Food Microbiology 35 (2013) 92–98

Contents lists available at SciVerse ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

# Metabolic activity and symbiotic interactions of lactic acid bacteria and yeasts isolated from water kefir

Jasmin Stadie, Anna Gulitz, Matthias A. Ehrmann, Rudi F. Vogel*

*Lehrstuhl für Technische Mikrobiologie, Technische Universität München, Weihenstephaner Steig 16, 85350 Freising, Germany*

ARTICLE INFO

*Article history:*
Received 26 January 2013
Received in revised form
17 March 2013
Accepted 27 March 2013
Available online 11 April 2013

*Keywords:*
Water kefir
Lactobacilli
Yeasts
Trophic interaction
Symbiosis
Mutualism

ABSTRACT

Water kefir is a mildly sour and alcoholic drink fermented by a stable microbial multispecies community. With its high sugar content and low amino acid concentration water kefir medium represents a demanding habitat. In this ecological niche only well adapted microorganisms which are fit to the consortium are able to grow and mutually provide essential nutrients. The synergism between main representatives of water kefir yeasts and lactobacilli was studied in a co-culture model system. Co-cultivation of yeasts and lactobacilli in water kefir medium significantly increased cell yield of all interaction partners, delineating the interaction of these water kefir isolates as mutualism. The support of *Zygotorulaspora (Z.) florentina* was due to the acidification of the medium by the lactobacilli, whereas lactobacilli are improved in growth by the disposal of essential nutrients produced by yeasts. The trophic interaction between *Lactobacillus (Lb.) hordei* and yeasts is constituted by the release of amino acids and Vitamin $B_6$ from yeasts, whereas *Lb. nagelii* is supported in growth by their production of amino acids. The interaction of *Z. florentina* and *Lb. nagelii* was further examined to reveal that co-cultivation induced the yeast to release arginine, which was essential for *Lb. nagelii*.



## 1. Introduction

Symbiosis was firstly defined in 1879 by the German mycologist Heinrich Anton de Bary, who defined it as “the living together of unlike organisms”. It includes mutualism (both organisms benefit), commensalism (one benefits and the other isn't adversely affected), and parasitism (one benefits and the other is harmed) (De Bary, 1879). Interactions between different organisms can emerge in different ways. Physical associations can occur in loose communities which are based on special signaling molecules (quorum sensing) or in symbiotic associations with adhesion factors like proteins or polysaccharides (biofilm). These associations can be based on different molecular interactions like the adjustment of the physiochemical environment (change of pH), trophic interactions (organisms benefit from metabolic agents of the other), metabolite exchange of different organisms resulting in molecules that neither partner can produce alone (cooperative metabolism), protein secretion and gene transfer (Frey-Klett et al., 2011). Symbiosis in the forms of mutualism or commensalism is wide-spread in fermented foods, for example in yogurt, milk kefir or sourdough. Backgrounds of interactions are hard to determine, especially since there are a variety of microorganisms in the different consortia (De Vuyst and Neysens, 2005; Farnworth, 2005; Gulitz et al., 2011). The mutualism of yogurt cultures *Streptococcus (St.) thermophilus* and *Lactobacillus (Lb.) delbrueckii* subsp. *bulgaricus* appears to be well established. While *Streptococcus* is provided with branched chain amino acids (namely valin) containing peptides by the proteolytic activity of *Lb. bulgaricus*, in return the *Lactobacillus* is stimulated by production of formic and pyruvic acid caused by *St. thermophilus* (Zourari et al., 1992). Gobbetti et al. studied the interaction between yeast and lactobacilli of sourdough microbiota. They found commensalism in co-culture, where final yields and growth rates of lactobacilli increased, however, yeasts were unaffected (Gobbetti et al., 1994a, 1994b). Another example for the symbiosis of microorganisms is milk kefir, whenever the interactions of participating microbes are not fully understood. Until today, a reconstruction of milk kefir grains out of a mixture of isolates has not been possible, yet. Certain enlightenments were presented by Cheirsilp et al., who described the interaction between *Lb. kefiranofaciens* and *Saccharomyces (S.) cerevisiae* in mixed culture concerning the enhancement of kefiran production, especially capsular kefiran (Cheirsilp et al., 2003a; 2003b). Similar to milk kefir, water kefir is also a consortium of different microorganisms which is used for preparing a homemade fermented beverage. Grains are plunged in a

* Corresponding author. Tel.: +49 8161 713663; fax: +49 8161 713327.
*E-mail address:* rudi.vogel@wzw.tum.de (R.F. Vogel).

