTGGCTCAG-3') with a binding position 7 according to (Brosius et al., 1981) and 609R (5'-ACTACYVGGGTATCTAAKCC-3') with a binding position 1099 according to (Brosius et al., 1981). The PCR program was 94 °C for 2 min, 32 cycles of 94 °C for 45 s, 52 °C for 90 s, 72 °C for 2 min and a last step at 72 °C for 5 min. The reaction mixture (50 µl) consisted of 0.1 mM of each deoxynucleoside triphosphate, 0.75 U *Taq* polymerase, 5 pmol of each primer and 1 µl of the genomic DNA. The amplified DNA had a length of 800 bp and was purified with the cycle pure kit (Omega bio-tek) according to manufacturer's instructions.

The sequencing was done by a commercial provider (GATC Biotech, Germany). The identification of the bacteria was done with the BLAST program.

2.2.2.2.2 Yeast identification isolated with culture-dependent procedures

The isolated yeasts were identified by FTIR as described by (Kümmerle et al., 1998). In the case of ambiguous FTIR results strains were identified by partial sequencing of the 26S rDNA as described by (Kurtzman and and Robnett, 2003). For the identification the GenBank/ EMBL/DDBJ accession numbers used were U72165 (*Zygotorulaspora florentina*) and U73596 (*Hanseniaspora valbyensis*).

2.2.2.3 DNA isolation from water kefir grains for culture-independent procedures

The water kefir grains of three water kefirs (A, F and W) were propagated under standardized conditions at least two times prior to analysis. One water kefir (water kefir I) was analyzed directly after arrival from the supplier by post.

For the DNA isolation of water kefir grains 10 g of each water kefir grain was diluted with 90 ml ¼ Ringer's reagent and mechanically homogenized with a bag mixer for 60 s. Then, 5 ml of this solution was centrifuged at 5000 g for 10 min. The pellet was washed with 2 ml TE buffer and centrifuged again.

For the DNA isolation of the supernatant of the water kefir 50 ml were centrifuged as described above and the pellet was washed with 5 ml TE buffer and then treated the same way as above described.

The pellets were stored at -20 °C until further use.

The DNA isolation was performed with the E.Z.N.A.^{TM} Bacterial DNA kit according to manufacturer's instruction (also see 2.2.2.1).

2.2.2.3.1 16S rRNA gene amplification for high-throughput Pyrosequencing

The DNA isolated from each water kefir was used as a template for the amplification of the V1 to V4 hyper-variable regions of the bacterial 16S rRNA gene with the Ba27 f (Lane, 1991), (5'- AGAGTTTGATYMTGGCTCAG-3') with a 16S binding position 8-27 according to Brimacombe et al., 1990 and Ba519 r primers (Lane, 1991), (5'- TATTACCGCGGCKGCTG-3') with a 16S binding position 519-534 according to Brimacombe et al., 1990. The reaction mixture (50 µl) consisted of 0.1 mmol l^{-1} of each deoxynucleoside triphosphate, 0.75 U *Taq* polymerase, 5 pmol of each primer and 1 µl of the genomic DNA, and amplification was carried out using a PCR

program of 94 °C for 2 min; 32 cycles of 94 °C for 45 s, 52 °C for 90 s and 72 °C for 2 min; and a final step at 72 °C for 5 min.

After the PCR reaction, the quality of the amplified PCR products (approximate length: 520 nt) was confirmed by electrophoresis, and the products were purified using a Cycle Pure Kit (Omega Bio-Tek) according to the manufacturer`s instructions. A 20 µg sample of each amplicon was sequenced in a 454 Genome Sequencer FLX Titanium by LGC Genomics (Berlin, Germany).

2.2.2.3.2 ARDRA analysis

DNA samples from the four water kefirs (see 2.2.2.3) were used as templates for amplification with the specific bifidobacteria primers Bif164_mod_for (Langendijk et al., 1995) with the sequence 5'- GGGTGGTAATGCCGRATG-3' and a 16S binding position of 164-181 according to Brimacombe et al., 1990 and LM3_mod_rev (Kaufmann et al., 1997) with the sequence 5'- GGTGCTNCCCACTTTCATG-3' and a 16S binding position of 1412-1432 according to Brimacombe et al., 1990.

The reaction mixture (50 µl) consisted of 0.1 mM of each deoxynucleoside triphosphate, 0.75 U *Taq* polymerase, 5 pmol of each primer and 1 µl of the genomic DNA, and the amplification was carried out using a PCR program of 94 °C for 5 min; 32 cycles of 94 °C for 1 min, 45 s of 60 °C and 72 °C for 2 min; and a final step at 72 °C for 5 min. The amplified DNA had a length of approx. 1400 nt and was purified using the PeqGOLD Gel Extraction Kit (PeqLab, Germany) according to the manufacturer's instructions. The DNA was eluted in 30 µl elution buffer and stored at 4 °C until further use.

Amplicons were cloned in *E. coli* Top 10 using the AccepTor™ Vector Kit (Novagen). Restriction analysis was performed on 100 clones from each kefir sample after colony PCR using the above primers with *mbo*I (GATC, Fermentas) according to the

manufacturer's instructions. Fragments were electrophoresed in a 1.3 % (w/ v) agarose gel (0.5 x Tris-borate-EDTA buffer [45 mmol/ l Tris-borate, 1 mmol/ l EDTA]), followed by staining with dimidium bromide.

Amplicons from the DNA of "*B. crudilactis"* LMG 21775, *B. psychraerophilum* DSM 22366^T, *B. longum* DSM 20088^T, *B. animalis* subsp. *lactis* DSM 10140^T, *B. lactis* BB12 and *B. breve* DSM 20213^T were also included in this analysis.

2.2.2.3.3 16S rDNA analysis of *Bifidobacteriaceae*

For the analysis of the full 16S rDNA sequence of the novel uncultivated *Bifidobacterium* species the universal 16S primers 616V with the sequence 5'-AGAGTTTGATYMTGGCTCAG-3' (Brosius et al., 1981), 630R with the sequence 5'-CAKAAAGGAGGTGATCC-3' (Juretschko et al., 2002) with the binding position of 7 and 15282 according to the 16S genes of *E. coli* respectively were combined with newly designed specific primers Bif_spec_for (5'-GGATGTGGGACCCATTC-3') and Bif_spec_rev (5'-GAACCCGTGGAATGGGTC-3') with the binding position 829-845 and 837-856 according to *E. coli*. The DNA from water kefir A was used as a template. From this amplicon, 1525 nt were sequenced and submitted to the EMBL database under the accession number HE804184.

For the identification of *B. psychraerophilum* the primers LM3_mod_rev with the sequence 5'-GGTGCTNCCCACTTTCATG-3' (Kaufmann et al., 1997) and Bif164_mod_for with the sequence 5'- GGGTGGTAATGCCGRATG-3' (Langendijk et al., 1995) were used with the binding position 1412-1432 and 164-181 according to the 16S gene of *E. coli* respectively.

2.2.2.4 Influence of the composition of water kefir microbiota by changing growth conditions

The water kefir grain (W) was grown under standardized conditions so that the procedure could be done from one batch water kefir grain. These water kefir grains were cultured under five different conditions (Table 14). The different extracts were prepared by mixing 480 g of the dried fruit in 1 l of mineral water (Residenzquelle, naturell). The fruits were cut into small pieces and then manually shaken for 20 min. The extracts were centrifuged at 17 000 g for 3 h and sterilized using a 0.20 µm membrane filter unit (Sarstedt, Germany). The extracts were stored at -20 °C until further use. The concentration of the extract was 100 ml/l. The amount of kefir grain was 29 g for each preparation. The different kefir samples were incubated at the different temperatures whereas the standard, cranberry and the apricot sample was incubated at 21 °C, one sample was incubated at 37 °C and one sample at 12 °C. The grains fermented three days and every third day the decrease or increase of the wet grain masse was measured. Therefore the grains were washed with tap water, strained and weighed. This procedure was repeated over 24 days.

After 24 days the cfu was enumerated by plating the different samples on MRS, GM, YPG and TP agar plates with appropriate serial dilutions.

Table 14: Different culture conditions for water kefir grains.

standard	***CE**	****AE**	**37 °C**	**12 °C**
8 % sucrose solution	8 % sucrose solution	8 % sucrose solution	8 % sucrose solution	8 % sucrose solution
fig extract	cranberry extract	apricot extract	fig extract	fig extract
grains	grains	grains	grains	grains
21 °C	21 °C	21 °C	37 °C	12 °C

*CE= cranberry extract, ** AE= apricot extract

2.2.2.4.1 Ribosomal intergenic spacer analysis (RISA)

At the end of the fermentation (after three days) the DNA was isolated from the grains and the supernatant of the different samples. DNA isolation see 2.2.2.3. With the DNA a PCR was performed with the universal primer sets 23S r (5'-GGGTTBCCCCATTCRG-3') and 1406 f (5'-TGYACACACCGCCCGT-3'). The primers were respectively complementary to positions 1320 and 1336 of the 16S rRNA and131 and 115 of the 23S rRNA genes of *E. coli* (Cardinale et al., 2004). The reaction mixture (50 µl) consisted of 0.1 mM of each deoxynucleoside triphosphate, 0.75 U *Taq* polymerase, 5 pmol of each primer and 1 µl of the genomic DNA. The PCR program was 94 °C for 2 min and 30 cycles of 94 °C for 15 s, 55 °C for 15 s and 75 °C for 45 s followed by a final step at 75 °C for 5 min.

With the PCR solution a 15 % acrylamide gel was performed with the components 5 xTBE, deionized water and acrylamide. The amount of 16 µl PCR product was mixed with 6 µl 10 x loading dye. The analysis of the band patterns was done after running the gel 5 h at 100 V. The gel was stained with dimidium bromide.

2.2.2.5 Freeze-drying experiment with water kefir grains

For the freeze-drying experiment water kefir grains were cultured under standardized conditions to guarantee that the different set up of the experiment were done from one batch of water kefir grains.

The water kefir grains grown under standardized conditions were frozen over night at different temperatures at -12 °C, -20 °C and -80 °C respectively. One sample was quick-frozen by liquid nitrogen directly before the freeze-drying procedure. The water kefir grains were put into 50 ml Falcon tubes and in the caps holes were punctured with sterile canula to ensure sublimation. The freeze-drying took place over 24 h with FreeZone Plus 2.5 l Cascade Benchtop Freeze Dry System (Labconco) with a

vacuum set point of 0.0 mBar and a drying chamber set point at -40 °C with a transmission rate of 10 s.

After the freeze-drying the viable cell count was determined by plating serial dilutions of water kefir grains on different media (MRS, GM, YPG and TP) and incubate them. The rest of the water kefir grains were regrown under standardized conditions with the same amount of grains (40 g). Every third day the increase or decrease of the grain amount was measured and with the grains a new fermentation was started. After a few fermentation steps the viable cell count was determined again.

From each medium approximately 50 single colonies were picked and streaked on new plates for obtaining pure single cultures. The identification of the microorganisms was done by MALDI-TOF MS see 2.2.4.

2.2.3 Data processing and 16S rDNA sequence analysis

The data obtained from high-throughput pyrosequecing was analyzed as follows.

The initial sequence analysis was performed using the classifier software (RDP Naïve Bayesian rRNA Classifier Version 2.4 (Wang et al., 2007)) provided in Ribosomal Database project II (Cole et al., 2009) to estimate the microbial diversity at the genus/family level. Sequences shorter than 150 nt were removed. The confidence threshold was at least 80 %. Sequences with less than 97 % similarity to the sequences deposited in RDP II were classified as unidentified.

Taxon assignment for the 16S rDNA sequences was performed in the ARB software package (http://www.arb-home.de; (Ludwig et al., 2004; Westram et al., 2011)and using the SILVA (http://www.arb-silva.de; (Pruesse et al., 2007) and LTP (all-species Living Tree Project; http://www. arb-silva.de/projects/living-tree;(Munoz et al., 2011; Yarza et al., 2008)) databases. Only sequence reads of at least 400 nucleotides were

included for comparative data analysis. Preliminary taxon assignment above the species level was roughly achieved by applying the classifier software (Wang et al., 2007) provided by the RDP (Ribosomal Database Project; (Cole et al., 2009)and the PT-server (Positional Tree server;(Ludwig et al., 2004; Westram et al., 2011))-based 'next relative search' of the ARB package.

For a more detailed taxon assignment, the species differentiation capacity of the 16S rRNA marker for the relevant taxonomic groups extracted from the LTP database was evaluated. The current species threshold is set at 98.7 % identity over the entire 16S rRNA sequence (Stackebrandt and Ebers, 2006). As there are many type strains that share higher similarities, 16S rRNA-based assignment is only possible for closely related species groups in such cases. The species threshold value cannot be applied directly to analyse partial rDNA sequence data, such as that derived in the present study. This analysis required that the (non-linear) correlation of full and partial sequence-derived similarities be determined for the individual type strain groups and that the respective species-(group) thresholds be delineated. These similarity-based thresholds were used to define the respective score value ranges obtained by applying the ARB PT-server approach (Ludwig et al., 2004; Westram et al., 2011). This suffix tree-based search for common heptanucleotide occurrences in the source (pyrosequencing) and reference (type strain) data allows a phylogenetic similarity analysis without the need for sequence alignment. The underlying principle is based on the finding that multiple occurrences of identical heptanucleotide stretches are highly unlikely in 16S rRNA primary structures (our unpublished data). Thus, sequences from diverse sources that contain identical heptanucleotides can be regarded as homologous. PT-server score thresholds were defined for two levels: the species or (lowest) species-group level and the lowest level that clearly separates the respective species (group) from all other type species.

The resulting three score ranges allow a sequence to be assigned to its respective species or species-group, its phylogenetic lineage, or to higher taxa, respectively (Table 19). The high error rate in sequence data obtained by high-throughput methods (Balzer et al., 2010) must be taken into account when interpreting these score ranges. Lower scores may represent as-yet undescribed species or simply low-quality data.

2.2.4 MALDI-TOF MS analysis

2.2.4.1 Sample preparation

For the identification of microorganisms single colonies on agar plates were needed. The bacteria or yeasts were grown over night on the appropriate agar plate in the way that single colonies were obtained. These single colonies were analyzed by MALDI-TOF MS.

2.2.4.2 Preparation of MALDI matrix

For the preparation of the MALDI matrix A-Cyano-4-hydroxy-cinnamic acid (sigma-Aldrich Chemie GmbH), acetonitrile (ChemLab GmbH), trifluoroacetic acid (Merck KGaA and deionzied water were used.

2.2.4.3 Sample application onto MALDI target

The preparation of the MALDI target was done under sterile work flow. Single colonies were picked from the plates and streaked on the target. This sample was overlaid with 1 µl of acetic acid and dried. After that the sample was overlaid with 1 µl matrix and dried again for co-crystallization.

2.2.4.4 Cleaning of MALDI target

The cleaning of the MALDI target was done according to manufacturer’s instructions. The target was overlaid with 70 % ethanol for 5 min, then washed with hot tap water

and wiped with 70 % ethanol subsequently. The target was overlaid with 100 µl 80 % trifluoroacetic acid and wiped again. A final cleaning was done with deionized water and dried by wiping.

2.2.5 Fluorescence in situ hybridization (FISH) analysis

For the analysis the water kefir was grown under standardized conditions. The water kefir grain and the supernatant were analyzed with FISH.

The whole water kefir grain and the supernatant were and fixed with Ethanol (50 %) and 4 % PFA for 12 h separately. The following steps were done according to (Pavlekovic et al., 2009). The water kefir grain was smashed onto a microscopic slide for hybridization.

The probes used are shown in Table 15. The probe EUB338-I for the water kefir grain was labeled with FLUOS (5(6)-carboxyfluorescein-N-hydroxysuccinimde ester), the probe HGC 69a with the hydrophilic sulphoindocyanine dye Cy5. The supernatant was analyzed with the probe probe HGC 69a labeled with the hydrophilic sulphoindocyanine dye CY3. The probes were ordered from Thermo Electron GmbH, Ulm, Germany.

Table 15: Probes used for the FISH analysis of water kefir

probe	target	sequence (5'-3')	reference
EUB 338-I	all bacteria	GCTGCCTCCCGTAGGAGT	(Amann et al., 1990a)
HGC 69a	actinobacteria	TATAGTTACCACCGCCGT	(Roller et al., 1994)

For the visualization a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany) was used equipped with two Helium/ Neon Lasers, one Argon-ion Laser and one UV-Laser.

The experiment was done on the Department of Microbiology (TU München) at the Lab of Microbial Systems Ecology under the guidance of Dr. NM Lee.

3 Results

3.1 Microbial diversity of water kefir grains analyzed with culture- dependent procedures

3.1.1 Quantification of bacteria and yeasts

The microbiota of three independent water kefir grains (water kefir A, F and W) from different origin were analyzed. The bacteria grown on the MRS and GM agar were enumerated and the colony forming units were determined.

The viable cell numbers (cfu/g) of the bacteria in the three water kefirs ranged from $1.2*10^6$ to $5.6*10^8$, the cell count on MRS medium was in the same range in all three water kefirs ($1.6*10^8$ in water kefir A, $1.3*10^8$ in water kefir F and $1.3*10^8$ in water kefir W) whereas the cell count on GM showed differences, water kefir A and F had a cell count of $3.7*10^6$ and $1.2*10^6$ but the cell count of water kefir W was higher ($5.6*10^8$) (Table 16).

The viable yeast cells counted on water kefir A and F were similar with a cell count of $6.4*10^6$ in water kefir A and $5.8*10^6$ in water kefir F. The viable yeast cell count in water kefir W was also higher with $2.7*10^7$ cells.

Table 16: Viable cell counts (cfu/ g) of the three water kefirs obtained on different media

media	viable cell count wk A	viable cell count wk F	viable cell count wk W
MRS	$1.6*10^8$	$1.3*10^8$	$1.3*10^8$
GM	$3.7*10^6$	$1.2*10^6$	$5.6*10^8$
YPG	$6.4*10^6$	$5.8*10^6$	$2.7*10^7$

3.1.2 Identification of microorganisms

The microbiota of the three different water kefir grains were analyzed, therefore 453 bacterial isolates were obtained and the bacteria showing differences in their RAPD patterns were identified by 16S rDNA. The 453 bacterial isolates showed 28 different RAPD patterns and eight different bacterial species could be identified as *Lactobacillus casei*, *Lb. hordei*, *Lb. nagelii*, *Lb. hilgardii*, *Leuconostoc mesenteroides, Lc. citreum*, *Acetobacter fabarum* and *Ac. orientalis*. These species showed differences in their RAPD patterns, and differences in the patterns could be detected also among the identified single strains. These differences were mostly not very pronounced and might represent subspecies or biotypes of the identified species (Figure 2) but there were strains of the same species, which showed completely different patterns like *Lc. mesenteroides* TMW 2.1076 and TMW 2.1073, *Lb. nagelii* TMW 1.1826 and 1.1825, *Lb*. *hordei* 1.1907 and TMW 1.1959 and *Ac. fabarum* 2.1192, 2.1197 and 2.1198.

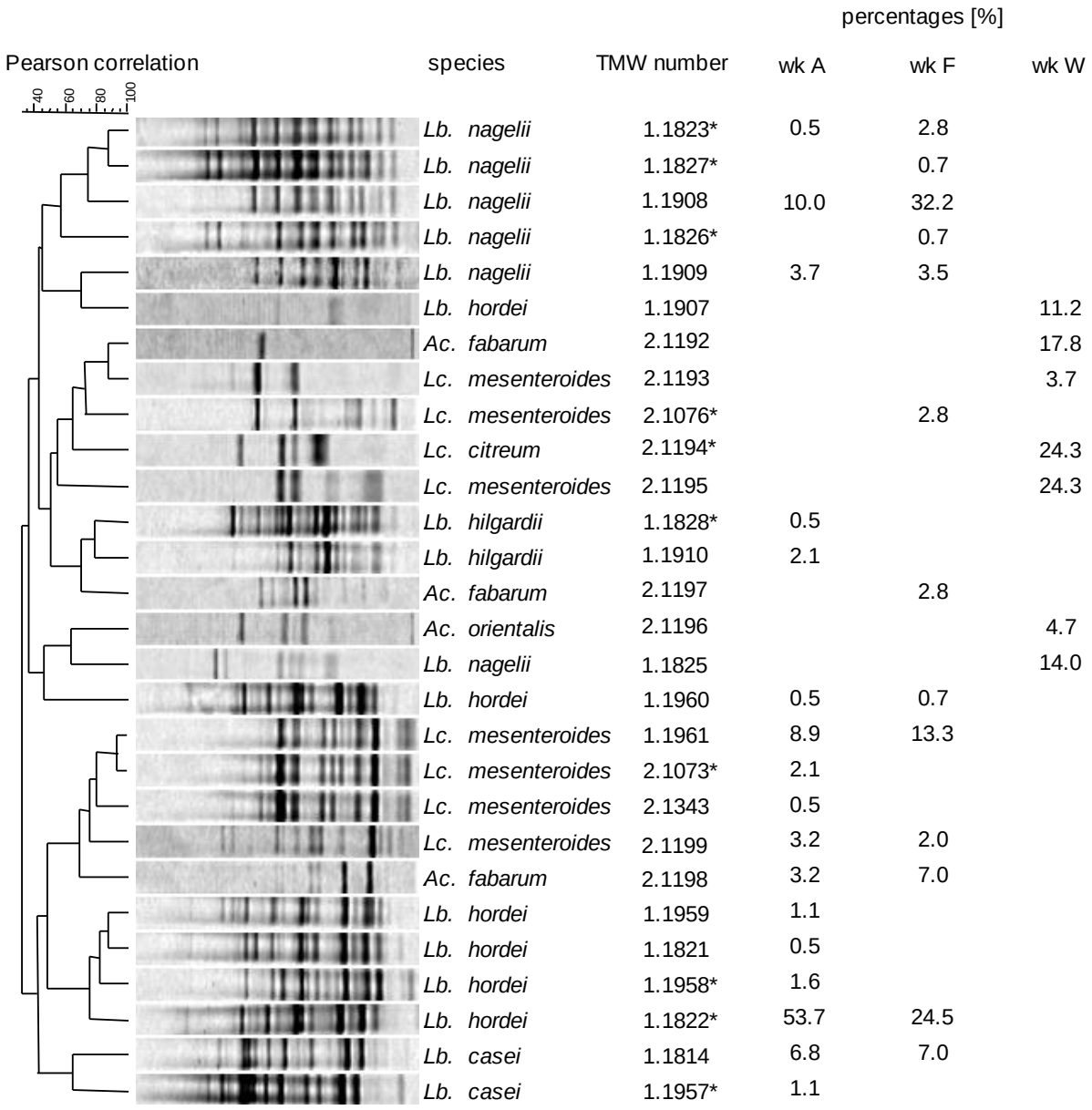

Figure 2: UPGMA cluster analysis of RAPD fingerprint patterns of the bacteria isolated from three water kefirs (A, F and W) of different origin which were grown identically.
Shown are the 28 different RAPD patterns obtained out of 453 bacterial isolates from the three water kefirs. The identification of the 28 species were done with 16S rDNA sequencing The TMW numbering displays numbers of the strain collection of "Technische Mikrobiologie Weihenstephan". The labeling shows the numbering in our study. Wk = water kefir, the marked species (*) are species producing exopolysaccharides which were grown on MRS containing saccharose instead of glucose.

