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The microbial diversity of water kefir

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ABSTRACT

The microbial diversity of water kefir, made from a mixture of water, dried figs, a slice of lemon and sucrose was studied. The microbial consortia residing in the granules of three water kefirs of different origins were analyzed. A collection of 453 bacterial isolates was obtained on different selective/differential media. Bacterial isolates were grouped with randomly amplified polymorphic DNA (RAPD)-PCR analyses. One representative of each RAPD genotype was identified by comparative 16S rDNA gene sequencing. The predominant genus in water kefirs I and II was *Lactobacillus*, which accounted for 82.1% in water kefir I and 72.1% in water kefir II of the bacterial isolates. The most abundant species in water kefirs I and II were *Lactobacillus hordei* and *Lb. nagelii* followed by considerably lower numbers of *Lb. casei*. Other lactic acid bacteria (LAB) were identified as *Leuconostoc mesenteroides* and *Lc. citreum* in all three water kefirs. The most abundant species in water kefir III was *Lc. mesenteroides* (28%) and *Lc. citreum* (24.3%). A total of 57 LAB belonging to the species of *Lb. casei*, *Lb. hordei*, *Lb. nagelii*, *Lb. hilgardii* and *Lc. mesenteroides* were able to produce exopolysacchrides from sucrose. Non LABs were identified as *Acetobacter fabarum* and *Ac. orientalis*. The *Acetobacter* species were more prevalent in consortium III. Cluster analyses of RAPD-PCR patterns revealed an interspecies diversity among the *Lactobacillus* and *Acetobacter* strains. Aditionally, *Saccharomyces cerevisiae*, *Lachancea fermentati*, *Hanseniaospora valbyensis* and *Zygotorulaspora florentina* were isolated and identified by comparison of partial 26S rDNA sequences and FTIR spectroscopy.

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1. Introduction

Water kefir is a homemade fermented beverage based on a sucrose solution with different dried and fresh fruits. In the traditional process of kefir preparation, the kefir grains are put into a solution containing 8% sucrose, dried fruits (typically figs) and some slices of lemon. Fermentation for one or two days at room temperature results in a cloudy, carbonated and straw colored drink, which is acidic, poor in sugar and slightly alcoholic.

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. 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).

Generally, the microbiota of water kefir is known to be a stable association 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). But 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.

The grains of water kefir are described to contain dextran, an α 1–6 linked glucose polymer (Galli et al., 1995; Horisberger, 1969; Pidoux, 1989). We have identified a strain of *Lactobacillus hilgardii* producing large amounts of the granule-forming dextran in water kefir and characterized the glycosyltransferanse responsible for the production of this dextran (Waldherr et al., 2010). The molecular background for the formation of a stable consortium is unknown and the comprehensive composition of the microflora is not scientifically defined yet.

The knowledge on the composition of the microflora and the quantitative abundance of the different organisms of water kefir are basic prerequisites for understanding the formation and the

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interactions of a stable consortium of these microorganisms. The aim of this study was to characterize the microbial community with state of the art molecular methods.

2. Materials and methods

2.1. Preparation of water kefir

The origin of the three water kefirs was from different long term traditional household preparations. The procedure to propagate water kefir was the same. Each water kefir was propagated under standardized conditions for at least two times to eliminate influences resulting from different cultivation procedures of the supplier.

The water kefir was prepared in a sucrose solution (100 g/l) in 1 l tap water containing two dry figs and a slice of organic lemon. The figs used were from local supermarket and from the company Seeberger (non sulfurated and no preservatives). The figs provide nutrients by diffusion and they are removed before consumption of the beverage as well as the lemons. The fermentation took place at 21 °C for three days. The supernatant was discarded and the kefir grains were strained, washed with tap water and strained again. The grains were used for further treatment.

2.2. Media and growth of microorganisms

For the preparation of serial dilutions 10 g of the washed and strained water kefir grains were diluted with 90 g of Ringer reagent and then 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 Ringer reagent. The different serial dilutions were plated on MRS agar plates, containing 2 g/l meat extract, 4 g/l yeast extract, 10 g/l peptone from casein, 1 ml Tween 80, 2.5 g/l K_2HPO_4, 5 g/l sodium acetate, 2 g/l diammoniumhydrogen citrate, 0.2 g/l magnesiumsulfate heptahydrate, 0.038 g/l manganesesulfate monohydrate, 20 g/l glucose, 15 g/l agar. The pH was adjusted at 5.7, the glucose was autoclaved separately and on *Gluconobacter* media containing 25 g/l mannitol, 5 g/l yeast extract, 3 g/l peptone, 15 g/l agar. To both media cycloheximide was added (150 μg/ml) after cooling the agar to 50 °C. The MRS agar plates were incubated anaerobically at 30 °C and the *Gluconobacter* agar plates were incubated aerobically at 30 °C for 3 days.