0740-0020/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fm.2013.03.009

sucrose solution (8%) supplemented with dried or fresh fruits, best figs (Reiß, 1990) and fermented at room temperature for two or three days. The resulting beverage is fizzy and cloudy, low acid, somewhat sweet and slightly alcoholic, depending on how long it was fermented. Mainly, the consortium of water kefir is comprised of $10^8$ lactobacilli, $10^6$–$10^8$ acetic acid bacteria and $10^6$–$10^7$ yeasts per gram granules (Gulitz et al., 2011). Microorganisms are embedded in transparent, cauliflower-shaped granules which mainly consist of an insoluble dextran (Horisberger, 1969; Pidoux et al., 1988). The origin of water kefir is unknown. First description of similar grains called “ginger beer plant” was made by Ward in 1892 (Ward, 1892). Until today various synonyms are known, thus this symbiosis is also called “California bees”, “African bees”, “Ale nuts”, “Balm of Gilead”, “Japanese Beer Seeds” or “Sugary kefir grains” (Kebler, 1921; Pidoux et al., 1988).

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

In a previous study we demonstrated the microbial diversity regarding dominant species of our water kefir consortia (Gulitz et al., 2011). The aim of this study was to get novel insights into the type of symbiotic interactions between microbes within the “organism“ water kefir. We compared the growth of main representative water kefir isolates in a model system in co-cultivation and pure culture.

## 2. Materials and methods

### 2.1. Strains, media and pre-cultivation

Predominant water kefir organisms, isolated and characterized by Gulitz et al. (2011), were chosen for co-cultivation experiments. Used isolates are *Lb. hordei* TMW 1.1822, *Lb. nagelii* TMW 1.1825, *Z. florentina* TMW 3.220 and *S. cerevisiae* TMW 3.221.

Lactobacilli were pre-cultured in a modified MRS medium and yeasts in YPG medium (Gulitz et al., 2011). Overnight cultures were centrifuged (5000 g, 5 min), washed twice with Ringer reagent and afterwards the cells were re-suspended in Ringer reagent to an absorbance at 590 nm ($OD_{590}$) of 2.5 (stock culture). $OD_{590}$ 2.5 is equal to $10^9$ cfu/ml lactobacilli and $10^8$ cfu/ml yeasts, respectively. All experiments were carried out in biological triplicates and technical duplicates.

The following experiments were executed in water kefir medium (WKM). For this medium 48 g dried figs were extracted in 100 ml still mineral water (Residenz Quelle naturell, Bad Winzheim, Germany) by shaking for 20 min. Big solids were removed by sieving and the smaller parts by centrifugation (17,000 *g*, 3 h) and sterile filtration (0.2 μm). WKM is comprised of 100 ml fig extract and 80 g sucrose per liter still mineral water.

### 2.2. Co-cultivation experiments

Co-cultivation experiments were executed in the Corning Transwell® culture system (Corning, Lowell, USA; (Gobbetti et al., 1994b)). This system consists of a 6 well plate with two separated parts in every well. The lower compartment (reservoir) is related with the upper compartment (insert) by a polycarbonate membrane (0.4 μm), which ensures diffusion of metabolic products but prevents mixture of cells. The reservoir resp. insert of the system were filled with 2.5 ml WKM inoculated with 4% of the above mentioned stock culture of yeast resp. *Lactobacillus* (start OD 0.1) and incubated at 30 °C. Furthermore each organism was singly cultured in 5 ml WKM in 6-well plates (Tissue Culture Plate, Flat Bottom, BD Falcon, New York, USA) with the same inoculation ratio as in co-culture. Pure WKM was filled in a 6 well plate as a sterile control and as a blank for OD measurement. After 24, 48 and 72 h 100 μl of each re-suspended culture was mixed with 400 μl WKM and measured at 590 nm. Each possible combination between yeast and *Lactobacillus* was tested. Preliminary tests (data not shown) showed the best effects with yeasts cultivated in the reservoir and lactobacilli in the insert. An acid-base diffusion assay with bromophenol blue as indicator could demonstrate metabolic diffusion after 10 min incubation time.