16S rDNA sequence comparisons of the strains isolated from the three different water kefirs with the reference sequences are shown in Table 17.

Table 17: Sequence similarity of isolated strains with the reference sequence found in gene bank

species	strain	accession numbers of sequences used as references	sequence of similarity [%]
Lb. nagelii	1.1823	AB162131	99.6
Lb. nagelii	1.1827	AB162131	99.2
Lb. nagelii	1.1908	AB162131	99.6
Lb. nagelii	1.1826	AB162131	99.6
Lb. nagelii	1.1909	AB162131	99.6
Lb. nagelii	1.1825	AB162131	99.6
Lb. hordei	1.1907	EU074850	100
Lb. hordei	1.1959	EU074850	100
Lb. hordei	1.1821	EU074850	99.9
Lb. hordei	1.1958	EU074850	99.9
Lb. hordei	1.1822	EU074850	99.0
Lb. hordei	1.1960	EU074850	100
Lb. casei	1.1814	HQ293086	99.5
Lb. casei	1.1957	HQ293086	99.5
Lb. hilgardii	1.1828	AY241664	99.2
Lb. hilgardii	1.1910	AY241664	99
Lc. citreum	2.1194	GU470983	99.9
Lc. mesenteroides	2.1195	HM058688	99.5
Lc. mesenteroides	2.1193	JF733808	99.7
Lc. mesenteroides	2.1076	HM058688	99.6
Lc. mesenteroides	1.1961	HM058688	99.5
Lc. mesenteroides	2.1073	HM058688	99.6
Lc. mesenteroides	2.1343	HM058688	100
Lc. mesenteroides	2.1199	HM058688	99.4
Ac. fabarum	2.1198	AM905849	99.1
Ac. fabarum	2.1192	AM905849	100
Ac. fabarum	2.1197	AM905849	100
Ac. orientalis	2.1196	AB052707	100

3.1.3 Differences in the bacterial composition of water kefir microbiota

The composition of the bacterial microbiota in the three water kefirs was different. The main flora of water kefir A and F consisted of the same three bacterial species but with different abundance.

Lb. hordei was the most prominent species in water kefir A with 57.4 %, in water kefir F the percentage was 25.2. *Lb. nagelii* was the main component in the microbiota of water kefir F with 39.9 %, in water kefir A it was present with 14.2 %. *Lc. mesenteroides* was present at percentages of 14.7 % and 18.1 % in water kefir A and F, respectively. In comparison to that water kefir W mainly consisted of *Leuconostocs* (24.3 % *Lc. citreum*, 24.3 % *Lc. mesenteroides*) and *Ac. fabarum* with 17.8 %. The lactobacilli species had an overall percentage of 25.2 where 11.2 % were *Lb. hordei* and 14 % were *Lb. nagelii*.

Lb. hilgardii was only present in water kefir A (2.6 %) and *Lb. casei* could not be detected in water kefir W. *Ac. orientalis* and *Lc. citreum* were only present in water kefir W (4.7 % and 24.3 %) (Table 18).

Table 18: Overview of percentages of bacteria species in the three water kefirs

species	wk A	wk F	wk W
Lb. casei	7.9	7.0	-
Lb. hilgardii	2.6	-	-
Lb. hordei	57.4	25.2	11.2
Lb. nagelii	14.2	39.9	14
Lc. citreum	-	-	24.3
Lc. mesenteroides	14.7	18.2	28
Ac. fabarum	3.2	9.8	17.8
Ac. orientalis	-	-	4.7

3.1.4 Differences in the yeast composition of water kefir microbiota

Four different yeasts were identified by FTIR as *Hanseniaspora valbyensis, Lachancea fermentati*, *Saccharomyces cerevisiae* and *Zygotorulaspora florentina.* The identification of *H. valbyensis* and *Z. florentina* was validated by 26 s rDNA sequencing. For the identification of yeasts five colonies each with identical morphology on YPG agar supplemented with bromphenol blue were selected. Both *Z. florentina* and *S. cerevisiae* showed white colonies. The percentage of white

colonies was 94, 92 and 93 % of all yeast colonies, in water kefir A, F and W, respectively. *S. cerevisiae* was identified by FTIR in all three water kefirs but indistinguishable from *Z. florentina* on plates due to the same morphology, and therefore no precise quantification of each species could be achieved. Water kefir W was studied in a more detailed analysis where 44 white colonies were identified by FTIR and 16 colonies were identified as *S. cerevisiae* and 25 colonies were *Z. florentina*.

L. fermentati formed a white colony with a blue center and was present in water kefirs A and F with 6 % and 8 %, respectively. *H. valbyensis* formed a white colony with a blue center and a blue circle and was present to 7 % in water kefir W.

3.2 Bacterial diversity of water kefir grains analyzed with culture-independent procedures

3.2.1 Bacterial identification and distribution determined by high-throughput sequence-based analysis

The bacterial microbiota of four independently grown water kefir grains from different origins were analyzed by high-throughput sequence-based analysis. Three water kefirs (A, F and W) were grown at least two times under standardized conditions to ensure that they all are propagated under identical conditions and to eliminate possible influences of the microbiota resulting from cultivation procedures of the supplier. Water kefir I was analyzed directly after arrival from the supplier to detect any kind of differences in the microbiota resulting from growing conditions. A total of 43293 (kefir A), 33079 (kefir F), 25951 (kefir W) and 41404 (kefir I) reads of the V4 region of the 16S rRNA were analyzed.

After quality trimming, a total of 31795 (kefir A), 23909 (kefir F), 18305 (kefir W) and 25319 (kefir I) reads were used for taxon assignments. The distribution of the different bacteria at the family level was evaluated by the software tools provided by the Ribosomal Database project II (Cole et al., 2009), demonstrating the presence of *Acetobacteraceae*, *Bifidobacteriaceae*, *Clostridiaceae*, *Leuconostocaceae*, *Lactobacillaceae* and unclassified bacteria (Figure 3). The most abundant bacteria were *Lactobacillaceae* (A 69 %, F 67.9 %, W 57.4 % and I 27.3 %), followed by *Bifidobacteriaceae* (A 21.5 %, F 18.7 % and I 21.9 %), with the exception of water kefir W, which had only 3.8 %. Kefir W showed a higher percentage of *Leuconostocaceae* (29.1 %) than the other kefirs. Only kefir I had a high amount of *Clostridiaceae* (37.2 %). *Acetobacteraceae* were present in low amounts (A 2.8 %, F 5.6 %, W 3.3 % and I 5.8 %).

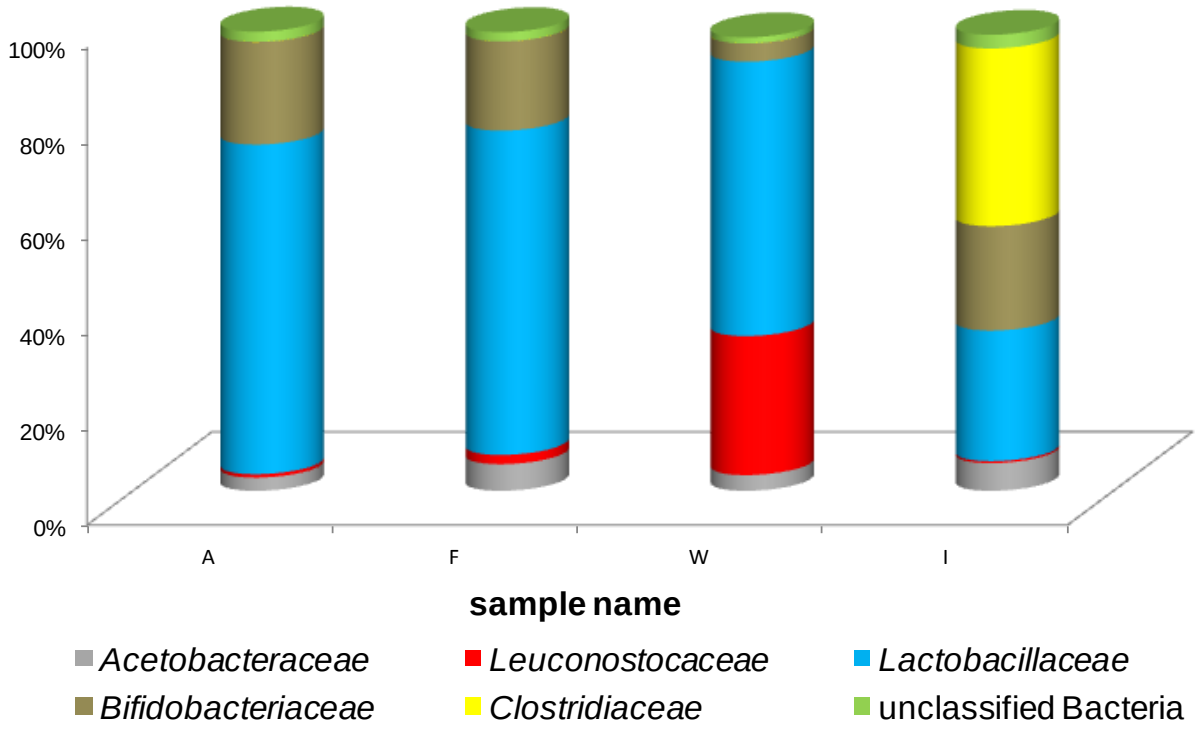

Figure 3: Microbial diversity of the four water kefirs on family level.
Each colour represents a different bacteria family.

The suffix tree-based search for the most similar type strain sequences was used to assign the sequences to genera, species groups or species (Table 19). With this method, we were able to identify 12 bacterial species and 12 bacterial groups. The main species of water kefirs A and F consisted of members of the *Lb. hordei* group, *Lb. nagelii*, and *Bifidobacterium* spp.; water kefir A also contained species from the *B. psychraerophilum* group; and water kefir F had a greater amount of *Ac. fabarum* group members. The most abundant sequences in water kefir I were from members of the *B. psychraerophilum* group, *Lb. hordei* group, *Lb. hilgardii*, *Lb*. *satsumensis, Ac. orientalis* group and *C. tyrobutyricum*. The core species of water kefir W were from the *Lb. hordei* group, *Lb. nagelii*, *Lb. hilgardii*, *Lc. citreum* group and *Lc. mesenteroides* group.

Table 19: Taxonomic assignment of 99328 analyzed sequences of four water kefir grains to genus, species group or species. Only taxa with >1 reads are shown. Species previously identified with culture-dependent methods are marked in bold letters. Numbers in parentheses show sum of reads of kefirs A, F, W and I assigned at genus level.

		Number of sequence reads											
genus	***species*(group)**[a]	**Kefir A (31795)**			**Kefir F (23909)**			**Kefir I (25319)**			**Kefir W (18305)**		
		genus[b]	group[c]	sp/gp[d]	*genus*	group	sp/gp	*genus*	group	sp/gp	*genus*	group	sp/gp
Acetobacter (3715)	*cerevisiae, cibinongensis, indonesiensis, malorum,* ***orientalis*** *orleanensis, tropicalis*	127	0	21	145	0	5	726	0	428	130	0	36
	Aceti		0	2		0	3		0	0		0	
	fabarum*, iovaniensis, ghanensis, syzygii*		183	157		492	544		185	92		264	163
	Peroxydans		2	0		0	0		5	1		4	0
Gluconobacter (359)	*albidus, oxydans, roseus, kanchanburiensis, sphaericus*	12	5	22	1	0	10	84	4	50	9	4	11
	cerinus, frateuri, japonicus, thailandicus wancheriae,		0	1		0	1		39	105		1	0
Gluconacetobacter (504)	*entanii, europaeus, hansenii, intermedius, kombuchae, nataicola, oboediens, rhaeticus, sacharivorans, sucrofermentans, swingsii, xylinus*	38	0	277	14	59	76	1	1	0	9	4	11
	liquefaciens, sacchari		0	0		0	0	0	0	0		5	7
	johannae		2	0		0	0	0	0	0		0	0
Bifidobacterium (18759)	*crudilactis, psychraerophilum*	1220	327	837	556	97	226	1655	659	3033	161	37	107
	subtile		31	0		0	0		51	0		0	0
	species		2540	2142		1881	2016		408	326		285	164
Lactobacillus (60561)	*aquaticus, sucicola, uvarum, capillatus*	12574	0	0	9184	2	0	3266	2	0	2732	9	0
	cacaonum, ***hordei,*** *mali*		405	1266		421	1492		316	1217		877	4612
	ghanensis		0	421		0	580		0	32		0	289
	nagelii		0	8057		0	5018		0	145		0	738
	satsumensis		1	19		8	91		36	509		2	43
	casei*, paracasei, zeae*		502	221		319	166		98	60		65	52
	hilgardii		188	0		128	0		3559	0		839	0

Leuconostoc (7293)	***citreum,*** *holzapfeli, lactis, palmae*	82	0	0	164	0	0	46	0	13	2278	0	3106
	holzapfelii		0	0		2	0		0	0		0	255
	mesenteroides, *pseudomesenteroides*		24	86		46	157		16	27		755	228
	kimchii		1	0		0	0		0	0		7	0
Clostridium (8137)	*arbusti*	0	0	0	0	0	0	0	337	0	0	0	0
	tyrobutyricum		0	2		0	5		7787	0		6	0

[a] The species groups (combined in the respective cells) unify valid species sharing over all 16S rDNA sequence similarities beyond the species threshold (Stackebrandt and Ebers, 2006). The species of a given genus are only listed if they are members of the groups represented in the table or newly determined data could be assigned to them.

[b] number of 16S rDNA sequences that could be assigned to valid taxa at (not below) the genus level.

[c] number of 16S rDNA sequences that could be assigned to the (phylogenetic) periphery of valid species or species groups (see [a]). The respective sequence similarities are within a range defined by the species threshold value and the sequence similarity for the respective species (group) and its closest type strain relative.

[d] number of the 16S rDNA sequences that could be assigned to valid species or species group (see [a]).

3.2.2 ARDRA analysis

With the ARDRA technique we focused on bifidobacteria because they were identified for the first time in water kefir, and we wanted to elucidate whether there are different bifidobacteria species in the water kefir. Using bifidobacteria genus-specific primers (LM3_mod_rev, Bif164_mod_for), we were able to amplify a 1253 nucleotide 16S rDNA sequence from all four water kefirs and from the reference strains. A clone library of 100 ARDRA profiles of each water kefir was also analyzed. *mbo*I restriction fragments of reference strains correspond to those as predicted by *in silico* analysis of the respective 16S amplicons. *"B. crudilactis"* (a hitherto not validated species) and *B. psychraerophilum* shared identical restriction patterns, as well as with *B. animalis* subsp. *lactis* and *B. lactis* that showed also same restriction patterns. *B. breve* shows a slightly different pattern, with one additional band in the 150-bp range and a high molecular weight band smaller than those of "*B. crudilactis"* and *B. psychraerophilum* of 480 bp. *B. longum* shows a unique restriction pattern.

Interestingly, in all kefirs, a unique profile was obtained consisting of 2 characteristic bands of 446 and 235 nt (lanes 8 to 11 in Figure 4) in common with *B. crudilactis* and *B. psychraerophilum* strains (lane 1-3) and one of 572 bp, indicating the dominant presence of one or more *Bifidobacterium* species sharing this patterns, non identified by the ARDRA profile in all four kefirs. This identical profile from all four water kefirs was different from that one of other bifidobacteria, including the closest 16S relatives "*B. crudilactis"*, *B. psychraerophilum* DSM 22366^T and the *B. psychraerophilum* strains isolated in this study.

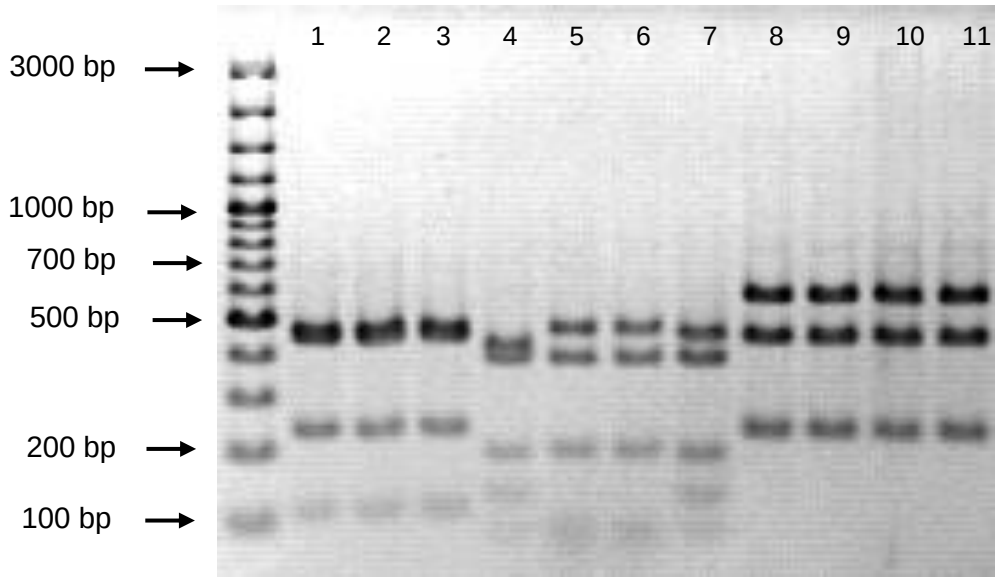

Figure 4: Agarose gel electrophoresis of 16S rRNA ARDRA profiles after restriction of 16S amplicons with *mbo*l of *Bifidobacterium* species.
The agarose gel shows the restriction pattern of "*B. crudilactis*" LMG 21775 (lane 1), *B. psychraerophilum* DSM 22366^T (lane 2), *B. psychraerophilum* from water kefir (lane 3), *B. longum* DSM 20088^T (lane 4), *B. animalis* subsp. *lactis* DSM 10140^T (lane 5), *B. lactis* DSM 58 (lane 6) and *B. breve* DSM 20213^T (lane 7). Lane 8-11 show the ARDRA profiles obtained from the four water kefirs A (lane 8), F (lane 9), W (lane 10) and I (lane 11) representing respectively 100 colonies restricted. A Generuler 100-bp ladder was used as size-marker.

3.2.3 Cultivation and quantification of bifidobacteria

The water kefirs W and I were analyzed representatively for *Bifidobacteriaceae*. The bacteria were cultured on a modified tryptone-phytone (TP) agar, and the viable cell numbers (cfu/ g) were enumerated. The number of *Bifidobacteriaceae* cells in water kefir I was $4.9*10^5$ cfu/g and that in water kefir W $1.5*10^6$ cfu/g. An analysis of representative colonies (at least 100 colonies of each water kefir) by PCR with the species-specific primer (Bif_spec_for and Bif_spec_rev) (data not shown) showed that all isolates grown on these plates belonged to the species *B. psychraerophilum.* bifidobacteria cell numbers were not analyzed for the water kefirs F and A because they were not physically available at that time.

3.2.4 Phylogeny of *Bifidobacterium* sp.

The 16S rRNA gene sequence was amplified from the DNA samples isolated from water kefir A in two fragments using a combination of a species-specific primer of the 'new' water kefir species (Bif_spec_for, Bif_spec_rev) and universal primers (616V, 630R). The almost complete 16S rRNA sequences (1525 nt) was determined. Comparison of this sequence with those available in public databases revealed *B. psychraerophilum* as the closest relative, with a sequence similarity of 96.4 %. The subsequent phylogenetic classification supports a new candidate species of the genus *Bifidobacterium*. The 16S rRNA gene sequence was submitted to the EMBL database under the accession number HE804184.

3.3 Influence of the composition of water kefir microbiota by changing growth conditions

3.3.1 Determination of the wet mass of water kefir grains

The influence of different fermentation parameters was analyzed and the alteration of the microbiota from the water kefir grains and the supernatant were detected. Therefore the water kefir grains of water kefir W were fermented under standardized conditions to enlarge the amount of water kefir grains. From one batch water kefir the different fermentations were started to ensure that the possible alteration of microbiota results from the different fermentation parameters and not from the initial start point. The decrease or increase of the wet mass of water kefir grains was measured after three days of fermentation and a new fermentation was started. This was done over 24 days and the grain mass is shown in Figure 5. The initial mass of water kefir grains was 29 g in 40 ml fermentation solution.