After the incubation the viable cell count was enumerated on a proper serial dilution. Single colonies were picked from each plate, streaked on the different media and incubated for 3 days. From every suitable serial dilution half of the colonies were picked. Single colonies were grown in liquid MRS or GM media and incubated overnight at 30 °C. Isolates on GM were shaken at 180 rpm (only).

For isolation of yeasts the serial dilutions were plated on YPG agar plates containing 10 g/l peptone from casein, 5 g/l yeast extract, 15 g/l agar, 20 g/l glucose and 0.01 g/l bromphenol blue. The pH was adjusted to 6.5. The glucose solution was autoclaved separately. After cooling the solutions to 50 °C the two solutions were mixed and chloramphenicol was added (100 mg/l). The agar plates were incubated at room temperature for 3 days.

2.3. Determination of EPS production

To verify if the bacterial isolates are able to produce exopolysaccharides they were grown on MRS agar containing 80 g/l saccharose instead of glucose. They were incubated at 30 °C over night. The phenotype of the EPS forming strains was slimy and glossy or ropy on the sucrose plates, whereas the ones not forming EPS showed normal colony morphology (Waldherr et al., 2010).

2.4. DNA isolation

For the DNA isolation of bacteria overnight cultures were centrifuged at 5000 *g* for 10 min, the pellet was washed with 1 ml TE-buffer containing 1 mM EDTA, 10 mM Tris, pH 8 and centrifuged again. The pellets were stored at −20 °C.

The total DNA was isolated from single colonies grown and washed as described before. The isolation was done with the Bacterial DNA kit (D3350-02, e.Z.N.A™, OMEGA bio-tek, USA) according to the instruction. The pellets were resuspended with 200 μl TE-buffer containing Lysozyme (10 mg/ml) the mixture was incubated at 37 °C for 60 min. 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.

DNA isolation of yeasts was performed with overnight cultures from single colonies and for the isolation of the yeast DNA the YeaStar™ Genomic DNA kit (Zymo Research, Germany) was used according to the instruction (protocol 1).

2.5. RAPD PCR

The primer used was the oligonucleotide primer M13V (5′-GTTTTC-CCA-GTC-ACG-AC-3′). The PCR reaction (25 μl) contained 25 pmol of Primer M13V, 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 (MWG AG Biotech, Ebersberg, Germany). 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 6X DNA Loading dye (Fermentas) and then electrophoretically separated in a 1.3% (wt/vol) agarose gel (0.5X Tris-borate-EDTA buffer [45 mM Tris-borate, 1 mM EDTA]). Registration of the PCR patterns, normalization of the densitometric traces, patterns storage, grouping of the strains using the Pearson product moment correlation coefficient and UPGMA cluster analysis were performed using BioNumerics Version 6.50 (Applied Maths, Belgium).

2.6. 16S rDNA sequence analyses

Strains showing different RAPD patterns were analyzed by comparative 16S rDNA sequencing. 16S rDNA was amplified with the universal primer 616 V (5′-AGA-GTT-TGA-TYM-TGG-CTC-AG-3′) (binding position: 7 according to Brosius et al., 1981) and 609R (5′-ACT-ACY-VGG-GTA-TCT-AAK-CC-3′) (binding position: 1099 according to (Brosius et al., 1981)) and a PCR program of 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 the 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.

2.7. Yeast identification

Yeast colonies were identified by FTIR as described by Kümmerle et al., 1998. In case of ambiguous FTIR results strains were identified by partial sequencing of the 26S rDNA as described by Kurtzman and Robnett, 2003.

The GenBank/EMBL/DDBJ accession numbers are:

- U72165 (*Zygotorulaspora florentina*)
- U73596 (*Hanseniaspora valbyensis*)

Table 1
Viable cell counts (cells/g) of the three water kefirs obtained on different media.

Media	Water kefir I	Water kefir II	Water kefir III
mMRS	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. Results

3.1. Quantification of the bacteria and yeasts

The microbiota of three independent water kefir grains (water kefirs I, II and III) from different origin was analyzed. The bacteria grown on the MRS and GM agar were enumerated and the viable cell count was determined.

The viable cell numbers (cells/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 I, 1.3×10^8 in water kefirs II and 1.3×10^8 in water kefir III) whereas the cell count on GM showed differences, water kefirs I and II had a cell count of 3.7×10^6 and 1.2×10^6 but the cell count of water kefir III was higher (5.6×10^8) (Table 1).

The viable yeast cells counted on water kefir I and II were similar with a cell count of 6.4×10^6 in water kefir I and 5.8×10^6 in water kefir II. The viable yeast cell count in water kefir III was also higher with 2.7×10^7 cells.