### 2.3. pH optimum of water kefir yeasts

The pH of YPG was adjusted with hydrochloric acid to pH 8.0, 7.0, 6.0, 5.0, 4.0 and 3.0. *Z. florentina* and *S. cerevisiae,* respectively, were cultivated in 5 ml of each medium with an inoculation of 4% stock culture for 72 h at 30 °C.

### 2.4. Modification of simplified chemically defined medium (SCDM)

Essential nutrients for water kefir isolates were identified in simplified chemically defined medium (SCDM) (Hebert et al., 2000). This medium contains 20 proteinogenic amino acids, vitamins and bases as single substances instead of an extract base. Glucose was used as carbon source. Growth behavior in full medium was compared to medium with one nutrient omitted. This method and following growth experiments were executed in a 96 well plate (Flat Bottom, Sarstedt, Nümbrecht, Germany) with 250 μl medium per well, an inoculation of 10 μl stock culture and 75 μl sterile paraffin oil (Sigma Aldrich, Steinheim, Germany) as cover for anaerobiosis. Measurements were done in a photometer (sunrise, tecan, Crailsheim, Germany) every 30 min for 48 h at 590 nm. Co-cultivation experiments in the model system were executed as described above in SCDM without pyridoxal (SCDM-Vit$B_6$), SCDM without L-arginine (SCDM-Arg) and SCDM without L-Isoleucine, L-leucine, L-methionine, L-phenylalanine, L-tryptophan, L-tyrosine and L-valin (SCDM-7AS), respectively. Lactobacilli and yeasts were additionally singly cultivated in the prepared media and SCDM as control. Since *Z. florentina* could not grow in SCDM-7AS this experiment was executed in seven different media where only one of the amino acids mentioned was omitted. Subsequent growth experiments were executed with *Lb. nagelii* in SCDM-Arg in mixture (1:1) with sterile filtrated supernatant of pre-fermented SCDM-Arg. Pre-fermentation was performed with singly cultivated *Z. florentina*, with *Z. florentina* and *Lb. nagelii* in mixed-culture with cell contact (inoculation with 4% stock culture of each strain), with *Z. florentina* and dead cells of *Lb. nagelii* (cell death induced by pasteurization, 10 min 78 °C), each in 6 well plates for 24 h. Additional media were SCDM-Arg with 10% yeast cell extract resp. yeast cell debris ($OD_{590}$ 0.2). For yeast cell extract stationary phase cells of *Z. florentina* were washed with dest. water, disrupted with a FastPrep-24 (MP Biomedicals, Heidelberg, Germany) and glass beads, pasteurized (10 min, 78 °C) and centrifuged (14,000 *g*, 30 min). The supernatant was used as yeast cell extract, the pellet as cell debris.

### 2.5. Modification of WKM

WKM was supplemented with all essential nutrients for *Lb. hordei* and for *Lb. nagelii*, respectively, in two different concentrations (amino acids 0.01 resp. 0.05 g/100 ml; pyridoxal 0.2 resp. 1.0 mg/100 ml). The growth of both lactobacilli was determined in these media in comparison to pure WKM after 20 and 40 h of fermentation. In addition the influence of 24 h pre-fermented (pre-fermentation with *Z. florentina* and *S. cerevisiae*, respectively) WKM was tested therefore sterile filtrated supernatant was used for further growth experiments with *Lb. hordei* and *Lb. nagelii*.