The grain mass over the 24 days of the different samples was stable, the increase of grain mass could be detected with the apricot extract and 12 °C sample. The first measurement of the grain mass showed an increase of all fermentation samples. After six days (2 fermentation starts) the increase of grains was lower. The cranberry extract sample showed the lowest increase of grain mass over time, the grain mass stayed nearly the same. The increase of the grain mass of the different fermentations was not that high as the grain mass increase of the standard water kefir.

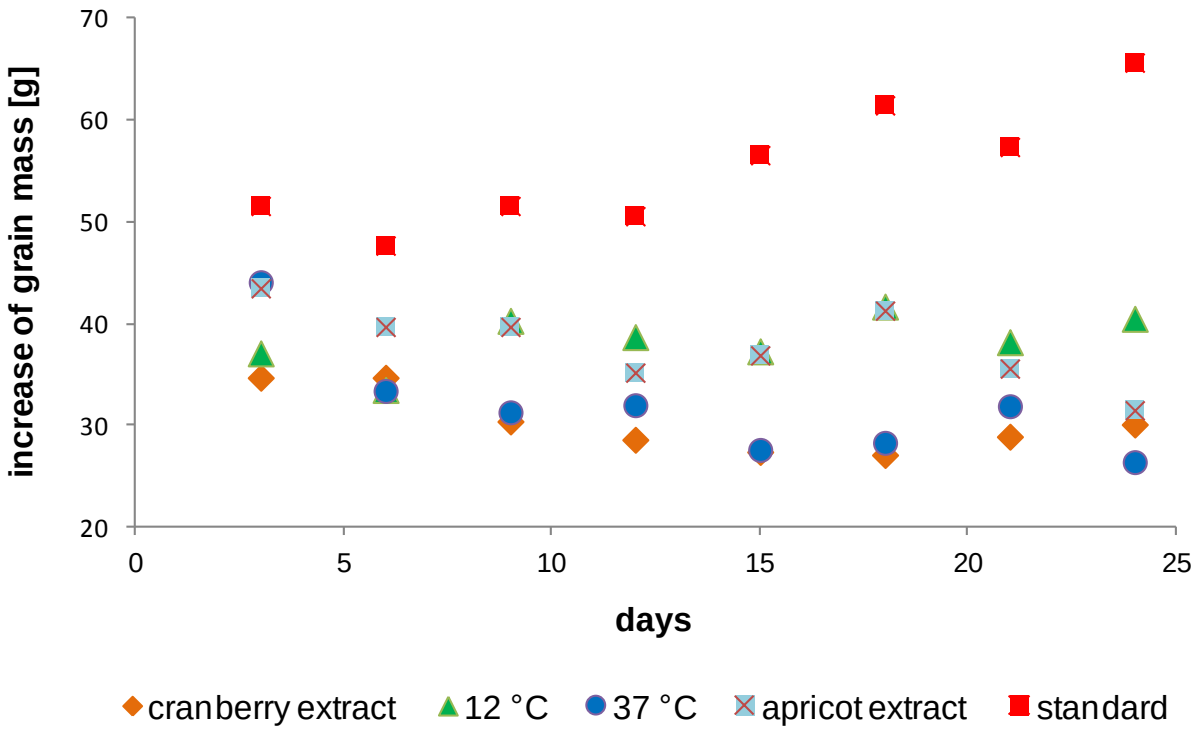

Figure 5: Measurement of the wet mass of water kefir grains.
The water kefir grains were first grown by standard procedure and from one grain batch new fermentations were started with different growth conditions. All samples had a 8 % sucrose solution and at the start of the new fermentation the same amount of grains. One sample was fermented with cranberry extract, one with apricot extract both at 21 °C. Two preparations had a different incubation temperature at 12 °C and 37 °C. The standard water kefir was used as reference. After three days of fermentation at the different parameters the wet grain mass was measured and a new fermentation was started with the washed and strained water kefir grains. This was done over 24 days.

3.3.2 Detection of the viable cell count

The water kefir grains were fermented with different parameters and after 24 days (8 “new” starts of fermentation) the viable cell count was enumerated on the different media (mMRS, GM, YPG and TP). The viable cell count on the mMRS media ranged from $7.5*10^5$ to $8.2*10^7$ whereas the lowest number showed the CE (cranberry extract) sample with $7.5*10^5$ the highest number was detected in the standard water kefir. The AE (apricot extract), 37 °C, 12 °C and the standard water kefir sample showed only small differences in their viable cell count. The cell count in the GM medium was in the same range ($1.0*10^7$-$7.8*10^7$) in all samples. The YPG medium showed that the viable cell count in the CE sample was the lowest with $7.8*10^5$, the AE sample had the highest number with $3.8*10^7$ the other three samples did not show high differences. No growth of colonies could be detected on the TP agar and the 37 °C sample, the highest number of bifidobacteria showed the CE sample with $1.0*10^7$, the AE-, the 12 °C- and the standard water kefir sample showed nearly the same numbers.

Table 20: Determination of the viable cell count of water kefir grown under different conditions. After 24 days (8 starts of “new” fermentations) in comparison with the standard water kefir. (CE= cranberry extract, AE= apricot extract).

media	CE [cfu/g]	AE [cfu/g]	37 °C [cfu/g]	12 °C [cfu/g]	standard [cfu/g]
mMRS	$7.5*10^5$	$2.7*10^7$	$6.3*10^7$	$7.8*10^7$	$8.2*10^7$
GM	$1.0*10^7$	$2.2*10^7$	$7.6*10^7$	$7.8*10^7$	$6.8*10^7$
YPG	$7.8*10^5$	$3.8*10^7$	$5.5*10^6$	$7.4*10^6$	$3.1*10^6$
TP	$1.0*10^7$	$5.9*10^6$	nd	$5.6*10^6$	$4.1*10^5$

3.3.3 Ribosomal intergenic spacer analysis (RISA)

The water kefirs grown under different fermentation parameters were analyzed also by culture-independent procedure. One procedure was the ribosomal intergenic spacer analysis (RISA) with PCR and the primers 23S r and 1406 f. The PCR samples were separated by acrylamide gelelectrophoresis. Five different fermentation terms were analyzed (different extracts like cranberry extract and apricot extract, different fermentation temperatures (12 °C, 37 °C)) in comparison with the standard water kefir. The water kefir grains and the supernatant were analyzed.

Figure 6 till Figure 10 show the RISA patterns from the water kefir grains and the supernatant of the different fermentation procedures. The samples were taken after 3 days of fermentation whereas a new fermentation was prepared after these three days with the same water kefir grains. The patterns were compared regarding the change of pattern in one kind of fermentation over time. The samples were taken over 12 days with four samplings of water kefir grain and supernatant and following DNA isolations. In all samples differences could be obtained regarding the patterns over time.

The RISA patterns of the fermentations with cranberry extract and apricot extract instead of fig extract is demonstrated in Figure 6. After 3 new starts of fermentation with cranberry extract (lane 3) more bands disappeared (four bands in the range of 500-750 bp and one band at ca. 200 bp). Lane 5 shows three bands and in comparison to the next samples one band is missing at ca. 150 bp and two bands in the higher molecular range at ca. 680 bp and 710 bp which represents the apricot sample.

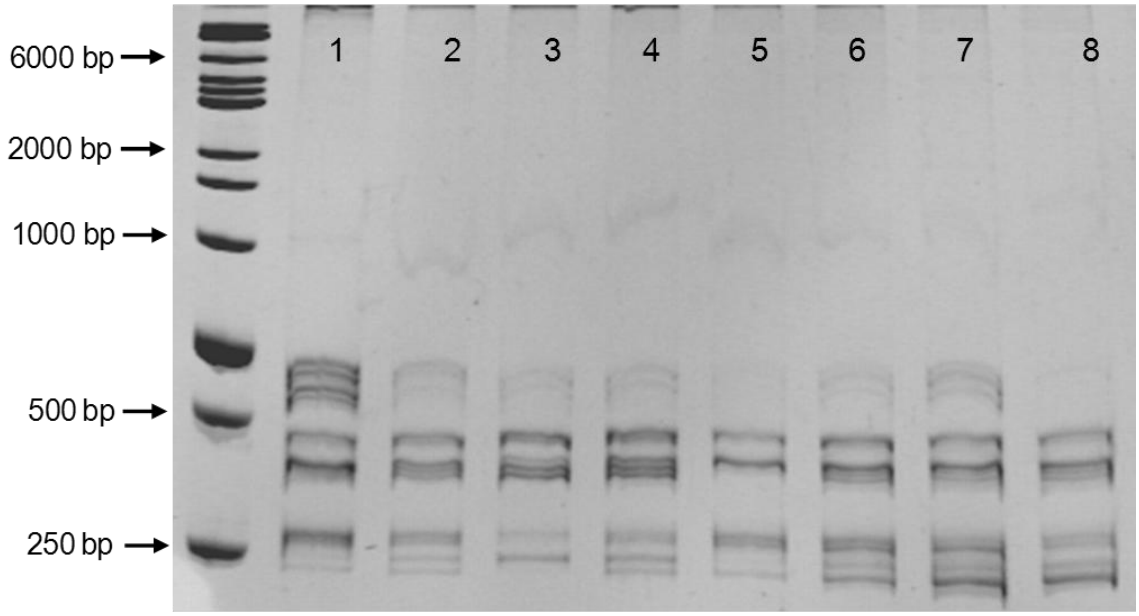

Figure 6: RISA patterns of the water kefir grains fermented with cranberry extract or apricot extract.
Water kefir grains from one batch (water kefir W) were portioned and fermented under different parameters. The acrylamide gel shows the RISA patterns of the cranberry and the apricot extract samples where fig extract was replaced by either cranberry or apricot extract. Each fermentation was done over 3 days and samples were taken from the grains after these 3 days of fermentation. New fermentations were started with the washed and strained water kefir grains respectively. This procedure was done over 12 days. DNA was isolated from the samples taken and a PCR was prepared with the RISA primers 23S r and 1406 f. Lane 1-4 shows the RISA patterns of the cranberry extract fermentation whereas 1 reflects the sample after the first (3 days) and 2 after the second fermentation period etc. Lane 5-8 shows the apricot extract fermentation and 1 also reflects the RISA pattern derived from the first sample taken after 3 days of fermentation and 2 after another 3 days of fermentation etc.

The supernatant analysis of the cranberry and apricot fermentation (Figure 7) shows differences in lane 3 and 4 which are showing three additional bands in comparison to lane 1 and 2 (bands at 1200 bp, 2000 bp and at ca. 570 bp are missing). Lanes 6-8 have two additional bands in comparison to lane 5 at ca. 1200 bp and 2000 bp.

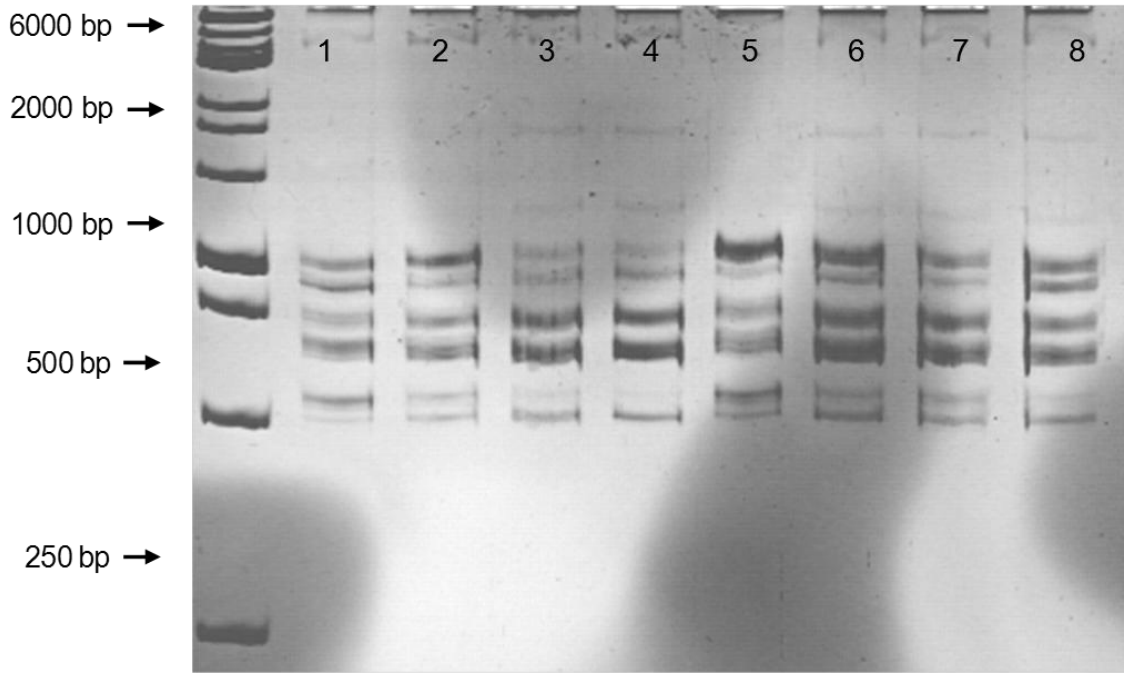

Figure 7: RISA patterns of the water kefir supernatant fermented with cranberry extract or apricot extract.
Water kefir grains from one batch (water kefir W) were portioned and fermented under different parameters. The acrylamide gel shows the RISA patterns of the cranberry and the apricot extract samples where fig extract was replaced by either cranberry or apricot extract. Each fermentation was done over 3 days and samples were taken from the supernatant after these 3 days of fermentation. New fermentations were started with the washed and strained water kefir grains respectively. This procedure was done over 12 days. DNA was isolated from the samples taken and a PCR was prepared with the RISA primers 23S r and 1406 f. Lane 1-4 shows the RISA patterns of the supernatant of cranberry extract fermentation whereas 1 reflects the sample after the first (3 days) and 2 after the second fermentation period etc. Lane 5-8 shows the supernatant of the apricot extract fermentation and 1 also reflects the RISA pattern derived from the first sample taken after 3 days of fermentation and 2 after another 3 days of fermentation etc.

Figure 8 shows the patterns of the standard water kefir fermentation (lane 1-4) and the fermentation at 37 °C (lane 5-8). The grains of the standard water kefir shows in the first fermentation step a lack of two bands one at ca. 500 bp and one at 1000 bp. No distinction could be obtained in lane 2 to lane 4. Within the 37 °C samples three bands disappeared in sampling 3 (lane 7).

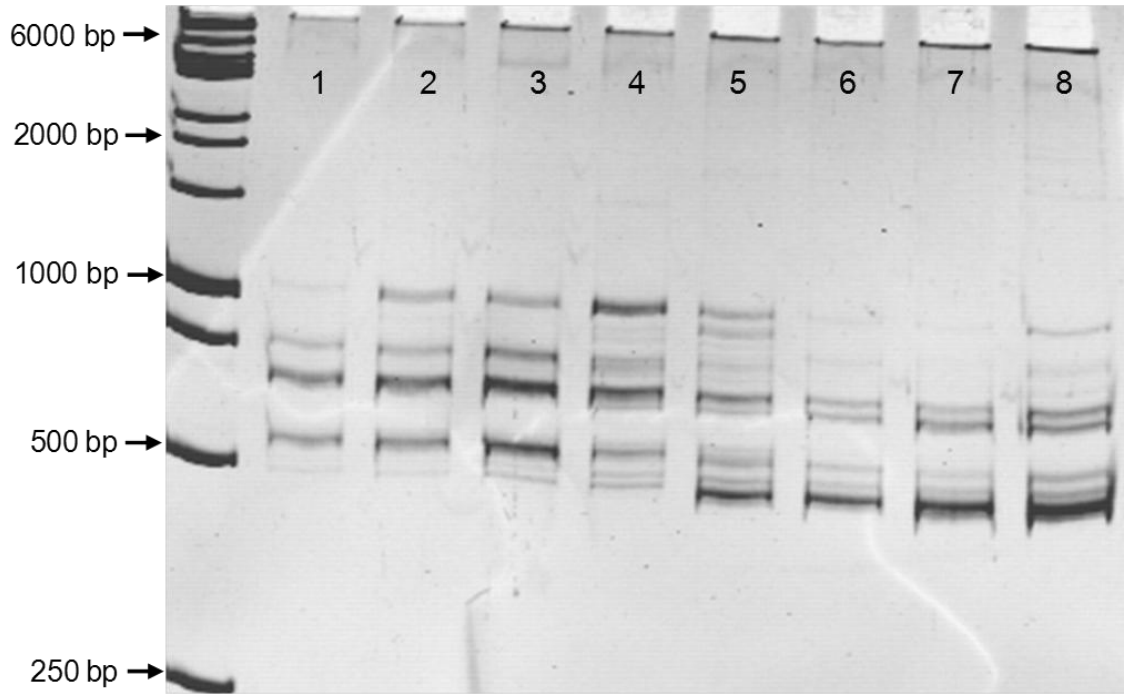

Figure 8: RISA patterns of water kefir grains fermented under standardized conditions and with a fermentation temperature of 37 °C.
Water kefir grains from one batch (water kefir W) were portioned and fermented under different parameters. The acrylamide gel shows the RISA patterns of the standard water kefir the water kefir fermented at 37 °C instead of 21 °C. Each fermentation was done over 3 days and samples were taken from the grains after these 3 days of fermentation. New fermentations were started with the washed and strained water kefir grains respectively. This procedure was done over 12 days. DNA was isolated from the samples taken and a PCR was prepared with the RISA primers 23S r and 1406 f. Lane 1-4 shows the RISA patterns from standard water kefir whereas 1 reflects the sample after the first (3 days) and 2 after the second fermentation period etc. Lane 5-8 shows the 37 °C sample and 1 also reflects the RISA pattern derived from the first sample taken after 3 days of fermentation and 2 after another 3 days of fermentation etc.

In Figure 9 the fermentation samples of the supernatant from standard water kefir and the 37 °C preparation is shown. Lane 3 (standard kefir, 3. preparation of new fermentation) shows two additional bands at app. 1300 bp and 1800 bp whereas one of the band (1300 bp) could also be detected in lane 2. Lane 5 (37 °C fermentation) shows more bands than lane 6-8, two bands in the higher molecular region (1500 bp and 1800 bp) and one band at ca. 800 bp.

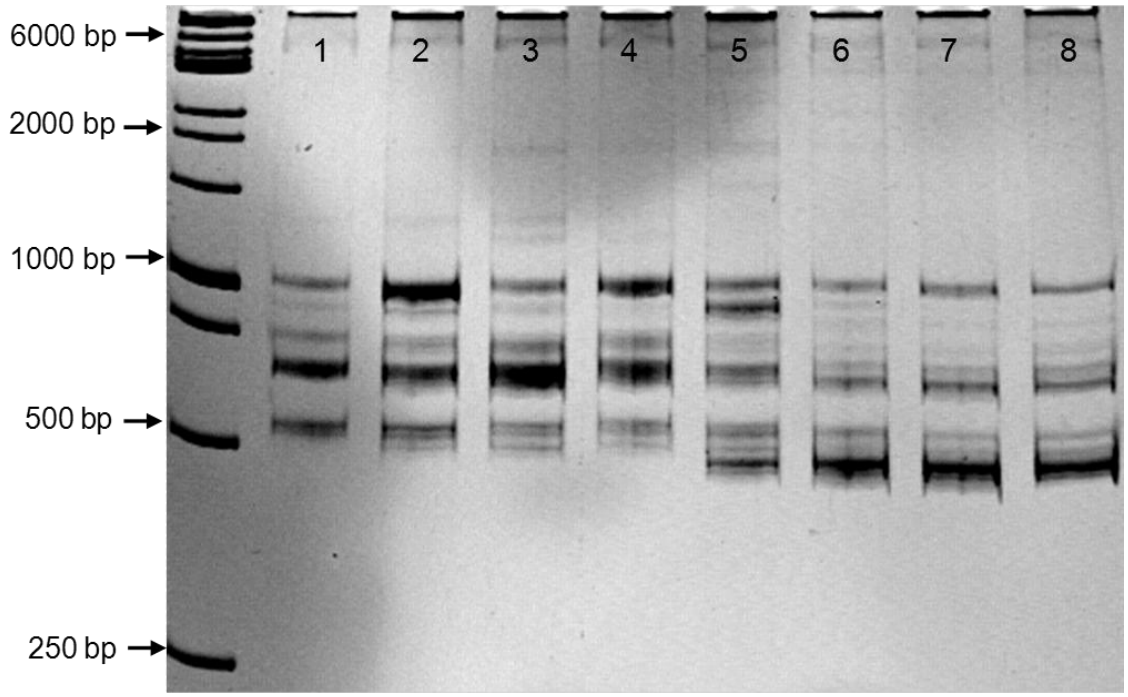

Figure 9: RISA patterns of the water kefir supernatant fermented under standardized conditions and with a fermentation temperature of 37 °C.
Water kefir grains from one batch (water kefir W) were portioned and fermented under different parameters. The acrylamide gel shows the RISA patterns of the standard water kefir the water kefir fermented at 37 °C instead of 21 °C. Each fermentation was done over 3 days and samples were taken from the supernatant after these 3 days of fermentation. New fermentations were started with the washed and strained water kefir grains respectively. This procedure was done over 12 days. DNA was isolated from the samples taken and a PCR was prepared with the RISA primers 23S r and 1406 f. Lane 1-4 shows the RISA patterns from standard water kefir whereas 1 reflects the sample after the first (3 days) and 2 after the second fermentation period etc. Lane 5-8 shows the 37 °C sample and 1 also reflects the RISA pattern derived from the first sample taken after 3 days of fermentation and 2 after another 3 days of fermentation etc.