3.2. Identification of microorganisms

The microflora of the three different water kefir grains was 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*, *Lactobacillus hordei*, *Lactobacillus nagelii*, *Lb. hilgardii*, *Leuconostoc mesenteroides, Leuconostoc citreum*, *Acetobacter fabarum* and *Acetobacter 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 (Fig. 1) 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.

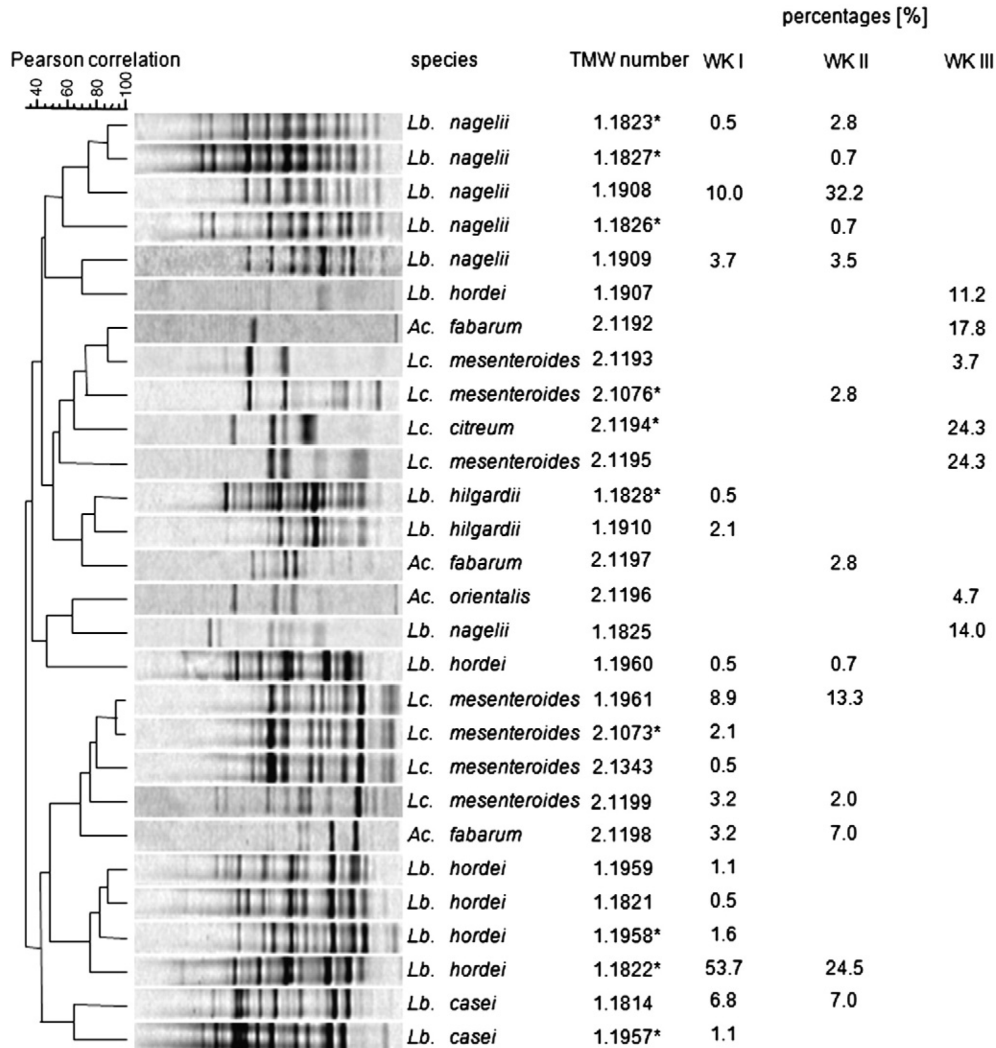

Fig. 1. UPGMA cluster analysis of RAPD fingerprint patterns of the bacteria isolated from three water kefirs of different origin which were grown identically. Shown are the 28 different RAPD patterns obtained out of 453 bacterial isolates from the three water kefirs. The identification of the 28 species were done with 16S rDNA sequencing The TMW numbering displays numbers of the strain collection of "Technische Mikrobiologie Weihenstephan". WK=water kefir, the marked species (*) are species producing exopolysaccharides upon growth on MRS containing saccharose instead of glucose.

Table 2
Sequence similarity of isolated strains with the reference sequences 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

16S rRNA gene sequence comparisons of the strains isolated from the three different water kefirs with the reference sequences are shown in Table 2.