## 3. Results

### 3.1. Co-cultivation experiments in the model system

Metabolic interactions between single water kefir isolates without cell–cell contact were investigated in the Transwell® system. Each co-cultivation of yeasts and lactobacilli tested showed an improvement of growth compared with single cultivation of the individual organisms (Figs. 1 and 2). Both lactobacilli showed equal positive effects in growth for the two yeasts (Fig. 1 A and B), whereas the co-cultivation of *Lb. hordei* with *Z. florentina* showed a significantly better improvement than the co-cultivation with *S. cerevisiae* (Fig. 2 A). $OD_{590}$ of stationary phase of *Lb. nagelii* was similar in co- and in single cultivation but growth rate in the exponential phase was higher in co-cultivation than in single cultivation (Fig. 2 B).

### 3.2. pH optimum of water kefir isolated yeasts

To determine the influence of the physiochemical environment on yeasts the growth in media with different starting pH values was examined. *Z. florentina* showed its pH optimum for reproduction after 72 h incubation at a starting pH 4 (end $OD_{590}$ 2.9), whereas starting pH 3 decreased growth of *Z. florentina* ($OD_{590}$ 1.8). Higher starting pH-values, from 5 to 8, displayed similar influence on growth, the $OD_{590}$ of *Z. florentina* rose to 2.6. *S. cerevisiae* was not influenced by acidification of the medium.

### 3.3. Essential nutrients in modified chemically defined medium

The metabolic interaction of water kefir isolates was determined in a simplified chemically defined medium (SCDM) with all proteinogenic amino acids, bases and vitamins as described by Hebert et al. (2000). Therefore, essential nutrients for used water kefir isolates were investigated. Growth of *Lb. hordei*, *Lb. nagelii*, *Z. florentina* and *S. cerevisiae* was tested in full medium (SCDM) in comparison to media where one nutrient was omitted. *Z. florentina* and *S. cerevisiae* did not show an auxotrophy for any of the substances tested. Lag phase of *Z. florentina* was prolonged but after 24 h exponential phases started in every medium. Lactobacilli tested showed an auxotrophy for L-leucine, L-isoleucine, L-methionine, L-phenylalanine, L-tryptophan, L-tyrosine and L-valine. *Lb. hordei* revealed an additional auxotrophy for pyridoxal, *Lb. nagelii* for L-arginine. To determine if water kefir yeasts can provide essential nutrients for *Lactobacilli*, three chemically defined media were prepared, first a medium without pyridoxal for interaction experiments with *Lb. hordei*, second a medium without L-arginine for *Lb. nagelii* and third a medium without the amino acids, which are essential for both lactobacilli. In co-cultivation both lactobacilli were able to grow in media without their essential nutrients within 48 h of fermentation in comparison to single cultivation in these media, the starting $OD_{590}$ 0.1 did not change (Fig. 3). The influence of *Z. florentina* was higher than that of *S. cerevisiae*, the $OD_{590}$ after 48 h of co-cultivation of *Lb. hordei* and *Z. florentina* in pyridoxal free medium was with 1.25 twofold higher than in co-cultivation with *S. cerevisiae* ($OD_{590}$ 0.63). Also the stimulation of *Lb. nagelii* in arginine free medium was 1.5 fold higher in the cultivation system with *Z. florentina* ($OD_{590}$ 0.68) than in the system with *S. cerevisiae* ($OD_{590}$ 0.41). *Z. florentina* was not able to grow in SCDM-7AS thus in this medium no interaction could be shown. Experiments where only one of the seven amino acids was omitted each medium showed that also *Z. florentina* was able to support both lactobacilli with these amino acids (Table 1). *Z. florentina* promoted *Lb. nagelii* with arginine in co-culture in the model system on arginine free medium. As we could not detect any arginine in a yeast-fermented medium, the yeast does not produce arginine as single substance, but may produce arginine-containing compounds that could be used by the *Lactobacillus*. To find out under which circumstances the yeast released arginine sources, growth of *Lb. nagelii* in different pre-fermented arginine free medium was determined. Table 2 displays results of growth tests of *Lb. nagelii* in modified SCDM − Arg. *Z. florentina* did not produce any arginine available components for *Lb. nagelii* in pure culture. Therefore, sterile filtered supernatant of a 24 h fermented mixed-culture with *Z. florentina* and alive or dead cells of *Lb. nagelii*, respectively, were chosen for further growth experiments. By dead *Lb. nagelii* cells yeast might be induced to produce arginine, but without any alive consumer inside of the fermentation vessel it should remain detectable. Mixed-culture incubation in comparison to co-cultivation of *Z. florentina* and *Lb. nagelii* in the cell-separating Transwell® system showed a