Figure 10 represents the RISA patterns of the 12 °C fermentation of the water kefir grains (lane 1-4) and the supernatant (lane 5-8). In lane 2 one additional band at ca. 750 pb appears and in lane 3 and 4 another band at ca. 1400 bp. The supernatant of the 12 °C sample shows differences after 3 new starts of fermentation (lane 7) with a band at ca. 480 pb, 1100 bp and 1500 pb (lane 8).

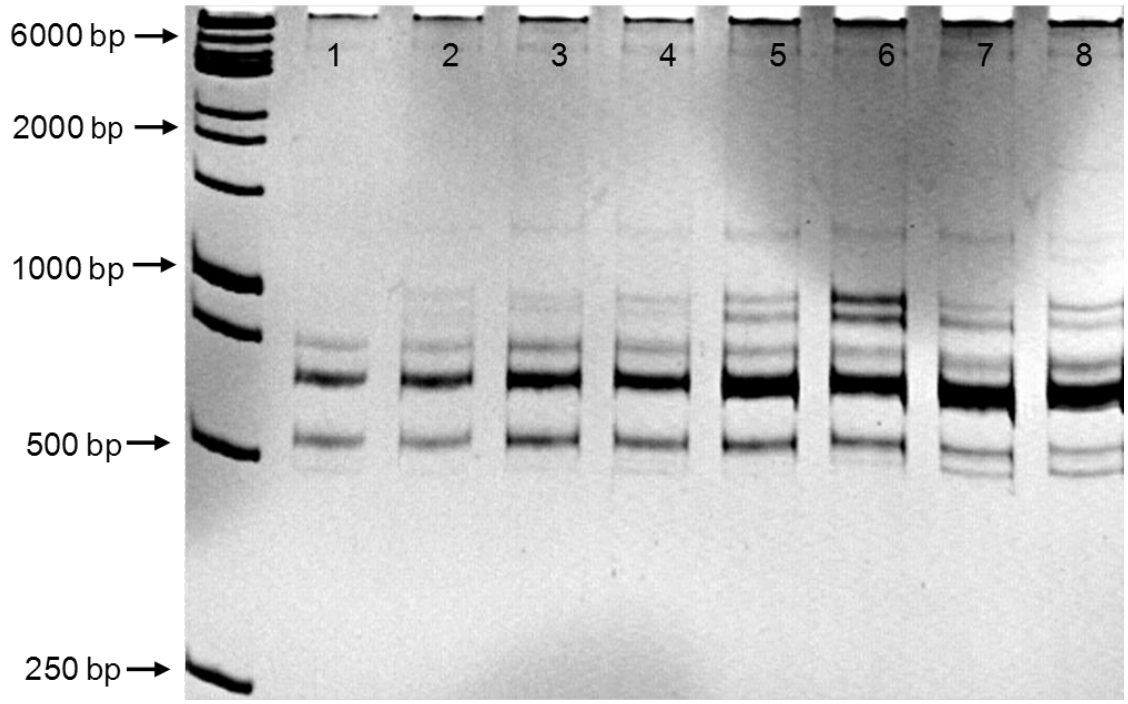

Figure 10: RISA patterns of the water kefir grains and the supernatant fermented at 12 °C.
Water kefir grains from one batch (water kefir W) were portioned and fermented under different parameters. The acrylamide gel shows the RISA patterns of the water kefir fermented at 12 °C instead of 21 °C. Each fermentation was done over 3 days and samples were taken from the grains and the supernatant after these 3 days of fermentation. New fermentations were started with the washed and strained water kefir grains respectively. This procedure was done over 12 days. DNA was isolated from the samples taken and a PCR was prepared with the RISA primers 23S r and 1406 f. Lane 1-4 shows the RISA patterns of the grains of 12 °C fermentation whereas 1 reflects the sample after the first (3 days) and 2 after the second fermentation period etc. Lane 5-8 shows the supernatant of the 12 °C fermentation and 1 also reflects the RISA pattern derived from the first sample taken after 3 days of fermentation and 2 after another 3 days of fermentation etc.

3.3.4 High-throughput sequencing of the water kefir samples grown under different conditions

For culture-indepent analysis of the water kefir samples grown with apricot extract, cranberry extract, at 37 °C and 12 °C and under standardized conditions. Based on the results of the RISA procedure (see 3.3.3) the samples of the third new fermentation from the five different parameter procedures were analyzed by high-throughput sequencing (LGC Genomics, Berlin, Germany). Two different primer pairs were used to analyze the influence of the primers and to compare the data sets obtained.

3.3.4.1 High-throughput sequencing analysis of the sequences obtained with the GM3 and 926R primer

The primers used for amplification were GM3 (5`-AGAGTTTGATCMTGGC-3`) targeting *E. coli* position 8-24 and 926R (5′-CCGTCAATTCMTTTRAGTTT-3′). A total of 9172 (standard kefir, water kefir grain), 6033 (standard kefir, supernatant), 9561 (apricot extract, water kefir grain), 16827 (apricot extract, supernatant), 10945 (cranberry extract, water kefir grain), 9782 (cranberry extract, supernatant), 16429 (37 °C, water kefir grain), 26567 (37 °C, supernatant), 12566 (12 °C, water kefir grain) and 28861 (12 °C, supernatant) reads of the V4 region of the 16S rRNA were used for taxon assignment. In all data sets the 16S rRNA chimeric sequences were determined by using Database Enabled Code for Ideal Probe Hybridization Employing R (DECIPHER) software (Wright et al., 2013). All data sets showed chimeric sequences (standard, water kefir grain 66, standard supernatant 70, apricot extract water kefir grain 232 and supernatant 571, cranberry extract water kefir grain 319 and supernatant 771, 37 °C water kefir grain 4 and supernatant 101, 12 °C water kefir grain 166 and supernatant 164). The distribution of the different bacteria at family level was evaluated by the software tools provided by the Ribosomal Database

project II (Cole et al., 2009), demonstrating the presence of *Acetobacteraceae*, *Lactobacillaceae*, *Leuconostocaceae*, *Enterobacteriaceae*, *Caulobacteriaceae*, *Pseudomonadaceae* and *Xanthomonadaceae* (Figure 11 and Figure 12). The most abundant bacteria in the water kefir grain were *Lactobacillaceae* (standard 36.6 %, 37°C 95.9 % and 12°C 24.7 % (Figure 12) apricot extract 45.9 % (Figure 11) the only exception was the sample with cranberry extract where no *Lactobacillaceae* could be detected but *Enterobacteriaceae* sequences were found in higher concentrations (31.5 %). *Leuconostocaceae* also showed a high abundance in the water kefir grain (standard 54.5 %, 12 °C 61.5 % (Figure 12) and apricot extract 35.3 % (Figure 11)) in the samples of cranberry extract and 37 °C no *Leuconostocaceae* were found. The supernatant differences in the abundance whereas a high abundance of *Acetobacteraceae* were found in the samples standard (72.9 %), *Lactobacillaceae* in the samples apricot extract (24.8%) and 37°C (92%). The samples standard and 12 °C showed a high concentration of *Leuconostocaceae* (23.3 % and 92.1 %, respectively). *Enterobacteriaceae* in the supernatant could be detected in high amounts in the samples apricot extract and cranberry extract (61.1 % and 64.1 %, respectively).

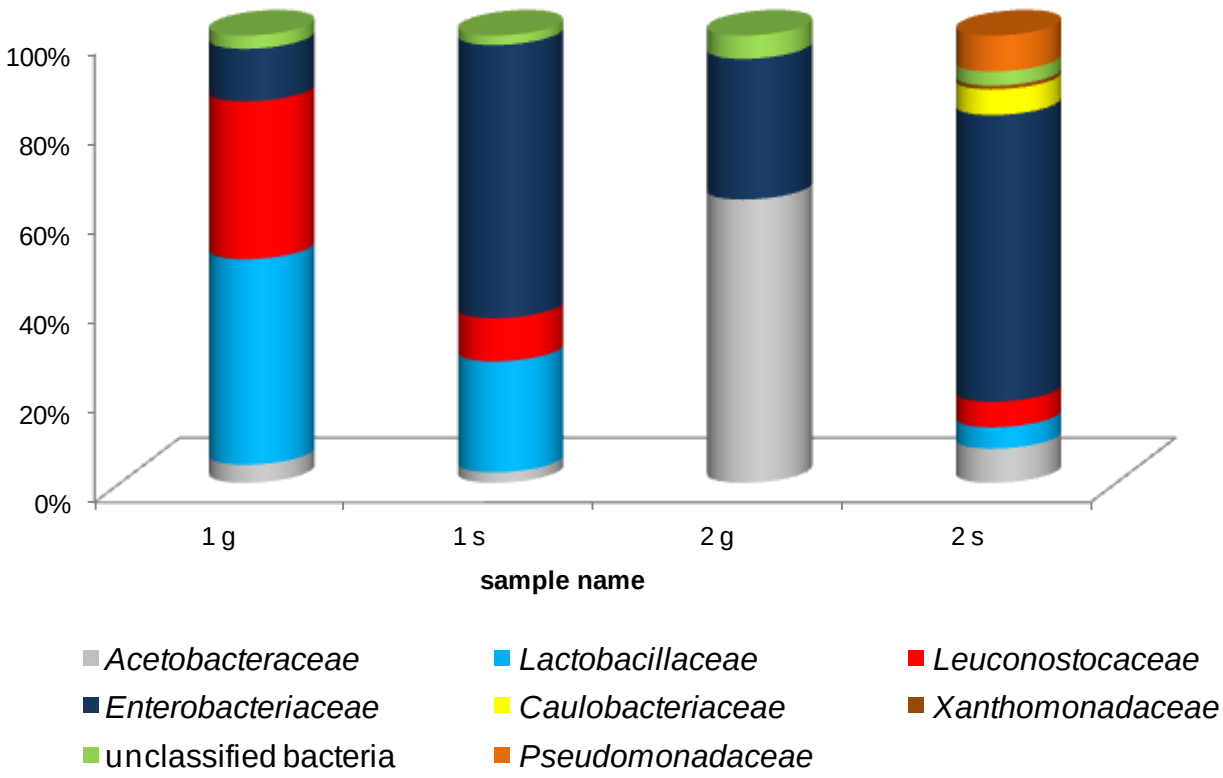

Figure 11: Microbial diversity of one water kefir grown with cranberry and apricot extract on family scale.
The water kefir grains used were from one batch and grown under following conditions: 1 = with apricot extract, 2 = with cranberry extract. g stands for water kefir grain and s for the supernatant analyzed. The DNA was isolated from the grains and supernatant after three fermentations and then amplified by LGC Genomics (Berlin, Germany), the primer used were GM3 and 926R.

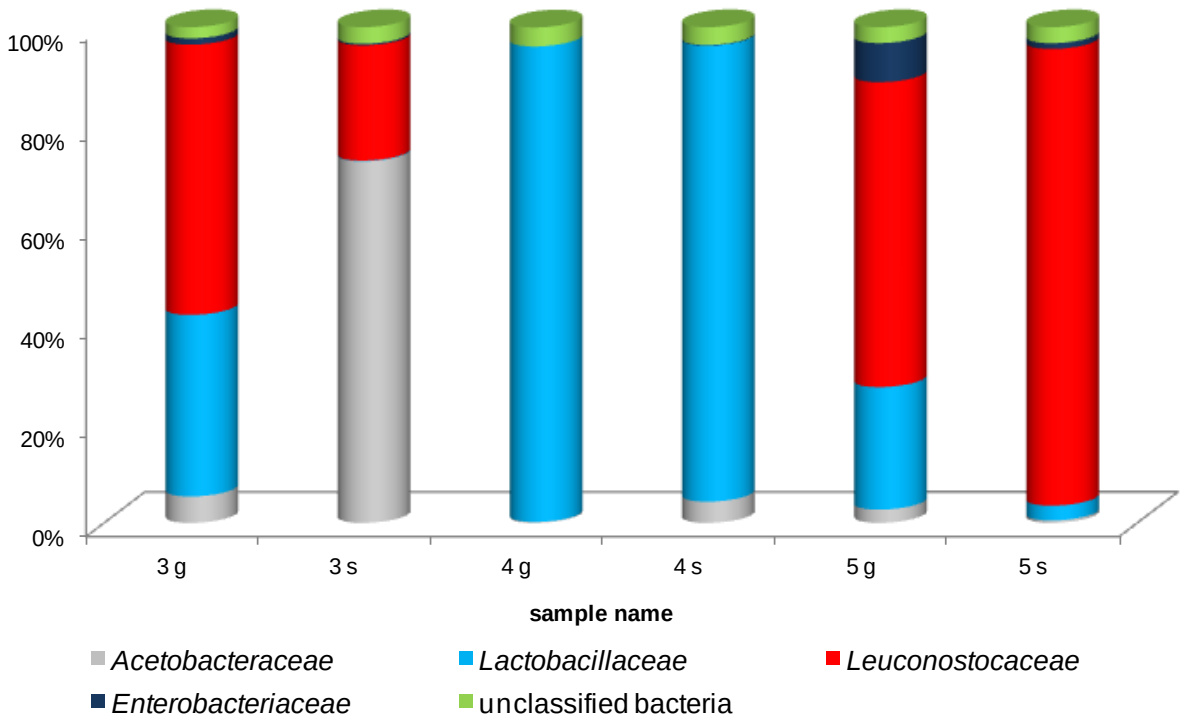

Figure 12: Microbial diversity of one water kefir grown under standard condition, at 37 °C and at 12 °C on family scale.
The water kefir grains used were from one batch and grown under following conditions: 3 = standard water kefir, 4 = grown at 37 °C and 5 = grown at 12 °C. g stands for water kefir grain and s for the supernatant analyzed. The DNA was isolated from the grains and supernatant after three fermentations and then amplified by LGC Genomics (Berlin, Germany), the primer used were GM3 and 926R.

3.3.4.2 High-throughput sequencing analysis of the sequences obtained with the 27 f and 519 R primer

For the second high-through put procedure the primer 27 f and 519 R were used. A total of 7540 (standard, water kefir grains), 24293 (standard, supernatant), 5678 (apricot extract, water kefir grains), 10455 (apricot extract, supernatant), 5138 (cranberry extract, water kefir grain), 6275 (cranberry extract, supernatant), 22517 (37 °C, water kefir grain), 24120 (37 °C, supernatant), 5346 (12 °C, water kefir grain), 29786 (12 °C, supernatant) reads of the V4 region of the 16S rRNA were used for taxon assignment. In all data sets the 16S rRNA chimeric sequences were determined by using Database Enabled Code for Ideal Probe Hybridization Employing R (DECIPHER) software (Wright et al., 2013). All data sets showed chimeric sequences (standard, water kefir grain 367, standard supernatant 9, apricot extract water kefir grain 124 and supernatant 12, cranberry extract water kefir grain 174 and supernatant 19, 37 °C water kefir grain 14 and supernatant 4, 12 °C water kefir grain 131 and supernatant 63. The distribution of the different bacteria at family level was evaluated by the software tools provided by the Ribosomal Database project II (Cole et al., 2009), demonstrating the presence of *Actinobacteraiceae*, *Acetobacteraceae*, *Lactobacillaceae*, *Leuconostocaceae*, *Enterobacteriaceae*, *Caulobacteriaceae*, *Pseudomonadaceae* and *Xanthomonadaceae* (Figure 13 and Figure 14). The most abundant species in the water kefir grain samples showed different species, *Lactobacilliceae* were most abundant in the samples standard, 37 °C, 12 °C and apricot extract, (34.1 %, 94.6 %, 21.6 % (Figure 14) and 30.7 % (Figure 13), respectively), in the apricot sample *Actinobacteriaceae* showed a high percentage with 34.6 %, *Leuconostocaceae* were found in high numbers in the samples standard, 12 °C and cranberry extract (57 %, 57.5 %, and 41 %, respectively).

Enterobacteriaceae were present in a high percentage in the cranberry extract sample with 32.5 %. In the supernatant samples *Lactobacilliceae* were present in high amounts in the samples standard, 37 °C and apricot extract and (18.6 %, 90.5 % and 32.2 %, respectively), the standard and 12 °C samples showed a high number of *Leuconostocaceae* with 69.5 % and 75.8 %, respectively. *Enterbacteriaceae* were found in the samples apricot extract (49.7 %) and cranberry extract (70.7 %).

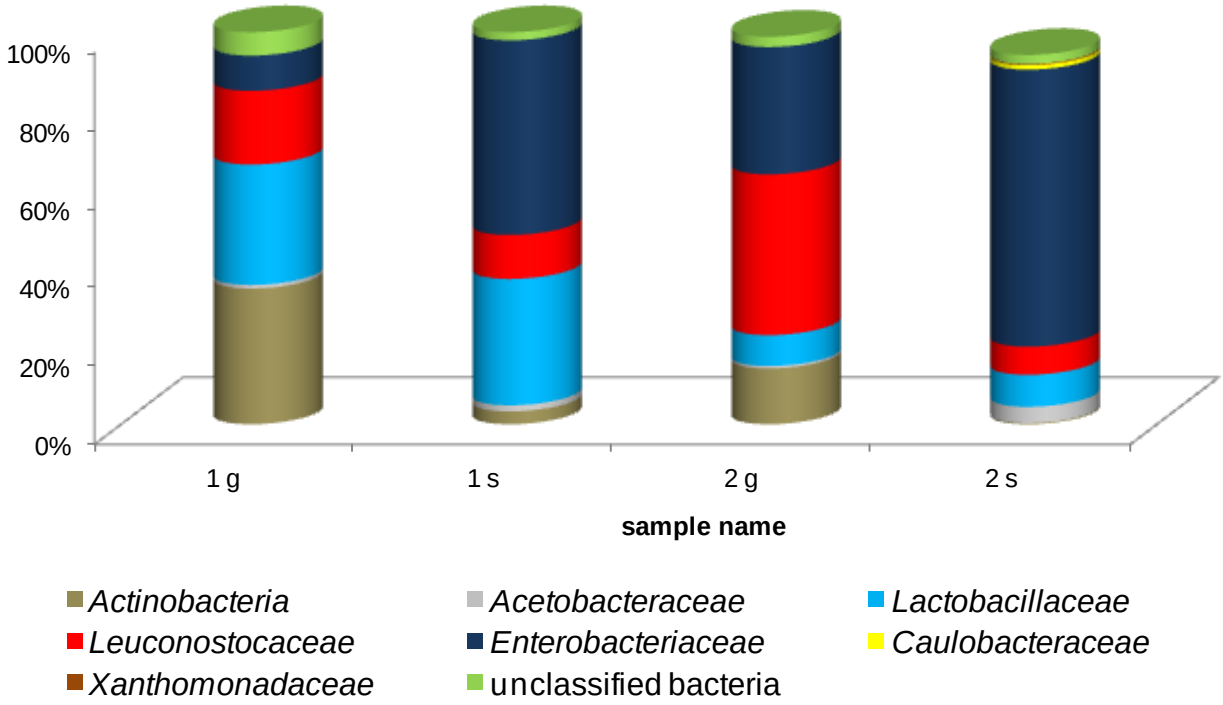

Figure 13: Microbial diversity of one water kefir grown with cranberry extract and apricot extract on family scale.
The water kefir grains used were from one batch and grown under following conditions: 1 = with apricot extract, 2 = with cranberry extract. g stands for water kefir grain and s for the supernatant analyzed. The DNA was isolated from the grains and supernatant after three fermentations and then amplified by LGC Genomics (Berlin, Germany), the primer used were 27 f and 519 R.

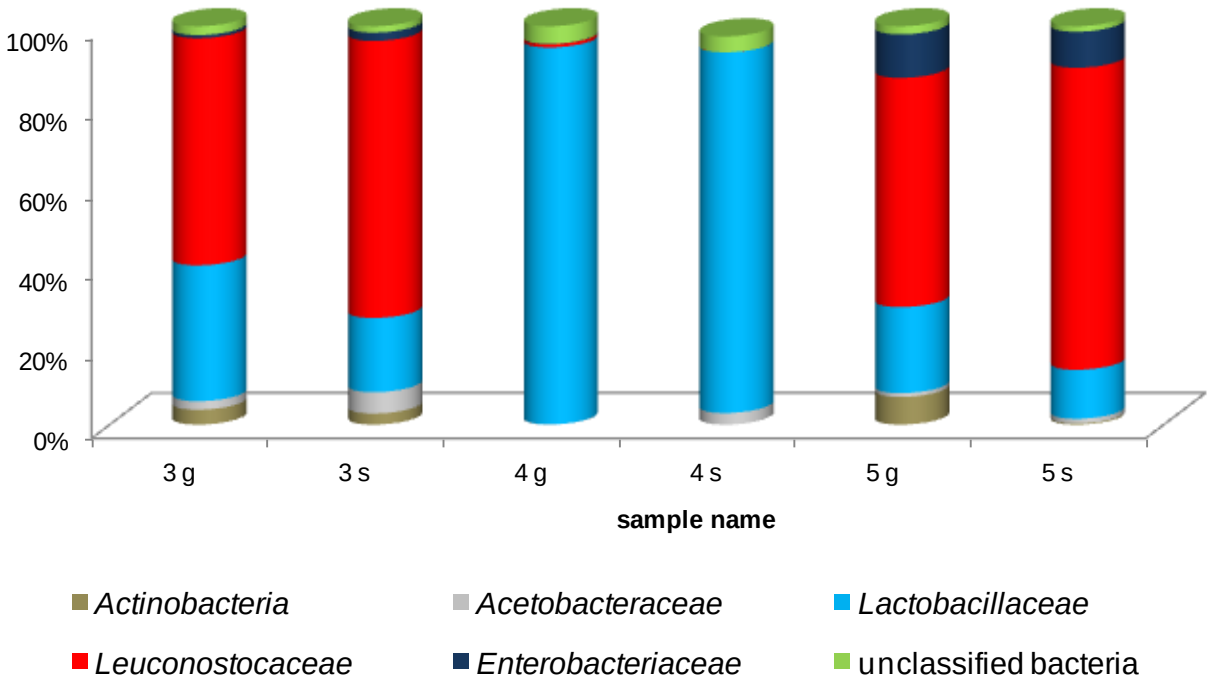

Figure 14: Microbial diversity of one water kefir grown under standard conditions, at 37 °C and 12 °C on family scale.
The water kefir grains used were from one batch and grown under following conditions: 3 = standard water kefir, 4 = at 37 °C and 5 = at 12 °C. g stands for water kefir grain and s for the supernatant analyzed. The DNA was isolated from the grains and supernatant after three fermentations and then amplified by LGC Genomics (Berlin, Germany), the primer used were 27 f and 519 R.