3.3. Differences in the composition of water kefir microbiota

3.3.1. Bacterial composition

The composition of the bacterial microbiota in the three water kefirs was different. The main flora of water kefirs I and II consisted of the same three bacterial species but with different abundance. *Lb. hordei* was the most prominent species in water kefir I with 57.4%, in water kefir II the percentage was 25.2. *Lb. nagelii* was the main component in the microbiota of water kefir II with 39.9%, in water kefir I it was present with 14.2%. *Lc. mesenteroides* was present at percentages of 14.7% and 18.1% in water kefir I and II, respectively. In comparison to that water kefir III 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 I (2.6%) and *Lb. casei* could not be detected in water kefir III. *Ac. orientalis* and *Lc. citreum* were only present in water kefir III (4.7% and 24.3%) (Table 3).

3.3.2. Composition of yeasts

Four different yeasts were identified by FTIR as *H. 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* colonies showed white colonies. The percentage of white colonies was 94, 92 and 93% of all yeast colonies, in water kefir I, II and III, 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 III 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 I and II 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 III.

Table 3
Overview of percentages of bacteria species in all three water kefirs.

Species	WK I	WK II	WK III
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	18.1	28
Ac. fabarum	3.2	9.8	17.8
Ac. orientalis			4.7

3.4. Production of exopolysaccharides from sucrose

A total of 57 LAB belonging to the species of *Lb. casei*, *Lb. hordei*, *Lb. nagelii*, *Lb. hilgardii* and *Lc. mesenteroides* were able to produce exopolysaccharides (EPS) from sucrose.

4. Discussion

In this study the use of state of the art molecular methods led to the identification of several additional and/or different species providing a revised view of the water kefir consortia. We found species in the water kefir, which have not been detected and identified in other water kefir studies so far and their microbiota were qualitatively and quantitatively different in composition from those previously described (Franzetti et al., 1998; Galli et al., 1995; Kebler, 1921; Lutz, 1899; Pidoux, 1989).

A typical consortium 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 is most probably *Z. florentina* as demonstrated for of water kefir III. In this analysis the phenotype of the white cell morphology was studied in detail and more cells were identified as *Z. florentina* showing this morhpology. The other water kefirs had a high percentage of cells showing white morphology and referred to the result of the detailed study of the phenotype showing white morphology water kefir III 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 Robnett, 2003). *S. cerevisiae* is able to convert 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.

In previous investigations of the water kefir microbiota *Lb. casei* was described as a very predominant species (Franzetti et al., 1998; Pidoux, 1989). In this study we did not find *Lb. casei* in high percentages. The presence of *Lb. casei* in waters kefir I and II could be explained by the ubiquitous occurrence of *Lb. casei* 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%. In our study the concentration of *Lb. hilgardii* was negligible and it was only found in water kefir I. 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 was able to produce EPS. Pidoux described that *Lb. brevis* is also an important LAB regarding the formation of gel-like granula (Pidoux et al., 1988) which we did not find in our water kefir consortia. On the other hand, we found EPS-producing strains of *Lc. mesenteroides* (TMW 2.1073, 2.1076), *Lb. nagelii* (TMW 1.1823, 1.1826, 1.1827) and *Lb. hordei* (TMW 1.1958) (Fig. 1) fulfilling the role described for *Lb. hilgardii* in the formation of granule EPS in other consortia.

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 also not be detected in the water kefir grains characterized in this study featuring the predominance of *Lb. nagelii*, *Lb. hordei* and *Lc. mesenteroides* in water kefirs I and II. This may be due to their absence in those samples. However, *Lb. nagelii* was described in 2000 by C. Edwards (Edwards et al., 2000) and *Lb. hordei* was first described in 2008 (Rouse et al., 2008) and their delineation from *Lb. brevis* long before that time was not possible due to the lack of molecular typing techniques. In water kefir III *Lb. hordei* and *Lb. nagelii* could also be detected but in much lower concentrations.

Leuconostoc species were found in all three water kefir consortia in significant percentages and were the main component in water kefir III. This high number of *Leuconostoc* species in the water kefir consortia indicates their competitiveness in the environment of water kefir.

Acetic acid bacteria were identified in previous studies in water kefir consortia but only in negligible quantity (Franzetti et al., 1998). In our study acetic acid bacteria were found in higher amounts, especially in water kefir III.

To some extent the differences in the composition of the water kefir consortia may be referred to different identification techniques used. The technique used for 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 identification this technique has clear limits towards a valid identification of *Lactobacillus* species closely related to each other (Dalezios and Siebert, 2001; Boyd et al., 2005; Singh et al., 2009). By RAPD genotyping and identification of the species with 16S rDNA analysis we were able clearly allot isolates to species and also identify microorganisms in water kefir, which have not yet been identified from this sources. 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 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.

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