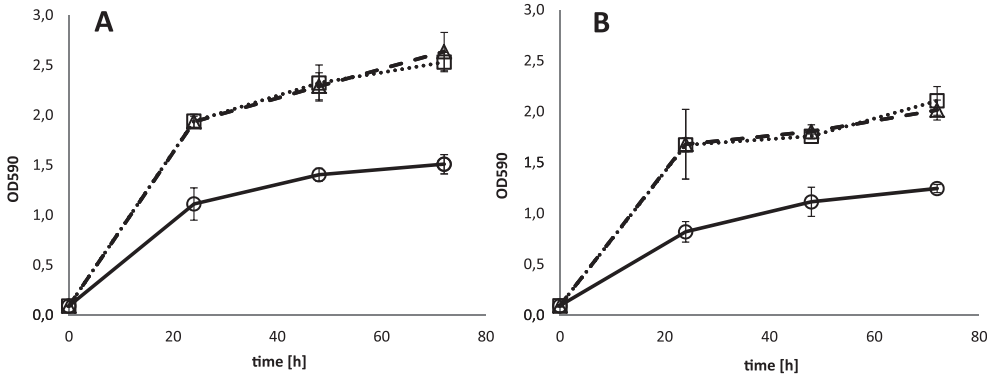


**Fig. 1.** Difference in growth of water kefir isolated yeasts in single- and in co-culture with lactobacilli in water kefir medium (WKM). Circles represent the single cultivation of *Z. florentina* (A) and *S. cerevisiae* (B), respectively. Dashed lines show growth of *Z. florentina* (A) and *S. cerevisiae* (B) in co-cultivation with *Lb. hordei*, dotted line the co-cultivation with *Lb. nagelii*.

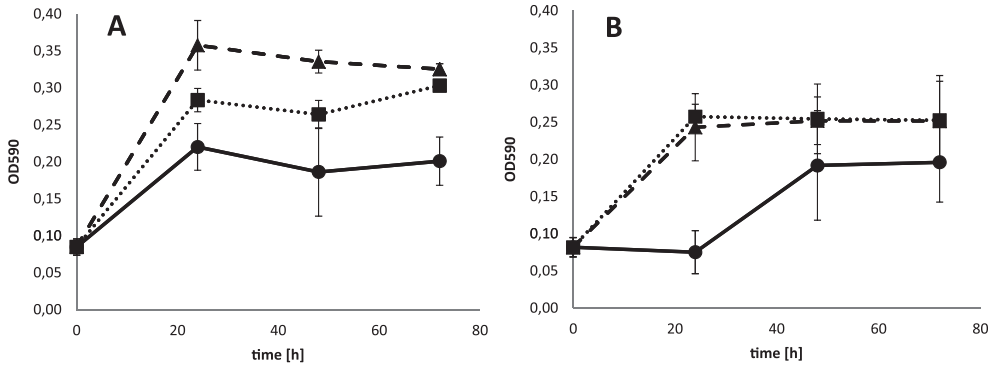


**Fig. 2.** Difference in growth of water kefir isolated lactobacilli in single- and in co-culture with yeasts in water kefir medium (WKM). Circles represent the single cultivation of *Lb. hordei* (A) and *Lb. nagelii* (B), respectively. Dashed lines show growth of *Lb. hordei* (A) and *Lb. nagelii* (B) in co-cultivation with *Z. florentina*, dotted line the co-cultivation with *S. cerevisiae*.

twofold higher growth rate (data not shown) thus, the influence of cell wall parts of the yeast on the *Lactobacillus* was determined. Since cell debris did not contain the stimulation factors for *Lb. nagelii,* yeast cells extract was added to arginine free medium. Addition of yeast cell extract yielded in stimulation of the *Lactobacillus* similar to SCDM with all essential nutrients.