3.4 Fluorescence in situ hybridization (FISH) of water kefir grains and the supernatant

For the fluorescence in situ hybridization analysis of water kefir grain and the supernatant three different probes were used. For detecting bacteria the EUB 338-I probe was used, EUB probe for detecting eukaryotes and HGC 69a for actinobacteria within the grains and the supernatant of water kefir. The water kefir grain analysis only showed fluorescing rods with the EUB 338-I probe labeled with the green fluorescing FLUOS dye (Figure 15).

The *Actinobacteriaceae* probe HGC 69a labeled with CY5 did not fluoresce. The blue small particles showing fluorescence are no bacteria but particles with autofluorescence.

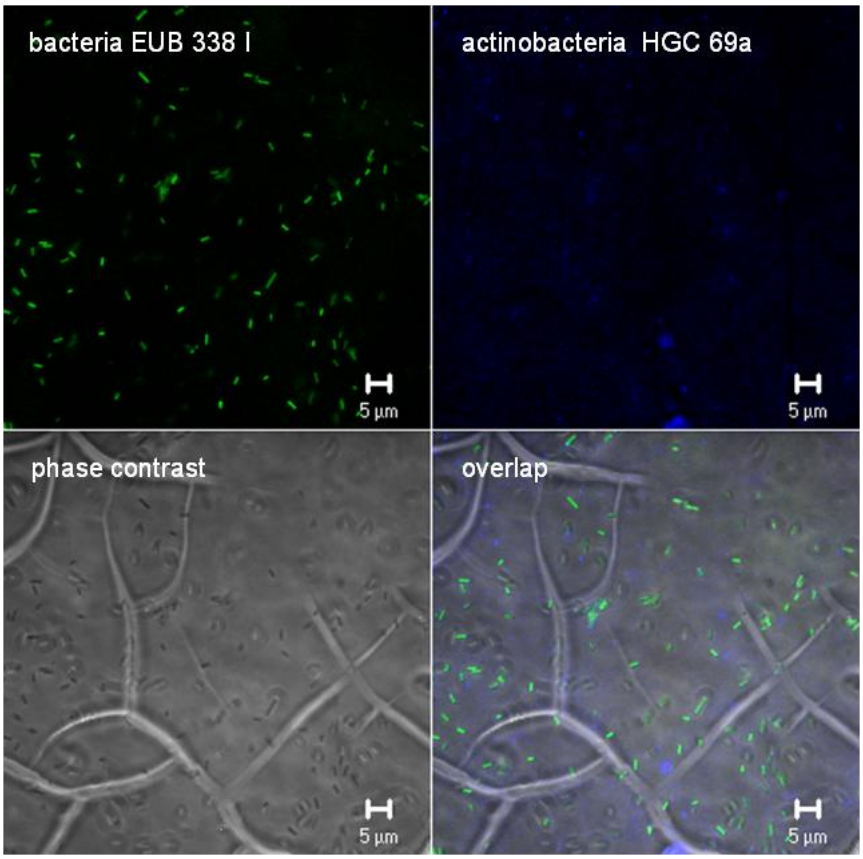

Figure 15: Microscopic image of FISH analysis of the water kefir grain with the EUB 338-I probe labeled with FLUOS dye. The fixation of the grain was done with ethanol for 2 h.

The supernatant of the water kefir was hybridized with the *Actinobacteriaceae* probe HGC 69a labeled with FLUOS Figure 16. The other probes used did not fluoresce.

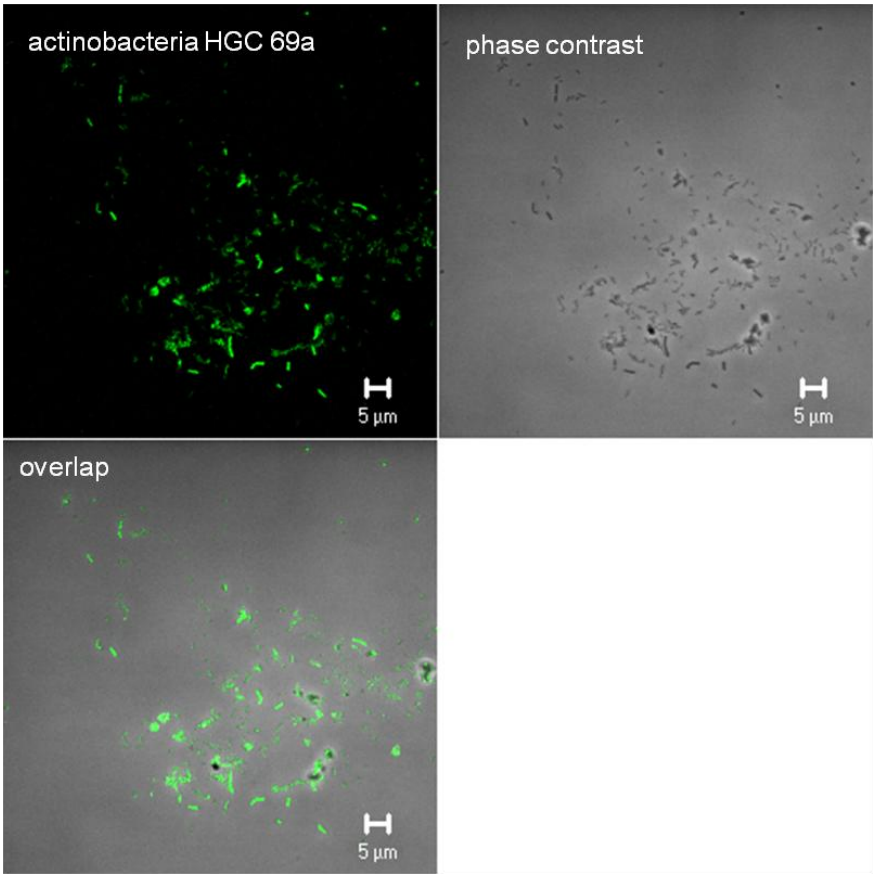

Figure 16: Microscopic image of FISH analysis of the water kefir supernatant with the actinobacteria probe HGC 69a labeled with FLUOS.

3.5 New bifidobacteria sequences

For detecting and identifying bifidobacteria the different water kefir samples were grown with suitable dilutions on the specific bifidobacteria medium (see 3.2.3). Single colonies were picked and streaked on new agar plates for gaining pure cultures. The identification was done by MALDI-TOF MS.

Two colonies could be identified having bifidobacterial sequences, which could not be assigned to any known bifidobacteria species. One colony was named “63” and showed the nearest homology to an uncultered bifidobacterial clone with 99 % identity (JN620478) isolated from activated sludge from a bioreactor treating for high organic synthetic wastewater in China (Beijing). The identity to the “new” bifidobacteria sequence with the accession number HE804184 was 98 %.

3.6 Freeze-drying of water kefir grains

3.6.1 Analysis of the revitalization of water kefir grains after freeze-drying

The water kefir grains from water kefir W were grown under standardized condition to perform this investigation from one batch of grains. The grains were splitted to equal amounts and frozen at different temperatures (-12 °C, -20 °C and -80 °C) for 24 h or quick-frozen with liquid nitrogen directly before the freeze-drying procedure. After the freeze-drying the water kefir grains were cultured under standardized conditions and every third day a new fermentation was started and the wet mass of water kefir grains was determined.

In Figure 17 water kefir grains are shown grown under standardized conditions with their normal appearance and after the freezing and freeze drying process.

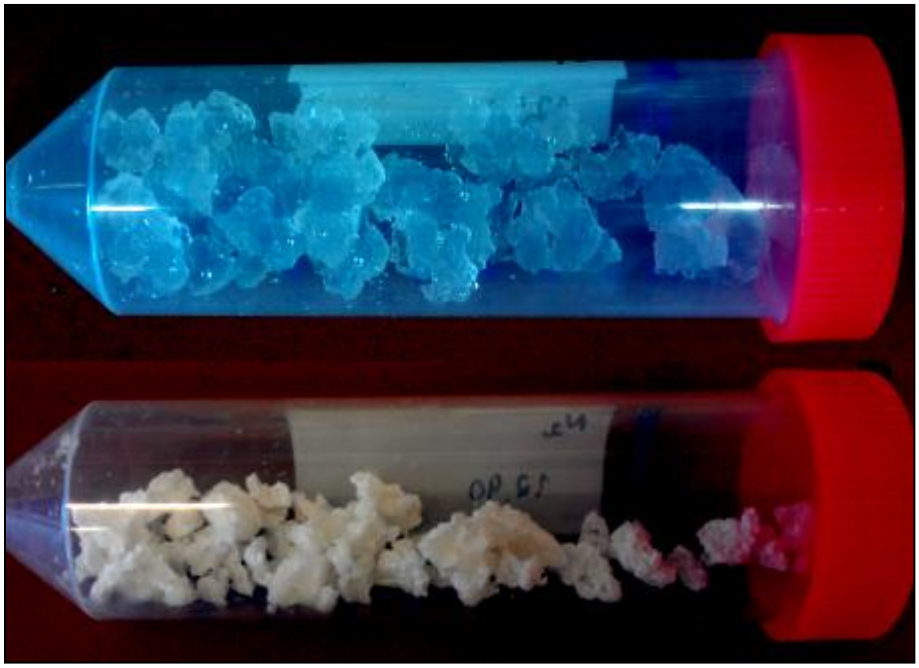

Figure 17: Falkon tubes with water kefir grains.
On the top of the figure the water kefir grain grown under standardized conditions are shown. On the bottom of the figure water kefir grains after freezing and freeze drying are shown.

Figure 18 shows the increase and decrease of the grain mass of the -12 °C, -20 °C and -80 °C sample. The difference of the inserted and the measurement after 3 days of fermentation of the water kefir grain mass is illustrated in the figure. The first two fermentations (6 days) show an increase of grain mass whereas the second fermentation only showed a slight increase. After 3 sets of fermentation the -12 °C and -20 °C sample showed a decrease of grain mass, the -80 °C sample showed a low increase. At the point of the fourth fermentation they all showed a decrease in the grain mass. The standard water kefir showed a stable increase of grain mass over the whole time of the experiment. After six fermentations the water kefir grain of the -12 °C, -20 °C and -80 °C were almost dissolved.

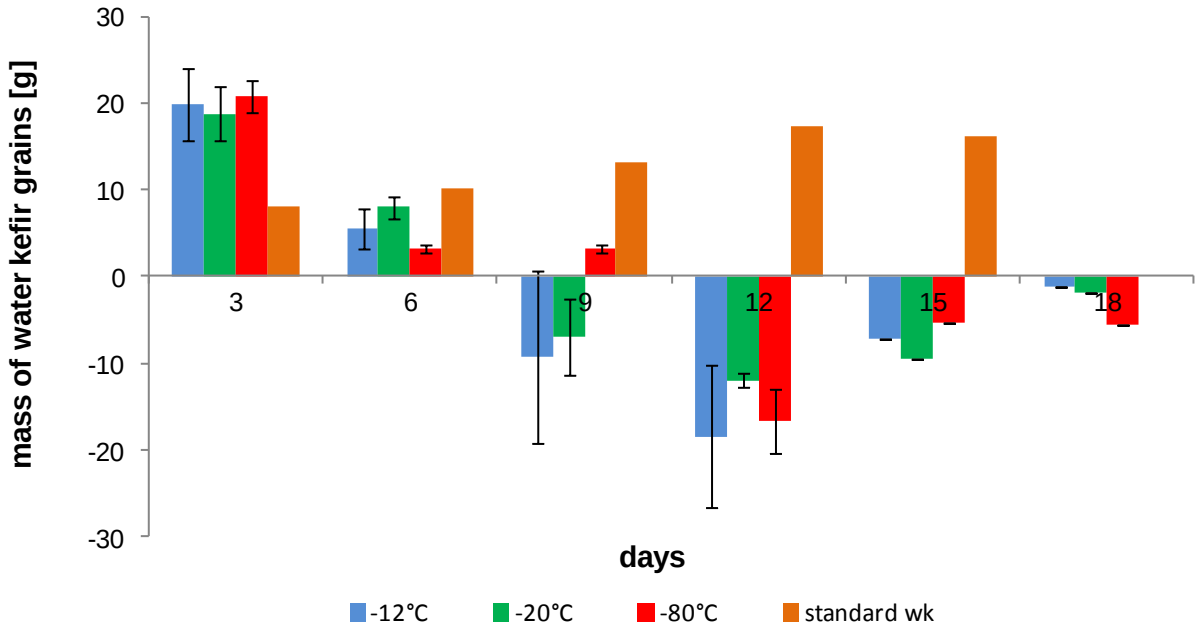

Figure 18: Reproduction of water kefir grain mass after different freeze-drying experiments and in comparison to the standard water kefir.
Water kefir grains from one batch were separated and the same grain amount were first frozen at -12 °C, -20 °C and -80 °C for 24 h. After that the freeze-drying procedure was done for 24 h. The freeze-dried water kefir grains were recultured under standardized conditions and after three days of fermentation the wet mass of grains were measured. This procedure was repeated six times (18 days) with the washed and strained water kefir grains. The standard water kefir was cultured under standardized conditions over the whole time of the experiment. The increase and decrease of grain mass compared to the grain mass inserted at the fermentation start is illustrated in the figure.

Figure 19 shows the appearance of the water kefir grains, grown under standardized conditions (left side) and after freezing at -12 °C and freeze-drying over night followed by regrowth under standardized conditions over three fermentations. The grain consistency changed drastically. The water kefir grains quick freeze-dried with N_2, freeze-drying over night and regrown under standardized conditions did show the same shape and consistency like the standard water kefir (shown on the left side of the picture).

Figure 19: Strained water kefir grains after growth.
On the left side water kefir grains were grown under standardized conditioned and strained through a sieve after three days of fermentation. On the left side strained water kefir grains are shown after freezing at -12 °C and freeze-drying over night. The water kefir grains were fermented three times (nine days).

The quick-frozen sample showed a stable increase of water kefir grain mass (Figure 20). To ensure the stability of this water kefir grains the experiment was enlarged and the wet grain mass was detected over 45 days (15 starts of new fermentations).

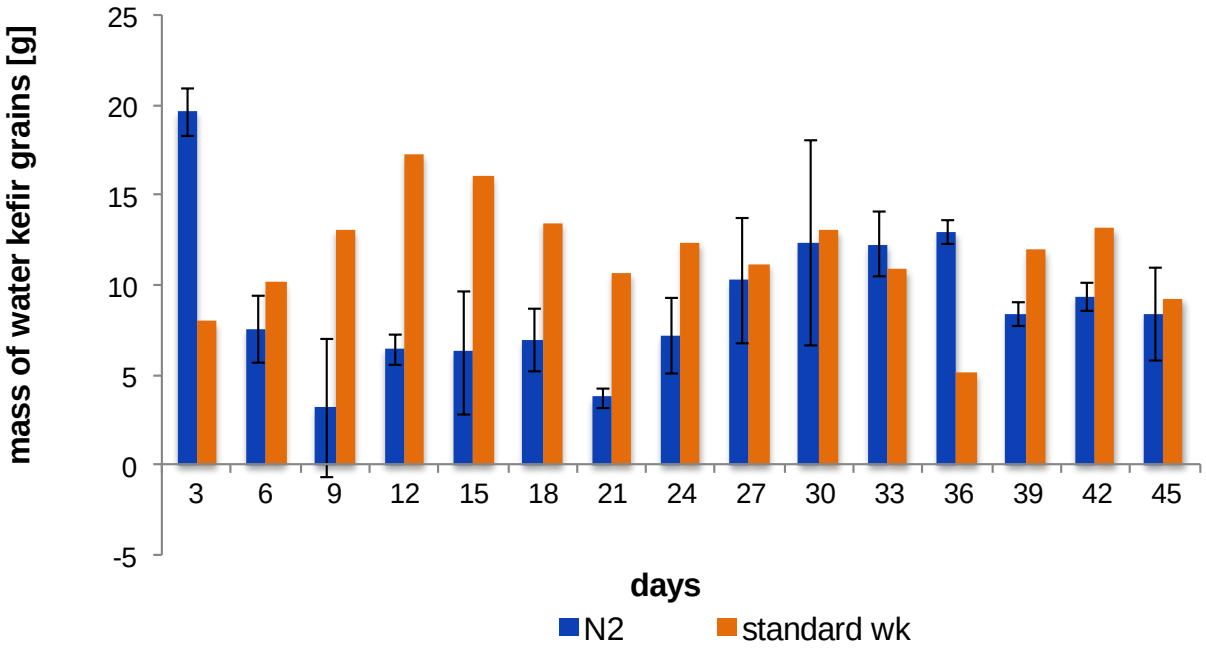

Figure 20: Reproduction of water kefir grain mass after different freeze-drying experiments. Water kefir grains from one batch were separated and the same grain amount was first quick-frozen with liquid N_2. After that the freeze-drying procedure was done for 24 h. The freeze-dried water kefir grains were recultured under standardized conditions and after three days of fermentation the wet mass of grains were measured. This procedure was repeated 15 times (45 days) with the washed and strained water kefir grains. The standard water kefir was cultured under standardized conditions over the whole time of the experiment. The increase and decrease of grain mass compared to the grain mass inserted at the fermentation start is illustrated in the figure.

3.6.2 Identification of bacteria and yeast species after freeze-drying and recultivation of water kefir grains

For the following analysis the -12 °C and the N_2 sample were selected due to the different behavior of the water kefir grains after the freezing and freeze-drying experiment. With the comparison of the different samples you might analyze a bacteria or yeast species or group which might be sensitive to the freezing and freeze-drying procedure.

Therefore, the viable cell count on different media (mMRS, GM, TP and YPG) were enumerated and 50 colonies of an appropriate dilution were identified by MALDI-TOF MS.

The viable cell count of the -12 °C sample was lower directly after the freeze-drying than after recultivation of the water kefir grains (Table 21). Differences could be detected within the comparison of the -12 °C sample where the cell number counted directly after the freeze-drying is much more lower than after regrowth (*Lb. hordei* with $2.9*10^3$ and $1.6*10^7$, *Lb. paracasei* with $4.5*10^3$ and $2.3*10^6$ respectively, *Lc. citreum* could not be detected after freeze-drying but after regrowth with $8.4*10^7$). On the other hand directly after freeze-drying three lactobacilli species were detected (*Lb. satsumensis*, *Lb. nagelii* and *Lb. hilgardii* all with a cell count of $2.2*10^2$) but after regrowth they could not be identified. The N_2 sample showed no significant difference between the two analyzes. No differences in the cell count of *Lb. hordei* and *Lb. paracasei* could be detected after freeze-drying and regrowth. *Lb. nagelii* could not be detected at all, *Lb. hilgardii* and *Lb. satsumensis* only directly after freeze-drying ($2.2*10^4$, $1.5*10^5$). *Lc. mesenteroides* was just identified after regrowth ($3.3*10^6$). Comparing the data obtained with the reference the viable cell count after regrowth is almost the same with the species *Lc. mesenteroides*, *Lb. hordei* and the cfu complete whereas *Lb. paracasei*, *Lb. satsumensis*, *Lb. nagelii* and *Lb. hilgardii* could not be identified.

Table 21: Identification and enumeration of lactic acid bacteria on mMRS media directly detected after freeze-drying and after recultivation of water kefir grains.

	-12 °C [cfu/g]		N_2 [cfu/g]		reference
	fz*	rg**	fz	rg	[cfu/g]
total cfu	$1.1*10^4$	$1.2*10^8$	$1.0*10^6$	$4.4*10^6$	$2.4*10^7$
Lc. citreum	nd	nd	nd	nd	$1.7*10^6$
Lc. mesenteroides	nd	$8.4*10^7$	nd	$3.3*10^6$	$8.2*10^6$
Lb. hordei	$2.9*10^3$	$1.6*10^7$	$6.0*10^5$	$9.1*10^5$	$1.4*10^7$
Lb. paracasei	$4.5*10^3$	$2.3*10^6$	$1.5*10^5$	$1.7*10^5$	nd
Lb. satsumensis	$2.2*10^2$	nd	$1.5*10^5$	nd	nd
Lb. nagelii	$2.2*10^2$	nd	nd	nd	nd
Lb. hilgardii	$2.2*10^2$	nd	$2.2*10^4$	nd	nd

*fz= samples analyzed directly after freeze-drying
**rg= samples analyzed after recultivation of water kefir grains under standardized conditions

The viable cell counts on the GM medium (Table 22) after regrowth is in the same range compared to the standard kefir. The -12 °C sample showed a lower cell number after freeze-drying whereas the N_2 sample showed nearly the same cfu after freeze-drying as well as after regrowth.

Ac. ghanensis could only be detected after regrowth of the N_2 sample ($1.1*10^5$) and *Ac. orientalis* was only identified in the standard water kefir ($7.4*10^6$). *Gb. oxydans* could be identified in the -12 °C but only after freeze-drying ($8.1*10^3$), in the N_2 sample the viable cell count after freeze-drying was just marginally lower ($1.6*10^4$) than after freeze-drying ($1.1*10^5$). The other species identified were lactobacilli. *Lb*. *satsumensis* was detected in the -12°C sample after regrowth ($2.5*10^6$) and in the N_2 sample only after freeze-drying ($9.7*10^4$) whereas the standard water kefir showed no presence of *Lb. satsumensis*. *Lb. hordei* showed the same amount after regrowth in all samples (-12°C $9.9*10^6$, N_2 $1.3*10^6$ and $6.1*10^6$ in standard water kefir). *Lc. citreum* was only identified in the standard water kefir ($3.5*10^5$).

Lc. mesenteroides showed the same concentration after regrowth compared to the standard with $7.4*10^7$ for -12°C sample, $2.6*10^6$ for N_2 sample and $1.4*10^7$ for standard water kefir. *Lb. hilgardii* was only identified after regrowth of the N_2 sample ($3.3*10^5$).

Table 22: Identification and enumeration of acetic acid bacteria on GM media directly detected after freeze-drying and after recultivation of water kefir grains.

	-12 °C [cfu/g]		N_2 [cfu/g]		reference
	fz*	rg**	fz	rg	[cfu/g]
total cfu	$1.1*10^4$	$1.1*10^8$	$6.0*10^5$	$5.0*10^6$	$3.1*10^7$
Ac. ghanensis	nd	nd	nd	$1.1*10^5$	nd
Ac. orientalis	nd	nd	nd	nd	$7.4*10^6$
Gb. oxydans	$8.1*10^3$	nd	$1.6*10^4$	$1.1*10^5$	$1.5*10^6$
Lb. satsumensis	nd	$2.5*10^6$	$9.7*10^4$	nd	nd
Lb. hordei	$4.2*10^2$	$9.9*10^6$	$8.1*10^4$	$1.3*10^6$	$6.1*10^6$
Lc. citreum	nd	nd	nd	nd	$3.5*10^5$
Lc. mesenteroides	nd	$7.4*10^7$	nd	$2.6*10^6$	$1.4*10^7$
Lb. hilgardii	nd	nd	nd	$3.3*10^5$	nd

*fz= samples analyzed directly after freeze-drying
**rg= samples analyzed after recultivation of water kefir grains under standardized conditions

The bifidobacteria were cultured on TP media (Table 23), the -12 °C and the N_2 sample showed no colonies directly after freeze-drying but after regrowth of the grains B. psychraerophilum could be detected. The cell numbers were in the same range with 3.4*105 for the -12 °C and 2.7*104 for the N_2 sample compared with 2.0*105 in the standard water kefir.

Table 23: Identification and enumeration of bifidobacteria on TP media directly detected after freeze-drying and after recultivation of water kefir grains.

	-12 °C [cfu/g]		N_2 [cfu/g]		reference
	fz*	rg**	fz	rg	[cfu/g]
total cfu	nd	$3.4*10^5$	nd	$2.7*10^4$	$2.0*10^5$
B. psychraerophilum	nd	$3.4*10^5$	nd	$2.7*10^4$	$2.0*10^5$

*fz= samples analyzed directly after freeze-drying
**rg= samples analyzed after recultivation of water kefir grains under standardized conditions

The yeast species were identified and enumerated on YPG medium. Four different yeast species could be detected (Table 24). The viable cell count after regrowth of the -12 °C and N_2 was in the same range like the standard water kefir ($5.4*10^6$, $3.0*10^5$ and $1.6*10^6$ respectively). The viable cell count after freeze-drying of the -12°C sample was lower with $4.8*10^3$ cfu/g compared to the N_2 sample with $2.1*10^5$ cfu/g. *Hanseniaspora valbyensis* showed lower numbers in the -12 °C sample after freeze-drying ($1.6*10^3$) but after regrowth the cell number was in the same range like N_2 and the standard water kefir ($3.1*10^5$, $1.4*10^5$ and $9.7*10^4$). *Pichia membranifaciens* could not be detected after freeze-drying of the -12 °C and N_2 sample whereas after regrowth the cell number showed no significant difference ($1.1*10^5$ and $1.7*10^4$ cfu/g) compared to the standard water kefir with $6.0*10^5$ cfu/g. *Saccharomyces cerevisiae* was first analyzed in lower numbers after freeze drying (-12 °C sample with $6.0*10^2$ and N_2 sample with $4.1*10^3$) but after regrowth the cell number was comparable to the standard water kefir ($3.7*10^5$) where the -12 °C had a cfu/g of $1.2*10^5$, N_2 $1.3*10^5$. *Zygotorulaspora florentina* showed the same behavior with a cfu/g of $8.0*10^2$ after freeze-drying and $7.6*10^5$ cfu/g after regrowth. The cell number of the N_2 sample showed no difference between the freeze-drying ($4.1*10^4$) and regrowth of water kefir grains ($3.4*10^4$).

Table 24: Identification and enumeration of yeast species on YPG media directly detected after freeze-drying and after recultivation of water kefir grains.

	-12 °C [cfu/g]		**N_2 [cfu/g]**		**reference**
	fz*	**rg****	**fz**	**rg**	**[cfu/g]**
total cfu	$4.8*10^3$	$5.4*10^6$	$2.1*10^5$	$3.0*10^5$	$1.6*10^6$
Hanseniaspora valbyensis	$1.6*10^3$	$3.1*10^6$	$1.6*10^5$	$1.4*10^5$	$9.7*10^4$
Pichia membranifaciens	nd	$1.1*10^5$	nd	$1.7*10^4$	$6.0*10^5$
Saccharomyces cerevisiae	$6.0*10^2$	$1.2*10^6$	$4.1*10^3$	$1.3*10^5$	$3.7*10^5$
Zygotorulaspora florentina	$8.0*10^2$	$7.6*10^5$	$4.1*10^4$	$3.4*10^4$	$2.4*10^5$

*fz= samples analyzed directly after freeze-drying
**rg= samples analyzed after recultivation of water kefir grains under standardized conditions

4 Discussion

The aim of this work was to analyze and characterize the water kefir microbiota regarding their microbial composition. Culture-dependent and –independent technologies showed the same core of microorganisms belonging to the bacterial genera *Lactobacillaceae*, *Leuconostocaceae*, *Acetobacteraceae* and *Bifidobacteriaceae* as well the yeast species *Saccharomyces cerevisiae* and *Zygotorulaspora florentina*.

According to the results obtained it is possible to produce a water kefir based beverage with species isolated from water kefir.

The analysis of water kefir fermented under different growth conditions showed that the temperature and the nutrient provided by plant material are important parameters for the stability and dynamics of the water kefir microbiota.

Water kefir grains could be preserved by shock-freezing with N_2 and freeze-drying over night whereas the water kefir species showed different viability.

4.1 Microbial diversity of water kefir grains analyzed by culture-dependent procedures

In this part of the thesis state of the art molecular culture-dependent methods were used for the identification of several additional and/ or different species providing a revised view of the water kefir microbiota. Species were found in water kefir which have not been detected and identified in other water kefir studies so far and the abundance of species in the water kefir microbiota were qualitatively and quantitatively different from those previously described (Franzetti et al., 1998; Galli et al., 1995; Kebler, 1921; Lutz, 1899; Pidoux, 1989).

A typical consortium regarding this type of culture-dependent investigation appears to consist of mostly lactic acid bacteria plus yeasts promoting alcoholic fermentation and some acetic acid bacteria, possibly oxidizing the ethanol formed.

The yeast predominant in all three water kefirs (A, F and I) is most probably *Z. florentina* as demonstrated for water kefir W. *Z. florentina* showed a white cell morphology in this analyses. Detailed studies of this white cell morphology showed that more cells were identified as *Z. florentina*. The other water kefirs had a high percentage of cells showing white morphology. Referred to the result of the detailed study of the phenotype showing white morphology with the water kefir W, it might indicate that *Z. florentina* is the most predominant species in all water kefirs.

All yeast species identified in the water kefirs were able to ferment glucose and this could indicate that they all show the same underlying metabolism (Esteve-Zarzoso et al., 2001; Kurtzman and and Robnett, 2003). *S. cerevisiae* is able to invert sucrose into the monosaccharides glucose and fructose by the enzyme invertase so that the yeast cells have glucose as a free metabolite (Ikram-Ul-Haq and Ali, 2007).

This might indicate that in the other water kefirs the composition of these two yeasts are nearly the same and that *Z. florentina* is the yeast species which is predominant.

The bacterial core species of the three water kefirs belonging to lactic acid bacteria were *Lb. hordei*, *Lb. nagelii* and *Lc. mesenteroides*, whereas in water kefir W *Lb. hordei* and *Lb. nagelii* were detected in much lower concentrations. The presence of these two lactobacilli in all water kefir consortia claims that they are important for the stability of the water kefir. It is not surprising that *Lb. nagelii* and *Lb. hordei* are part of the water kefir consortia as it is an alcoholic environment with high sugar concentration at the beginning of the fermentation and a low pH after three days of fermentation with increasing alcoholic concentrations. The description of *Lb. nagelii* was in 2000 by C. Edwards and it firstly was isolated from partially fermented wine (Edwards, 2000). *Lb. hordei* was firstly described in 2008 and isolated von malted barley (Rouse et al., 2008). These habitats (partially fermented wine and malted barley) exhibits specific requirements like an alcoholic environment with a low pH. *Lb. nagelii* is described to produce EPS from dextran which could be important for the stability and forming of water kefir grain. Different strains of *Lb. nagelii* (TMW 1.1823, 1.1826, 1.1827) and *Lb. hordei* (TMW 1.1958) could be identified as EPS-producer emphasizing the importance of these species.

Leuconostoc species were found in all three water kefir consortia in significant percentages and were the main component in water kefir W. This high number of *Leuconostoc* species in the water kefir consortia indicates their competitiveness in the environment of water kefir. *Leuconostocs* have been found to play an important role in fermentations like sauerkraut, cucumbers, finger millet, Boza and kimchi where they are predominant in the early stages of fermentation where they have to compete with other LAB (Dworkin et al., 2006).

Lc. mesenteroides species could also be identified as EPS-producer (TMW 2.1073, 2.1076) underlining the important role within the consortia.

Acetic acid bacteria were also a part of the bacterial core in this water kefir analysis, especially in water kefir W. *Ac. fabarum* was firstly described 2008 and isolated from Ghanaian cocoa bean heap fermentation. *Ac. fabarum* is also able to fermentate ethanol to acetic acid (Cleenwerck et al., 2008). During the water kefir fermentation ethanol is formed mostly by yeasts so that acetic acid bacteria have a high nutrient source. On the other hand it is not preferable to have to high concentration of acetic acid bacteria regarding the taste of the resulting beverage. *Ac. orientalis* was firstly isolated from canna flowers, starfruit and coconut and from traditional fermented foods (curd of tofu and tempe) (Lisdiyanti et al., 2001). The natural habitat of this species is fermented food where they could metabolize ethanol formed from yeasts and or acetate formed by LAB, so they are resistant for low alcoholic concentrations like in the water kefir environment. The fruits and flowers originate from exotic environments this might be a hint that the water kefir grain grew on leaves of cactus and the origin is from the orient.

The composition of the microorganisms in the water kefir consortia resulting from culture-dependent analysis differs to the investigations done previously. In previous investigations *Lb. casei* was described as a very predominant species (Franzetti et al., 1998; Pidoux, 1989). With culture-dependent methods *Lb. casei* could not be found in high percentages. The presence of *Lb. casei in* water kefir A and F could be explained by the ubiquitous occurrence of this species while it unlikely exerts a metabolic influence of the properties of the resulting beverage. The same is valid for *Lb. hilgardii*, which was predominant in the sugar kefir grains studied by Pidoux (1989) with a percentage of 30 %.

The concentration of *Lb. hilgardii* was negligible in this investigation and it was only found in water kefir A. There are some *Lb. hilgardii* strains, which produce EPS (Pidoux et al., 1990) and which are even described to play a major role in forming the stable grains of water kefir (Waldherr et al., 2010) while none of the *Lb. hilgardii* strains isolated in this study were able to produce EPS.

Pidoux described that *Lb. brevis* is also an important LAB regarding the formation of gel-like grain (Pidoux et al., 1988) which could not be identified in the different water kefir microbiota. Horisberger described the main flora of water kefir consisting predominantly of *Lb*. *brevis*, *Streptococcus lactis* and the yeast *S. cerevisiae* (Horisberger, 1969). Both bacterial species could not be detected in the water kefir grains characterized in this investigation featuring the predominance of *Lb. nagelii*, *Lb. hordei* and *Lc. mesenteroides* in water kefirs A and F. This may be due to their absence in those samples. That is one reason why they could not delineate *Lb. brevis* from the other LABs in their previous investigation long before that time due to the lack of molecular typing techniques.

Some acetic acid bacteria were identified in previous studies in water kefir consortia but only in negligible quantity (Franzetti et al., 1998).

To conclude the differences in the composition of the water kefir consortia may be to some extent referred to different identification techniques used. The technique used for the identification of lactic acid bacteria and acetic acid bacteria in the investigations done so far was API CH 50 (Franzetti et al., 1998; Pidoux, 1989). As strains of different species can behave the same phenotype in some physiological tests the identification with these tests has clear limits towards a valid identification of *Lactobacillus* species closely related to each other (Boyd, 2005; Dalezios, 2001; Singh et al., 2009). By RAPD genotyping and identification of the species with

16S rDNA analysis it was possible to clearly allot isolates to species and also identify microorganisms in water kefir, which have not been identified yet from this source. The core organisms like *Lb. nagelii*, *Lb. hordei*, *Lc. mesenteroides* and *Ac. fabarum* were isolated and identified in all three water kefirs. On the other hand, the different composition of the three water kefirs indicates that the specific percentages of each species are not the most important characteristic of a stable water kefir microbiota. It rather suggests a metabolic core comprising the overall metabolic capacity of the consortium, which may be more important than the species designation of the strains found.

4.2 Microbial diversity of water kefir grains analyzed by culture-independent procedures

In this study, the bacterial communities of four different water kefirs were analyzed by a culture-independent, high-throughput sequencing procedure employing 16S rRNA gene amplicon sequencing. The microbiota of all kefirs consisted of bacteria from three phyla: *Firmicutes* (79 %)*, Actinobacteria* (17.1 %) and *Proteobacteria* (3.7 %).

The *Firmicutes* were the dominant organisms in all kefirs, represented mainly by the genera *Lactobacillus* and *Leuconostoc*.

Kefir I, which was directly analyzed upon arrival without any propagation showed high numbers of clostridia (37.2 %), while clostridial sequences represented less than 0.03 % of the sequences in the other kefirs. Interestingly, *Clostridiaceae* assignments accounted for 0.82 % of reads in the starter grains of milk kefir (Dobson et al., 2011). The high clostridia content and a noticeable off flavour were obviously consequences of improper propagation or contamination during preparation and/or shipment.

Clostridium tyrobutyricum, which was the most abundant *Clostridium* species, is known as a food spoiling bacterium and a major cause of late cheese blowing (Klijn et al., 1995) but is also found in environments related to water kefir, such as in fruit pulp (Mayer et al., 2010). Therefore, we do not consider kefir I to harbour a typical kefir consortium and did not include the data from this sample in the subsequent analysis.

The phylum *Actinobacteria* is represented exclusively by the genus *Bifidobacterium,* and *Proteobacteria* are represented by *Acetobacter, Gluconobacter* and *Gluconacetobacter*. Kefirs A, F and W differed in their composition only marginally.

Due to sequencing artefacts such as sequence-specific errors, miscounted homopolymeric runs etc., the use of 454 pyrosequencing data for community profiling is known to occasionally inflate estimates of actual diversity when taxon assignments are made using alignment-based approaches (Kunin et al., 2010). To prevent overestimates of diversity using regions of the 16S rDNA, a clustering threshold of 97 % identity is widely used. This limits the assignment of sequences at the current species threshold of 98.7 %. In this study, we used a suffix tree-based search for common heptanucleotide occurrences in the pyrosequencing data and reference data allowing similarity analyzes without the need for sequence alignments of large data sets. This allows a more reliable assignment of reads to a single species or species group.

The data from this study corroborate the identification of species that have been described in previous studies of water kefir using culture-dependent techniques (Gulitz et al., 2011). The main core of bacteria described here is similar to that shown in our previous investigation with culture-dependent methods, in which *Lb. hordei*, *Lb. nagelii*, *Lc. mesenteroides* and *Lc. citreum,* as well as *Ac. fabarum,* represented

the core group of organisms (Gulitz et al., 2011). In this study, kefir W had the highest number of *Leuconostocaceae*, and high numbers of *Lb. nagelii* and *Lb. hordei* sequences were found in water kefirs A and F. We were able to detect only small numbers of *Lb. casei* in all four water kefirs, while *Lb. casei* (Franzetti et al., 1998) and *Lb. hilgardii* were identified as the predominant bacterial species in other water kefirs (Pidoux, 1989). A low percentage of the sequences in water kefir A and F were derived from *Lb. hilgardii,* whereas water kefirs I and W showed a slightly higher percentage. This is consistent with the data obtained using the culture-dependent approach (Gulitz et al., 2011).

Based on the data obtained from culture-dependent and –independent analyses it was possible to generate a starter culture with the most abundant species isolated from water kefir microbiota (Gulitz et al., 2013 and 2011). As a result a water kefir based beverage could be produced with the project partner at Hochschule Ostwestfalen-Lippe, Technikum für Getränketechnologie, Lemgo.

4.2.1 Identification and characterization of bifidobacteria found in water kefir

The detection of bifidobacteria in water kefir is unexpected and has not been described previously. In this context, it is worth mentioning that we were not able to observe cells with bifidobacterial morphology using light microscopy. Notably, previous studies using scanning electron microscopy have not identified bifidobacteria as elements of the water kefir consortium (Neve and Heller, 2002).

This might be due to the atypical morphology of *B. psychraerophilum*, which is described to show bifurcation only occasionally (Simpson et al., 2004).

In our hands, neither the type strain of *B. psychraerophilum* nor strains isolated from water kefir in the present study showed bifidobacterial morphology, even in pure cultures (data not shown).

B. psychraerophilum firstly was isolated from porcine caecum but the authors claimed that it is possible that they are no resident bacteria of the caecum but might passing through and originate from food (Simpson et al., 2004). This species is able to tolerate and even grow under aerobic conditions which are given in the water kefir microbiota. But the water kefir grain itself also could exhibit anaerobic niches.

Recently, Dobson et al. (2011) reported the finding of bifidobacterial sequences in a culture-independent analysis of milk kefir. In this consortium, the *Bifidobacterium* population, containing the species *Bifidobacterium breve*, *Bifidobacterium choerinum*, *Bifidobacterium longum*, and *Bifidobacterium pseudolongum* comprised just 0.2 % of total assigned taxa in the collective starter grain and 0.4 % in the kefir milk. However, culture-dependent methods failed to detect *Bifidobacterium* species in either sample. This underscores the fact that culture-independent analysis is a powerful tool for the better understanding of microbial consortia and that bifidobacteria with unknown taxonomy and physiology may contribute to various extents to such consortia. This underlines the difficulty to culture bacteria under laboratory conditions, this is especially true for *Bifidobacteriaceae* due to their special nutritional requirements (Arunachalam, 1999).

Interestingly, a large number of bifidobacterial reads from water kefir could not be assigned to any known species.

The *Bifidobacterium*-specific 16S ARDRA analysis of 100 clones per kefir showed a single restriction profile in all of the kefirs that could be clearly differentiated from that of the type strains *B. crudilactis* FR62/b/3^T and *B. psychraerophilum* LMG 21775^T.

Moreover, the sequences of all corresponding amplicons showed a similarity of less than 97 % to its closest relatives, *B. crudilactis* FR62/b/3^T and *B. psychraerophilum* LMG 21775^T.

This result was unexpected because the metagenomic data showed ratios of 3:1, 9.4:1, 1:6.8 and 1:2.1 of *B. spec*. to the bifidobacteria *B. psychraerophilum, B. crudilactis* and *B. subtile*, respectively. Because the template DNA samples used for pyrosequencing and ARDRA and primer binding sites were identical, this discrepancy may be ascribed to an artificial cloning bias.

4.2.1.1 Cultivation of bifidobacteria isolated from water kefir

The selective cultivation of bifidobacteria from natural habitats harbouring lactobacilli and other lactic acid bacteria is difficult because the cultural and biochemical properties of both genera overlap (Thitaram et al., 2005). Many attempts to isolate bifidobacteria from water kefir with previously described media, including mupirocin-containing media, failed in our hands because the bifidobacteria were overgrown by lactic acid bacteria or yeasts (data not shown). Our modified selective medium suppresses yeast and LAB growth due to the high amounts of cycloheximide and kanamycin, and the addition of oligofructose as bifidogenic carbohydrate allowed the selective cultivation of up to $1.5*10^6$ cfu g^{-1} *B. psychraerophilum* alone from kefir granules.

4.2.2 Characterization of the novel bifidobacteria species found in water kefir

The isolation of novel *Bifidobacterium* species, the 16S sequence of which was highly abundant in the pyrosequencing and ARDRA data, was not successful, suggesting that either we were unable to meet its optimal growth conditions or the pyrosequencing data do not necessarily reflect the true numbers of living cells. Nevertheless, this medium may also be helpful to allow the cultivation of bifidobacteria from other sources from which the isolation of bifidobacteria has not been successful to date.