### 3.4. Modification of WKM

As water kefir lactobacilli did not grow well in WKM hence, the influence of essential nutrients in WKM in different concentrations was determined. During 20 h of fermentation growth of both lactobacilli was higher in the modified medium than in pure WKM. After 40 h optical density (590 nm) of both lactobacilli increased only in the modified medium with five fold higher concentration of essential nutrients, the $OD_{590}$ in the lower concentration stagnated (Fig. 4).

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

## 4. Discussion

With a content of 31.3 and 22.7% of water kefir bacteria, *Lb. hordei* and *Lb. nagelii*, respectively, are the predominant bacteria in water kefir ascertained with state of the art methods. As main representatives of yeasts *Z. florentina* and *S. cerevisiae* were described. Thus, these apparent key players in water kefir were used for interaction experiments in this study. Inoculation with a bacteria : yeast ratio of 10% was similar to the average viable cell counts per gram water kefir grains (Gulitz et al., 2011).

In every co-cultivation experiment we could show an increase of cell yield for both interaction partners in comparison to pure cultures of single water kefir isolates from the beginning of fermentation (Figs. 1 and 2), delineating the interaction of these water kefir isolates as mutualism. The effect of interdependency of lactobacilli and yeast has also been observed in sourdough (Damiani et al., 1996; Gobbetti et al., 1994a, 1994b), in milk kefir (Cheirsilp et al., 2003a; 2003b), fermented milks (Gadaga et al., 2001) and in sugary kefir grains (synonym for water kefir; (Leroi and Pidoux, 1993a)). These studies just present a stimulation of co-cultured lactobacilli, yeasts were either unaffected or even decreased in growth for 65% (Leroi and Pidoux, 1993a). A support of yeast, as demonstrated in our study, has not been described, before. Merely, Leroi and Pidoux (1993b) could determine a small increase of yeast cell yield in mixed-cultures employing a bacteria : yeast ratio of 5%. Also, their study remains descriptive leaving the metabolic background unsolved. In our experiments we started with a bacteria : yeast cell ratio of 10%, thus an increase of yeast cell rate seems to enhance the interaction in water kefir.

Growth of *Z. florentina* was increased in pH lowered YPG at pH 4 in comparison to higher starting pH values. Lactobacilli produce lactic and acetic acid during fermentation and therefore, they optimize the milieu for *Z. florentina*. The growth of food fermenting yeasts is known to be improved at decreasing pH. For the brewing yeast *Saccharomyces carlsbergensis* Vosti and Joslyn (1954) described growth optimum at pH 3.83. The metabolic interaction for *Z. florentina* relies on the adjustment of the physiochemical environment.