4.2.2.1 Phylogenetic position of water kefir bifidobacteria

The phylogentic tree of *Bifidobacteriaceae* based on partial 16S rRNA gene sequences including bifidobacteria isolated from water kefir in this thesis is shown in Figure 21. The phylogenetic tree shows that the bifidobacteria isolated from water kefir originate from a monophyletic group. In this group *B. mongoliense* is included, this species was isolated from fermented milk a habitat also having slightly alcoholic concentration (Watanabe et al., 2009). In this group all species are able to grow under aerobic conditions and tolerate low pH (Delcenserie et al., 2007; Simpson et al., 2004; Watanabe et al., 2009). *B. psychraerophilum* is able to grow at low temperature even at 4°C growth could be detected. The minimal growth temperature of *B. mongoliense* is 15°C. The optimal growth temperature of bifidobacterial species normally ranges from 37-41°C and growth below 20°C normally does not occur (Biavati et al., 2000). Bifidobacteria were mainly isolated from faeces (human and animal) some from bumblebee intestine, sewage and dental plaque (Russell et al.).

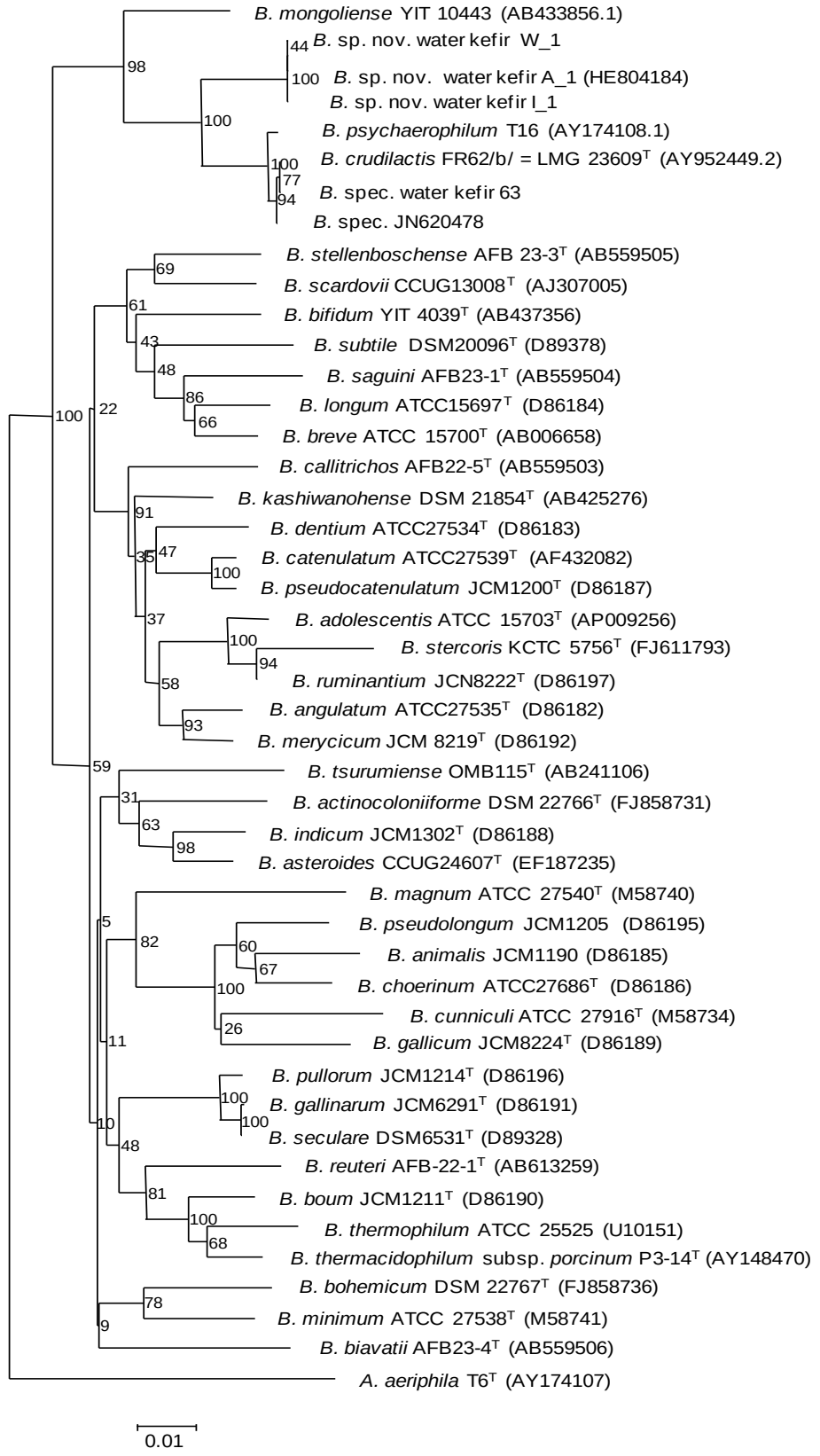

Figure 21: Neighbour-joining tree based on 16S rRNA gene sequence comparison, showing the phylogenetic relationships of the B. sp. nov. water kefir A_1 (HE804184), B. sp. nov. water kefir W_1, B. sp. nov. water kefir I_I and B. spec. "63", and reference type strains of other bifidobacterial taxa.
The tree was routed with *A. aeriphila* as an outgroupand bootstrap value calculated from 100 trees represented as percentages at each branch-point. For each species, the accession number is shown in parentheses.

In conclusion this investigation confirms previous data and broadens the available knowledge about the microbial consortium of water kefir. The unexpected presence of bifidobacteria in all samples and the difficulty of cultivating these species indicate that the role of bifidobacteria in other fermented foods may also be underestimated. The phylogenetic position of bifidobacterial 16S rRNA gene sequences with similarities of less than 96.4 % to known species suggests the identification of a new candidate species of the genus of *Bifidobacterium*. The large number of bifidobacteria sequences found in all samples indicates that these bacteria are part of the core species of water kefir.

4.3 Influence of the composition of water kefir microbiota by changing growth conditions

4.3.1 Measurement of the wet mass of water kefir grains

The growth of the grain mass during the time of fermentation under different conditions showed diverse increase.

The water kefir grains grown with cranberry extract, apricot extract and grown at 37 °C only showed a marginal increase in their wet mass after each fermentation. Comparing to that the grain mass grown under standardized conditions increased steadily. The consistency of the water kefir grains in all samples did not change during the time of the experiment. The standard water kefir was grown with the addition of dried figs and the result corresponds to investigation of Reiß, 1990. In that investigation different fruits were tested and dried figs showed the highest increase in grain mass upon 12.3 %, in this investigation the highest grain mass increase was detected after the seventh fermentation with 126.5 % increase.

The figs promote the greatest growth and this could be explained by their nutritive substances acting as growth promoting factors. The nutrients seems to be located in the fruit pulp and could be extracted with cold water (Reiß, 1990). In dried figs a high amount of phenolic compounds could be detected such as proanthocyanidins which are promoting the growth of bifidobacteria and lactic acid bacteria (Yamakoshi et al., 2001). The mineral content like potassium, calcium and iron is also higher in figs than in other fruits (Vinson, 1999). The high content of sugars, diverse saccharides and different amino acids might also promote growth of microorganisms. This might be a reason why the “traditional household” water kefir is prepared with dried figs. Cranberries have been shown to prevent urinary tract infections by disease causing organisms like *E. coli*, *Staphylococcus aureus*, *Enterobacter aerogenes*, *Salmonella* spp, *Listeria monocytogenes* and *Pseudomonas aeruginosa* (Rahbar and Diba, 2010). The antimicrobial effects were mostly shown on bacteria causing urinary tract infections which are gram negative bacteria. Lactic acid bacteria could not be shown to be negatively affected by the natural compounds such as polyphenols, anthocyanins and proanthocyanidins occurring in high amounts in cranberries (Cimolai and Cimolai, 2007). Some lactic acid bacteria even show the ability to degrade these natural compounds called tannic acid and could obtain energy from it like *Lb. plantarum* and *Lb. acidophilus* (Hervert-Hernandez et al., 2009). The influences of tannins on different species are dependent on the strain and the presence of tannins could result in inhibitory effects of some water kefir microorganisms even if it is unlikely due to the fact that mostly Gram negative bacteria are inhibited. The apricot extract might not supply enough nutrients for an increase in the grain mass, the 37 °C and 12 °C seems not to be the optimal growth conditions for the microorganisms in the water kefir as they did not show a mass increase as well.

4.3.2 Constitutions of the water kefir grain and supernatant grown under different conditions analyzed by high-through put sequencing with the primer 27 f and 519 R

The water kefir grain and supernatant of the standard sample showed nearly equal numbers of two bacteria families namely *Lactobacillaceae* and *Leuconostocaceae.* *Lb. nagelii* and *Lb. hordei* were identified as representatives belonging to the *Lactobacillaceae* family in the culture-dependent as well as in the –independent experiments as predominant species. There are many factors which could be acting as limiting growth factor one important parameter is the optimal growth temperature for each species. The optimal growth rate of *Lb. nagelii* is at 25 °C, over 45 °C the growth is inhibited, the minimal temperature where growth could be detected is 5 °C (Edwards, 2000). *Lb. hordei* shows an optimal growth rate at 30 °C and could grow up to 45 °C but not below 15 °C (Rouse et al., 2008). This could explain the high amount of *Lactobacillaceae* in the 37 °C sample where almost no other family is represented. In comparison to that the 12 °C sample shows less abundance of *Lactobacillaceae* where *Lb. hordei* is not able to grow according to Rouse et al., 2008. *Lb. nagelii* on contrary shows growth at this temperature. There are other *Lactobacillaceae* which were identified in the water kefir microbiota like *Lb. ghanensis* showing weak growth at 15 °C (Nielsen et al., 2007), some species of the *Lb. casei* group can grow at low temperature some even at 5 °C, *Lb. satsumensis* species show growth at 15 °C (Endo and Okada, 2005). Overall there are some *Lactobacillaceae* isolated and identified within the water kefir microbiota able to grow at 12 °C. There could be a shift in the composition of the *Lactobacillaceae* for showing specific alteration within this family a more detailed analysis would be necessary.

The samples with cranberry extract and apricot extract grown at 21 °C showed also a lower amount of *Lactobacillaceae* which could not be explained by temperature changes.

The *Leuconostocaceae* showed a higher abundance in the standard kefir, at 37 °C no leuconstoc species could be detected whereas at 12 °C the abundance was also high, the apricot and cranberry extract sample showed lower concentrations. *Leuconostoc mesenteroides* and *Lc. citreum* identified as water kefir species behave like psychrotrophic bacteria having an optimal growth temperature of 30 °C but they are showing growth even at 4 °C (Hamasaki et al., 2003). At 37 °C some species only show marginally growth that could be one reason why nearly no *Leuconostocaceae* could be identified in the 37 °C.

Actinobacteriaceae are present in low concentrations in all samples except the 37 °C sample where no *Actinobacteriaceae* were detected. *Bifidobacterium psychraerophilum* was the species identified within this investigation as well as a possible new species (B. sp. nov. water kefir A_1 (HE804184)). *B. psychraerophilum* has its growth optima at 37 °C which is contrary to the findings in this investigation. One explanation why no bifidobacteria could be identified within the 37 °C sample is that the yeast species *Zygotorulaspora florentina* is not able to grow at this temperature and *Saccharomyces cerevisiae* showed a limited growth rate over time (personal communication, Jasmin Stadie). It might be that bifidobacteria need the yeast for interaction and symbiosis and that there is a lack of nutrients which are normally supplied by yeasts. Another reason could be the pH due to the high amount of lactobacilli present and the resulting fast pH decrease. The lactobacilli do not compete with the yeast species for the nutrients and so the metabolic activity of lactobacilli increases.

B. psychraerophilum needs a minimum initial pH of 4.5 for growth and below pH 4 inhibitory growth effects occur (Simpson et al., 2004). After 24 h of fermentation at 37 °C the pH was 3.4 and after 72 h the pH was 3.2 which are lower than the minimal pH needed for growth (personal communication; Jasmin Stadie).

The family of *Enterobacteriaceae* was represented in high numbers in the cranberry extract and apricot extract sample. These bacteria are not a member of the traditional water kefir microbiota as they could cause spoilage of food or beverages where the genera *Escherichia*, *Erwinia*, *Enterobacter*, *Citrobacter*, *Serratia* and *Proteus* are representatives (Sperber and Doyle, 2009). These bacteria also might have pathogenic traits. They are present in the environment like soil and freshwater and were found in a lot of food products like raw milk concerning food, dairy product, fish products, meat, poultry, fresh fruit and vegetables (Lawley et al., 2008). Some species like *Citrobacter freundii*, *C. koseri* and *Klebsiella pneumonia* might be resistant to extreme conditions as they were identified from dried infant formula so that they should be able to survive desiccation for some time. The cranberry fruit itself is unlikely to be a suitable habitat for these microorganisms as they have antibacterial effects against *E. coli*, *Samonella* spp. and *Listeria monocytogenes* (Rahbar and Diba, 2010). Cranberry extract could contain benzoic acid which also exhibit antimicrobial effects and is used in beverages against spoilage and therefore growth of bacteria (also lactic/acetic acid bacteria) is inhibited (González et al., 1998). Another source could be the tap water or other contamination during preparation and fermentation.

Pseudomonadaceae are only present in small percentages in the cranberry and 37 °C sample. *Aeromonas* species are belonging to the *Pseudomonadaceae* they are thought to be a main contaminant in drinking water, there are species which can

grow at low temperature (below 10 °C) and some species are mesophilic. One contamination source in the preparation of water kefir is the tap water used for washing the grains, after the washing these bacteria might grow under certain conditions like within the cranberry extract sample where the pH is not as low as in the other samples. The pH optima of these bacteria is between 6.5 and 7.5 (Lawley et al., 2008).

The apricot and cranberry sample showed the highest diversity of bacteria species including food and beverage spoiling bacteria. By comparing these two samples with the other samples one obvious characteristic is different, the pH in these samples is much higher. The parameters pH, temperature and water activity are driving forces for selective pressure on the natural food microbiota (Sperber and Doyle, 2009). The pH of the cranberry extract sample is not changing over the three days of fermentation the lowest pH after three days of fermentation was 6.6. The apricot extract sample showed a pH after three days of fermentation of 4.6 whereas the pH of the standard sample and 37 °C decreases over the first 24 h of fermentation to 3.7 and 3.4, respectively. The lowest pH measured was 3.2 with the 37 °C sample after three days of fermentation (personal communication, Jasmin Stadie). The 12 °C sample showed the lowest pH after three days of fermentation of 5.2, but in this sample the metabolic activity is decreased due to the low temperature and some species of different bacterial families are inhibited in growth. The fast decrease in pH due to metabolic activity results in biopreservation of water kefir. Fermentation is used widely as a natural food preservation either by natural occurring fermentation or by adding specific bacteria (Caplice and Fitzgerald, 1999). Lactic acid bacteria play an important role in fermentation as they can grow at a wide range of temperature and some species are metabolically active even at pH 3.2.

The metabolism of hexoses results in formation of lactic acids and ethanol protecting the natural microbiota by lowering the pH. The natural acidification protects the food or beverage for food spoiling bacteria like *Enterobacteriaceae* and *Pseudomonadaceae*. The standard and 37 °C water kefir sample showed the biopreservation by LAB, especially the 37 °C where no *Enterobacteriaceae* were identified as a result of the fast pH decrease. In the apricot and cranberry extract samples there were LAB present but the pH is not decreasing in a way to inhibit growth of unwanted bacteria.

As a conclusion it could be stated that the water kefir grown under standardized conditions showed stability in the composition of the microbiota, whereas the cranberry, apricot extract and 12 °C samples were contaminated in different concentrations by *Enterobacteriaceae*, *Pseudomonadaceae*, *Caulobacteriaceae* and *Xanthomonadaceae* as a result of high pH. The 37 °C sample lack of *Leuconostocaceae* which are also important for organoleptic characteristic as well as *Actinobacteriaceae* which might also have health benefit effect when consuming water kefir. With this experiment it could be shown that by changing different parameters alteration in the microbiota of water kefir could be seen.

4.4 Comparison of culture-dependent and culture-independent method for detecting and identifying bacteria in microbiota

4.4.1 Advantages and disadvantages of culture-dependent procedures

Comparing the culture-dependent and –independent procedures for analyzing the water kefir microbiota, the main core of bacteria species identified is nearly the same.

Bacterial species identified in both analyses were *Lactobacillaceae*, *Leuconostocaceae* and *Acetobacteraceae*. But *Bifidobacteriaceae* were only found with culture-independent procedure and were present in a high percentage in three out of four water kefirs ranging from 18.7 % to 21.9 % whereas one water kefir only showed 3.8 % (see 3.2.1). Traditional techniques such as microscopy, cultivation and characterization according to physiological/ biochemical analysis for identification of microorganisms exhibits disadvantages. The characterization of microorganisms with microscopic features is nearly impossible due to their small appearance and lack of remarkable morphological characteristics (Muyzer, 1999). Cultivation of microorganisms from a natural microbiota is very difficult due to their special physiological requirements, which are not easy to imitate artificially. Over 99 % of microorganisms originating from natural habitats are not cultivated by standard techniques (Hugenholtz et al., 1998). The culture-dependent investigation for analyzing the water kefir microbiota was designed on the foundation of few previous studies which were mostly performed decades ago (Franzetti et al., 1998; Galli et al., 1995; Horisberger, 1969; Kebler, 1921; Lutz, 1899; Moinas, 1980; Neve and Heller, 2002; Pidoux, 1989; Pidoux et al., 1988; Reiß, 1990; Waldherr et al., 2010; Ward, 1892). Therefore three different media were applied for the enumeration of lactic acid bacteria, acetic acid bacteria and yeasts. Microorganisms in food fermentation coupled with alcoholic fermentation showed that they are in a viable but not culturable state and therefore could not be detected with culture-dependent procedures (Bokulich and Mills, 2012). Regarding these investigations no indication of the presence of bifidobacteria in the water kefir microbiota were possible leading to an incomplete characterization of the community as the growth parameters and culture media did not achieve the demands of this genus.

But there are some critical steps by interpreting data gained from high-throughput analyses. The high amount of sequences/reads has to be stored and computational power has to be adequate for handling the raw data.

4.4.1.1 Importance of primer design for analyzing bacterial microbiota by high-throughput sequencing

The read length of the 16S rRNA gene sequences is a very important characteristic for proper microbial identification. The 454 sequencing technique generates read length of approximately 450-700 bp (van Hijum et al., 2013). Therefore the oligonucleotide primer placement in the genome has to be selected carefully. Placement of the primer in different regions of the 16S rRNA gene can result in a completely different analysis of the community composition and the spreading of the microorganisms leading to ambiguous interpretation. This is especially true for the analysis of the water kefir consortium shown in this thesis.

All bacteria exhibit 16S rRNA genes consisting of nine hypervariable regions often adjoined by conserved nucleotide sequences (Chakravorty et al., 2007). This constitution permits the identification of certain bacterial species by PCR amplification of specific sequences with the help of universal primers (Chakravorty et al., 2007). Universal primer pairs bind to conserved regions of the 16S rRNA gene. But the design of a universal primer binding to all bacteria is not possible (Sim et al., 2012). The reason for that is that even the conserved regions show small differences in their consecutive nucleotide sequence. The efficiency of the primer binding to the template is dependent on the accurate matching of the primer to the template nucleotide sequence. Mismatches in the sequence of the primer and the template are often not tolerated and lead to bias in the amplification of all taxa in a heterogeneous community (Frank et al., 2008). Mismatches at the 3’ end lead probably to a higher

ineffective binding of the primer to the template than at the 5'end (Sim et al., 2012). To overcome this problem degenerated primer could be used. Another limit by using universal primers is that the sequence diversity of the 16S rRNA hypervariable regions of bacteria is not adequate for distinguishing all bacteria (Chakravorty et al., 2007). The annealing of the primer in a heterogeneous community is also a critical step regarding the bias for detecting specific taxa (Sim et al., 2012).

4.4.1.2 Comparison of the primer pairs used for high-throughput sequencing analysis of the water kefirs grown under different conditions

Overall the choice of the universal primer is one of the most critical factor in analyzing a community by 16S rRNA procedure (Wang and Qian, 2009). But it also shows a perspective for detecting species, which have not been cultivated yet (Frank et al., 2008). In this investigation it could be shown that the choice of universal primer for characterizing a whole community is very important. Using the universal primer GM3/926R *Actinobacteriaceae* including mostly bifidobacteria sequences could not be found at all, whereas with the primer 27 f/519 R *Actinobacteriaceae* sequences were detected. The primer 926R showed one mismatch at the 10^{th} position of the primer sequence and by changing the nucleotide sequence of the primer bifidobacteria species could be detected which was shown by Sim et al., 2012, where they used a "bifidobacteria-optimized" universal primer called 926Rb. The primer should be valid for the community analyzed so that the main bacteria species and the organisms of interests are successfully amplified. In the case of this investigation the usage of the primers 27 f and 519 R are the most suitable for analyzing the bacterial water kefir microbiota. The analysis with two different universal primers but with the same DNA template showed differences in the composition of the water kefir. The main differences were in the standard cranberry extract sample with the GM3/926R primers more *Acetobacteraceae* sequences were obtained whereas in the other

samples the numbers of this bacteria family only differ marginally with both universal primer pairs. In the standard sample in the supernatant no *Lactobacillaceae* sequences were obtained with GM3/926R but with 27 f/519 R they could be identified. With the usage of two primer pairs it could be shown that different results in the composition of the water kefir microbiota were obtained.

As a resume it could be shown that the choice of universal primer for detecting a microbiota is very important for analyzing the composition and making the right conclusion. The universal primer pair 27 f/519 R did amplify one bacterial family (*Actinobacteriaceae*), which was not amplified with the other primer pair used.

4.5 Characterization of the water kefir microbiota by Fluorescence in situ hybridization (FISH) analysis

The FISH analysis of the water kefir was done to use another common procedure without the need of pre-cultivation and for characterizing this microbiota.

The different probes were used for detecting bacteria in general (EUB 338-I) and *Actinobacteriaceae* with a specific probe (HGC 69a). The actinobacteria probe was used for detecting bifidobacteria which were identified as a water kefir microorganism by high-throughput sequencing and could also be cultured on a modified bifidobacteria medium. The FISH method is a procedure widely used for characterizing communities. With in situ hybridization and probes targeting rRNA it is possible to detect microorganisms without cultivation and it could be used for detecting domains but also subspecies within a community. A lot of investigations have been done analyzing the genetic diversity in bacterial communities (Amann et al., 1996; Amann et al., 1990b; Amann et al., 1995; Manz et al., 1993).

FISH is also used for detecting marker organisms within a community like in foods, waste water, rivers, drinking water, sewage or clinical investigations (Ootsubo et al., 2003). The FISH analysis with the water kefir was mainly done to visualize bifidobacteria. The water kefir grain analyzed with FISH only showed fluorescence with the universal EUB 338-I probe. In the water kefir grain no presence of bifidobacteria could be shown (blue fluorescence; probe labeling with CY5) only some autofluorescing background could be seen. The autofluorescing particles showed irregular forms and no bacterial appearance. By comparing the pictures with the different microscopic positions it could be shown that almost all bacteria of the phase contrast are reacting with the EUB 338-I probe. This indicates that no bifidobacteria are in the water kefir grain. But this result should be interpreted very carefully. The experiment set up was not optimized for the analysis of the very special consistency of the grain. During the FISH experiment a lot of biomass was lost, especially during the washing steps with ethanol. The fixation with PFA and ethanol is also a step which should be optimized. Another factor that influences the FISH experiment and the result is the accessibility of the target rRNA intracelluarly and this is dependent on the permeability of the target cell. The permeability is influenced by the cell structure like cell walls, membranes and capsules. The permeability of gram positive bacteria is more difficult than of gram negative bacteria due to their rigid cell walls (Roller et al., 1994). That could be a reason why the HGC 69a did not show any signal in the grain but cannot explain why the supernatant did show signals. The availability of the probe binding site could also influence the effectiveness of binding the probe to its target oligonucleotide sequence. The supernatant shows the opposite of the water kefir grain, all fluorescing bacteria are reacting with the HGC 69a probe showing green fluorescence.

This indicates that the supernatant contains bifidobacteria but it is difficult to interpret why no other bacteria are present. The loss of biomass was not as high as with the water kefir grain.

As a conclusion the FISH experiment is a state of the art procedure and should be improved for the analysis of water kefir. The most interesting characteristic that could be analyzed by FISH is the arrangement and the localization of the species within the grain. First investigations have been done by cryo-embeding the water kefir grain and cryosectioning but the problem with the loss of biomass could not be solved yet (data not shown). With the first FISH analyses it could be shown that bifidobacteria are a part of the water kefir microbiota but for a more detailed analysis the procedures has to be optimized.

4.6 Survival of water kefir microorganisms upon freeze-drying

The freeze-drying set up was done to analyze the ability of a possible conservation of water kefir grains for a potential industrial use. The important characteristics like microbial stability, appearance, consistency and size of the grains had to be guaranteed. Therefore water kefir grains were examined in different ways. The water kefir grains of the N_2 sample did not show alteration in the consistency. Some indications could be possible why the grains of these samples (-12 °C, -20 °C and -80 °C) alter their consistency and are dissolved after few fermentation steps (see Figure 19).

4.6.1 Possible effects of freeze-drying on the stability of water kefir grain formation

4.6.1.1 Water kefir as an example for a biofilm community

The water kefir grain could be seen as an example for a biofilm formation, where different bacteria and yeast species are enclosed by matrix consisting of various EPS. Biofilms consist not only of EPS but also of proteins, nucleic acids, lipids and/or humic substances (Mayer et al., 1999). The formation of biofilms by microbial community protect them from their environmental stresses (Vu et al., 2009). In the past some investigations have been done regarding the consistency of the water kefir grain and these authors described the grains to consist of dextran (Galli et al., 1995; Horisberger, 1969; Pidoux, 1989; Pidoux et al., 1988). These investigations were done some years ago and therefore not the latest techniques were used. Pidoux studied EPS formation of *Lb. brevis* and *Lb. hilgardii* which were identified as the main bacterial components in the water kefir microbiota at that time (Pidoux et al., 1990, Pidoux 1988). Compared to this investigations these two bacterial strains were not identified or indentified in low numbers with culture-dependent and –independent procedures, respectively. For the formation and the maintenance of a stable biofilm the cell-to-cell interaction and the cell density is an important characteristic. One factor which might be important for the control and interaction of the community is quorum sensing where extracellular signal molecules (autoinducers) are released and detected by microbes within a community (Bassler, 1999). The signal molecules control gene expression which regulate physiological actions as a response of alteration in the density of cells (Bassler, 1999). The changing structure of the water kefir grains after slow freezing, freeze-drying and rehydration by starting the fermentation under standard conditions could be an effect of the change of intact microbial composition. It might be possible that the closeness of the microbiota, especially after the rehydration (start of “new” fermentation) somehow changed and

therefore the grains dissolved because stabilizing factors (like quorum sensing) could not be organized any more. The understanding of the production and the whole consistency of the water kefir grains is not well understood yet and the functionality has not been described yet. The cell numbers directly counted after freeze-drying for the -12 °C as a representative of the frozen samples were much lower compared to the quick-frozen grains except for the bifidobacteria. The lower cell density in the -12 °C sample on mMRS, GM and YPG media supports the assumption that the cell-to-cell interaction might be negatively affected and the structure of the grain itself changes as well as the structure of the microorganisms organizing the stability and formation of the “biofilm” water kefir grain. Compared to this the quick-frozen sample showed a high stability in the cell concentration and grain formation at all time points of the experiment.

4.6.1.2 Effect of freezing temperature on the viability of water kefir microorganisms

The length of freezing or cooling of the sample is a very critical step for the viability of the cells. Cells could be damaged by slow cooling, because of the osmotic dehydration of the cell and intracellular water has time to flow out resulting in ice crystal formation extracellularly. An osmotic disequilibrium appears as the water is removed by ice crystals from extracellular environment and solutes are accreted (Zhao and Zhang, 2005). Channels could be formed in the unfrozen solution and the cells shrink as a respond to osmotic pressure (Mazur, 1984). These crystallization procedure and the increase in solute concentration might result in damages in the cell membrane leading to possible cell death (Zhao and Zhang, 2005). Microbial cells could also be harmed by the formation of intracellular ice crystals leading to lower viability of the cells. This happens when cooling is fast and the water has no time to flow out of the cell for maintaining equilibrium. Intracellular ice formation could also result in lethal damage (Zhao and Zhang, 2005). The quick-freezing process with

liquid nitrogen might not damage the cell membrane of microorganisms due to less time for building large ice crystals intracellularly. Freeze-drying with liquid nitrogen often shows good strain stability and high viability rates (Novik et al., 2008-2009) which was shown especially for different LAB cultures (Fonseca et al., 2006).

4.6.1.3 Influence of freeze-drying upon Gram positive and Gram negative bacteria

The viability of Gram negative bacteria after freeze-drying and rehydration is not as high as for Gram positive bacteria. The Gram negative bacteria might have an important role in the stability of the water kefir microbiota. This conclusion is supported by the results obtained on the GM medium. The Gram negative bacteria species *Ac. ghanensis* and *Ac. orientalis* could be important for the stability of the water kefir grain. Both species *Ac. ghanensis* and *Ac. orientalis* did not survive freeze-drying and rehydration in the -12 °C sample. In the N_2 sample no *Ac. orientalis* was identified but *Ac. ghanensis* could be detected after regrowth in high numbers. The standard water kefir did show high amounts of *Ac. orientalis* underlining the importance of this these species. One explanation for the stability of the water kefir grain formation of the N_2 sample could be that *Ac. ghanensis* might also fulfill the function of *Ac. orientalis*. This indicated that not the specific species is important but the overall microbial composition.

4.6.1.4 The water kefir grains as possible protectant for the microorganisms

Grains consisting of EPS, different proteins, amino acids or peptides could act as cryoprotectants during freezing and freeze-drying. These compounds could appear to act at the cell surface for stabilization or protection of the cells against solution effects (Mazur, 1970). Normally microbes benefit from cryoprotectants (Hubalek, 2003). It is very difficult to analyze the most suitable freeze-drying process for bacteria because even different strains of one species react different at freezing procedures

(Fonseca et al., 2000). The water kefir grain and the substances within this grain could act as cryprotectants and that might be a reason why bacteria and yeast species of the water kefir community survive freezing and freeze-drying at all. A lot of research has been done focusing on substances acting as protective agents to reduce damages of the cells during freezing as well as thawing summarized by (Morgan et al., 2006). These compounds often are said to prevent formation of ice crystals inter- and intracellularly by building hydrogen bonds with the membrane phospholipids. For lactic acid bacteria different protective agents were positively tested like amino acids and yeast extract. The viability could be improved by using these substances (Zhao and Zhang, 2005). The compounds stored within the grains might act as protective agents for some microorganisms during the stress of freeze-drying and rehydration. The grains could also be used from the microbes as nutrient resource even if it is not likely that bacteria use EPS as nutrients as described by (De Vuyst and Degeest, 1999). Nevertheless these complicated interactions are not cleared completely, especially not in the water kefir environment. The grains could also serve with amino acid or peptides stored within the grain so the inelastic form of the grains loosens.

In this experiment only a small percentage of the whole community was analyzed. Bacteria and yeast which could not be detected at any time point of the procedure could also be part of the microbiota, they are just under the detection limit. The experiment was done to analyze if one main group or species of bacteria/yeasts are mainly damaged by freezing, freeze-drying and rehydration. This would have been an evidence that they are mainly responsible for the formation of a stable water kefir microbiota. To analyze which bacteria or yeast group is affected the most and this damage leads to the dissolving of the water kefir grains a study in more detail has to be done.

The conclusion of this experiment is that the freeze-drying experiment with pretreatment of liquid nitrogen showed that the water kefir grains can be conserved and do not lose their characteristics in the fermentation. It could be shown that Gram negative bacteria could have an important impact on the water kefir stability and might be responsible for the insoluble grain formation. To get more specific information about the different species behavior during the freeze-drying process a more detailed experiment has to be done with single cultures.

Summary

Water kefir is a traditionally homemade beverage based on sucrose solution with dried fruits resulting in a fizzy and cloudy beverage after two or three days of fermentation at room temperature. The beverage is low in acid, poor in sugar and slightly alcoholic. Investigations of the water kefir grains did show that they consist of different lactic acid bacteria, acetic acid bacteria and yeasts (Franzetti et al., 1998; Galli et al., 1995; Horisberger, 1969; Lutz, 1899; Neve and Heller, 2002; Pidoux, 1989; Ward, 1892). These few investigations were done decades ago. There are only few data available on the analysis of the water kefir microbiota where identification and occurrence of microorganisms was not determined. The aim of this thesis was to characterize the microbiota of water kefir in detail by identifying core microorganisms important for a stable water kefir by culture-dependent and –independent procedures. The overall aim was to identify possible water kefir microorganisms which could be used as starter cultures for a water kefir based beverage.

Therefore the microbial diversity of water kefir, made from a mixture of water, dried figs, slices of lemon and sucrose was firstly studied by culture-dependent procedures. The microbial consortia residing in the grains of three water kefirs of different origins were analyzed. A collection of 453 bacterial isolates was obtained on different selective and differential media. Bacterial isolates were grouped with randomly amplified polymorphic DNA (RAPD)-PCR analyses. For the identification one representative of each RAPD genotype was identified by comparative 16S rDNA gene sequencing. The three water kefir studied showed nearly the same composition with the core species *Lactobacillus hordei*, *Lb. nagelii*, *Leuconostoc mesenteroides* and *Lc. citreum* representing lactic acid bacteria. Acetic acid bacteria were identified as *Acetobacter fabarum* and *Ac. orientalis*. A total of 57 LAB belonging to the species

Lb. casei, *Lb. hordei*, *Lb. nagelii*, *Lb. hilgardii* and *Lc. mesenteroides* were able to produce exoplysaccharides from sucrose. The cluster analyses of RAPD-PCR patterns revealed an interspecies diversity among the *Lactobacillus* and *Acetobacter* strains. Additionally, *Saccharomyces cerevisiae*, *Lachancea fermentati*, *Hanseniaspora valbyensis* and *Zygotorulaspora florentina* were isolated and identified by comparison of partial 26S rDNA sequences and FTIR spectroscopy whereas *S. cerevisiae* and *Z. florentina* were the predominant species in all water kefirs.

As a second procedure, water kefir microbiota of four water kefirs were analyzed by culture-independent methods 16S rRNA gene amplicon sequencing and amplified ribosomal DNA restriction analysis (ARDRA). The microbiota also consisted of different proportions of the genera *Lactobacillus*, *Leuconstoc* and *Acetobacter* validating results gained from culture-dependent methods. Additionally low numbers of *Gluconobacter* species were found. Surprisingly, varying but consistently high numbers of sequences representing members of the genus *bifidobacerium* were found in all water kefirs for the first time. Whereas part of the bifidobacterial sequences could be assigned to *Bifidobacterium psychraerophilum*, a majority of sequences identical to each other could not be assigned to any known species. The closest relative species was *B. psychaerophilum*. A *Bifidobacterium*-specific ARDRA analysis reflected the abundance of a novel *B*. species by revealing its unique restriction profile. Attempts to isolate the bifidobacteria were successful for *B. psychaerophilum* only.

To analyze the influence of growth parameters on the water kefir microbiota, water kefir grains were fermented under different growth conditions with cranberry extract and apricot extract, at 37°C and 12°C. The water kefir microbiota grown under

standardized conditions showed the highest stability in the bacterial composition. The cranberry and apricot extract samples as well as the 12 °C sample showed high numbers of contaminations with *Enterobacteriaceae*, *Pseudomonadaceae*, *Caulobacteriaceae* and *Xanthomonadaceae*. The microbiota grown at 37 °C did not show the abundance of *Leuconostocaceae* which might be important for organoleptic characteristics of the beverage.

On the basis of the results from culture-dependent and culture-independent procedures it was possible to generate a starter culture for a water kefir based beverage which might be produced industrially.

For the utilization of water kefir grains in an industrial scale freeze-drying preservation was analyzed. Water kefir grains quick-frozen with liquid nitrogen and a freeze-drying procedure afterwards could be conserved without losing their characteristics over the following fermentation process. The freezing of the water kefir grains at lower temperatures (-12 °C, -20 °C and -80 °C) showed dissolving of the water kefir grains. Hereby, the Gram negative bacteria like *Acetobacter ghanensis* and *Ac. orientalis* did not show a high viability after regrowth and might be important for a stable water kefir grain formation.

Zusammenfassung

Wasser Kefir ist ein Getränk, das traditionell durch Wasser Kefir Granula in Zuckerlösung und durch Zugabe von Trockenfrüchten im allgemeinen Hausgebrauch hergestellt wird. Die Fermentation findet über zwei bis drei Tage bei Raumtemperatur statt. Das entstehende Getränk weißt eine gelbliche Färbung auf, ist leicht sauer, enthält geringe Mengen an Zucker und Alkohol.

Vorhergehende Untersuchungen des Wasser Kefir Konsortiums identifizierten verschiedene Milchsäurebakterien, Essigsäurebakterien und Hefen als Hauptbestandteil dieses Konsortiums (Franzetti et al., 1998; Galli et al., 1995; Horisberger, 1969; Lutz, 1899; Neve and Heller, 2002; Pidoux, 1989; Ward, 1892). Diese Untersuchungen reichen teilweise sehr weit zurück und nur wenig ist über die mikrobielle Wasser Kefir Zusammensetzung, wie die Identifizierung der Mikroorganismen und die Verteilung dieser innerhalb des Konsortiums, bekannt.

Das Ziel dieser Arbeit war deshalb die mikrobielle Vielfalt des Wasser Kefir Konsortiums im Detail, durch kultur-abhängige und –unabhängige Methoden zu bestimmen und einzelne Mikroorganismen zu identifizieren, die für die Stabilität des Wasser Kefir Konsortiums wichtig sind. Das übergeordnete Ziel war Wasser Kefir Mikroorganismen zu identifizieren, die als Starterkultur für die Herstellung eines Wasser Kefir-basierenden Getränks eingesetzt werden können.

Hierfür wurde der Wasser Kefir zunächst mit Trockenfeigen und Zitronenscheiben in einer Zuckerlösung fermentiert. Es wurden drei Wasser Kefire verschiedener Herkunft mittels kultur-abhängiger Methoden untersucht. Hierbei wurden 453 einzelne Bakterienisolate auf verschiedenen, speziellen Kulturmedien angezogen und untersucht. Die Bakterienisolate wurden auf Grund ihrer RAPD-Muster (randomly

amplified polymorphic DNA) gruppiert und jeweils ein Repräsentant mittels 16S rDNA Analyse identifiziert. Die drei untersuchten Wasser Kefire zeigten eine ungefähr gleiche Zusammensetzung auf, mit den Hauptspezies der Milchsäurebakterien *Lactobacillus hordei, L. nagelii*, *Leuconostoc mesenteroides* und *Lc. citreum* und den Essigsäurebakterien *Acetobacter fabarum* und *Ac. orientalis*. 57 der verschiedene Spezies (*Lb. casei*, *Lb. hordei*, *Lb. nagelii*, *Lb. hilgardii* and *Lc. mesenteroides*) wurden als Exopolysaccharid-Produzenten identifiziert, bei Anzucht auf Saccharose-Agar. Die RAPD-PCR Gruppen-Analyse zeigte die Vielfalt der Spezies von *Lactobacillus* und *Acetobacter* auf. Zusätzlich wurden verschiedene Hefe Spezies mittels 26S rDNA und FTIR identifiziert (*Saccharomyces cerevisiae*, *Lachancea fermentati*, *Hanseniaspora valbyensis* und *Zygotorulaspora florentina*), wobei *S. cerevisiae* und *Z. florentina* die dominanten Spezies waren.

Als weiter Methodik wurden vier verschiedene Wasser Kefire mittels kultur-unabhängiger Prozesse durch 16S rRNA Gen Amplifikat Sequenzierung und ribosomaler DNA Restriktions Analyse (ARDRA) untersucht. Die verschiedenen Wasser Kefir Mikrobiota bestanden aus unterschiedlichen Anteilen der Bakterien-Gattungen *Lactobacillus*, *Leuconostoc* und *Acetobacter*. Dies bestätigt die Ergebnisse der kultur-abhängigen Methodik. Zusätzlich wurden geringe Mengen an *Gluconobacter* Spezies gefunden. Überraschenderweise wurden in allen Wasser Kefiren zum ersten Mal variierende, aber konstant hohe Konzentrationen an Bifidobakterien gefunden. Ein Teil der Sequenzen konnte dem schon beschriebenen *Bifidobacterium psychraerophylum* zugeordnet werden, ein Großteil der Sequenzen konnte jedoch keiner schon beschriebenen Spezies zugeordnet werden. Die nächst verwandte Spezies war *B. psychaerophilum*. Es wurde eine *Bifidobacterium* spezifische ARDRA Analyse durchgeführt, hierbei zeigten alle Proben ein spezifisches Restriktionsmuster auf. Dies unterstützt die Annahme, dass es sich

hierbei um eine neue Bifidobakterium Spezies handelt. Es war möglich *B. psychraerophilum* auf geeigneten Nährmedien erfolgreich zu kultivieren.

Um den Einfluss verschiedener Anzuchts-Parameter zu analysieren, wurde die Wasser Kefir Granula unter verschiedenen Anzuchtsbedingungen fermentiert und analysiert. Hierzu wurde die Granula mit Aprikosen- und Cranberryextrakt und bei 37 °C bzw. 12 °C fermentiert. Der Wasser Kefir, angezogen unter Standardbedinungen zeigte hierbei die höchste Stabilität in der Zusammensetzung und Verteilung der Bakterien. Die Wasser Kefir Proben, fermentiert mit Aprikosen- bzw. Cranberryextrakt und der Wasser Kefir, der bei 12°C fermentiert wurde, zeigten hohe Kontaminationen auf durch das Auftreten von *Enterobacteriaceae*, *Pseudomonadaceae*, *Caulobacteriaceae* und *Xanthomonadaceae*. Bei der 37 °C Probe konnten keine *Leuconostocaceae* detektiert werden, die aber für organoleptischen Eigenschaften des Getränkes wichtig sein könnten.

Basierend auf den erhaltenen Ergebnissen der kultur-abhängigen und -unabhängigen Untersuchungen des Wasser Kefir Konsortiums war es möglich eine Starterkultur, mit verschiedenen Wasser Kefir Isolaten, zu entwickeln. Der Einsatz dieser Starterkultur ermöglicht es ein Getränk auf Wasser Kefir Basis industriell zu produzieren.

Für die industrielle Nutzung von Wasser Kefir Granula wurde zusätzlich die Gefriertrocknung als Konservierungsmethodik untersucht. Wasser kefir Granula, die zunächst mittels Stickstoff schockgefroren und dann gefriergetrocknet wurden, zeigten bei Wiederanzucht bzw. über den Verlauf weiterer Fermentationszyklen keinen Verlust in den typischen Charakteristiken des Wasser Kefirs auf. Dies macht einen industriellen Einsatz der konservierten Wasser Kefir Granula möglich. Das Einfrieren der Wasser Kefir Granula bei niederen Temperaturen (-12 °C, -20 °C und

-80 °C) zeigte, dass das Wasser Kefir Konsortium sich änderte und dies über mehrere Fermenationszyklen zur Auflösung der Wasser Kefir Granula führte. Hierbei wurde festgestellt, dass die Gram negativen Bakterien Spezies *Acetobacter ghanensis* und *Ac. orientalis* keine hohen Überlebensraten zeigten und dies gibt Hinweis darauf, dass diese Spezies wichtig sind für die Stabilität des Wasser Kefir Konsortiums.

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