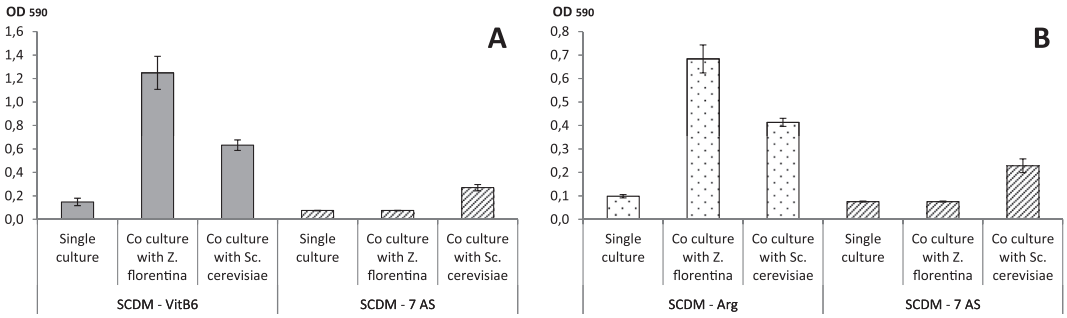


**Fig. 3.** Co-cultivation of water kefir isolated yeasts and lactobacilli in modified SCDM. $OD_{590}$ after 48 h cultivation of *Lb. hordei* (A) and *Lb. nagelii* (B) grown in SCDM-VitB6 (grey bars), SCDM-Arg (spotted bars) or SCDM3-7AS (striped bars), respectively, single cultivated or in co-culture in the model system with *Z. florentina* and *S. cerevisiae*, respectively.

**Table 1**
$OD_{590}$ after 72 h fermentation of *Lb. hordei* and *Lb. nagelii* in co-cultivation in the model system with *Z. florentina*, start $OD_{590}$ 0.1. As positive control lactobacilli were singly cultivated in SCDM containing all nutrients. For negative controls lactobacilli were singly cultivated in each SCDM without an essential amino acid. *Z. florentina* grew to $OD_{590}$ 0.75 in every medium.

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

[a] n.d. = not determined.

**Table 2**
Growth of *Lb. nagelii* under different conditions. Since *Z. florentina* was able to support *Lb. nagelii* in co-cultivation in the model system on SCDM − Arg, it was to find out under which circumstances *Z. florentina* released arginine sources. Growth of *Lb. nagelii* in different modified media was compared with its growth behavior in SCDM with all nutrients and SCDM − Arg. Single culture growth experiments were executed in microplates for 72 h of fermentation.

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

Pre-fermented media with yeasts could stimulate *Lb. hilgardii* in growth and lactic acid production, whereas free amino acids and vitamins did not show an effect (Leroi and Pidoux, 1993b). We could not confirm the support by pre-fermentation with our water kefir species tested, conversely, addition of vitamins and free amino acids play an important role, because *Lb. hordei* and *Lb. nagelii* show auxotrophies for some amino acids and vitamin $B_6$, respectively. Addition of essential nutrients to WKM in different concentrations showed the influence and lack of these substances in WKM. After 20 h of fermentation the content of essential nutrients in pure and lower modified WKM were already consumed because the optical density of both lactobacilli stagnated in these media. Only in the medium with higher concentrations of essential nutrients cells were able to replicate furthermore (Fig. 4). WKM contains glucose out of the fig concentrate as well as sucrose that is inverted to fructose and glucose, and yeasts are known to secrete vitamin $B_6$ during fermentation in the presence of glucose (Abbas, 2006). Thus, a part of trophic interaction between *Lb. hordei* and both yeasts is revealed as delivery of vitamin $B_6$ by *Z. florentina* and *S. cerevisiae*.

In co-cultivation with yeasts *Lb. nagelii* was able to grow in simplified chemically defined medium without addition of essential arginine. The support of *Z. florentina* showed 1.5 fold higher cell yield than in interaction with *S. cerevisiae*. With 52.5% of water kefir isolated yeasts *Z. florentina* is the predominant yeast in water kefir grains, thus it seems to play a more important role in mutualism therefore further interactions were only analyzed with this yeast. Addition of yeast fermentation broth, yeast cell debris, co-cultivation fermentation broth and pre-fermented medium with yeast and dead cells of *Lb. nagelii* did not support growth of *Lb. nagelii* in arginine free medium (Table 2). This accounts for the fact that *Z. florentina* only excretes amino acids essential for *Lb. nagelii* in co- or in mixed-culture, but not if they are singly cultivated. The effect that yeasts release essential nutrients for lactobacilli was observed in sourdough by Challinor and Rose (1954) and Gobbetti

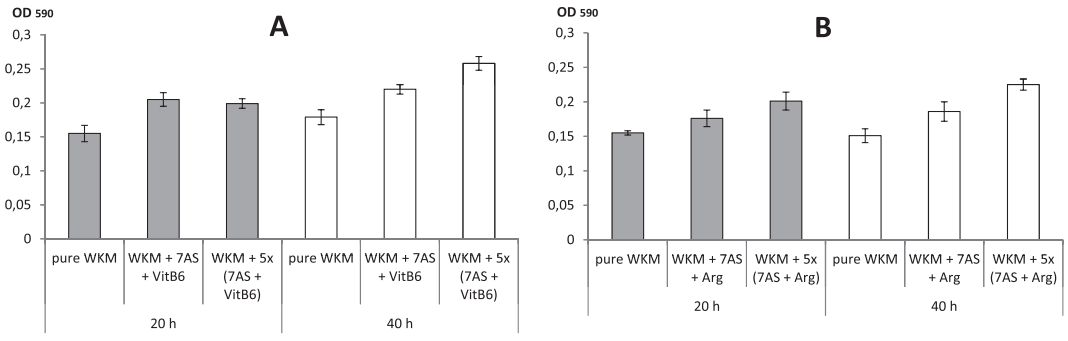


**Fig. 4.** Growth of *Lb. hordei* (A) and *Lb. nagelii* (B) in pure WKM and modified WKM supplemented with essential nutrients in different concentrations after 20 h (grey bars) and 40 h (white bars) of fermentation. Growth experiments were executed in microplates.

et al. (1994a). On the one hand release of amino acids by yeasts can be explained by the change in membrane permeability in presence of glucose (Lewis and Stephanopoulos, 1967) or by autolysis of yeast cells (Vosti and Joslyn, 1954). In our study we could demonstrate for the first time that the stimulation has to be caused by the *Lactobacillus*, because support only occurs in co-cultivation. The addition of yeast cell extract to arginine free medium resulted in growth of *Lb. nagelii* similar to medium with arginine. Therefore it is suggested that the co-culture of these two organisms partially affects autolysis in yeasts or triggers other mechanisms of (selective) nutrient release. Autolysis of yeasts can be induced e.g. by various proteins, peptides and amino acids leading to a change in membrane permeability (Babayan and Bezrukov, 1985), and thus *Lb. nagelii* may produce such molecules signaling the yeast to autolyze.

Fig. 5 displays an overview about the revealed metabolic interaction of main representative cultivable water kefir isolates. *Z. florentina* is depicted closer to the lactobacilli and the arrows are shown more intensive because of the higher influence of this yeast in comparison to *S. cerevisiae*. In co-cultivation of *S. cerevisiae* with lactobacilli the yeast is improved in growth but the reason has still to be ascertained. During this study we explained metabolic interactions of single water kefir isolates in a model system with planktonic cultures. Interaction experiments with organisms embedded in grains, closer to their natural association, are technically limited and have not been possible, yet. This may be due to the fact, that the water kefir consortium contains partly unculturable types of bifidobacteria (Gulitz et al., 2013). Interactions in water kefir are likely more complex than the interaction of only two organisms demonstrated here, and therefore, the role of other species, namely acetic acid bacteria, (yet unculturable) bifidobacteria and *Leuconostocaceae* remain to be elucidated.

WKM is a high sugar and low amino acid containing medium therefore, in this ecological niche only well adapted microorganisms are able to grow. *Lb. nagelii* was first isolated from slightly fermented grape juice that is a demanding habitat similar to water kefir, even the existence of yeasts implies that *Lb. nagelii* is well adapted in such ecological niches and lives in mutualism with yeasts. If we perceive water kefir as an organism rather than a mere association it forces itself that induction of autolysis of yeast cells on the one hand plays an important role for nutrient exchange and on the other hand for species regulation in the consortium, preventing organisms overgrow by others. Such social behaviors are known in other complex multicellular communities, namely biofilms, in terms of programmed cell death and lysis, while the control mechanisms are not fully understood (Rice and Bayles, 2008; Sadykov and Bayles, 2012). So it is appears likely that the water kefir consortium uses related mechanisms to regulate community composition and growth.

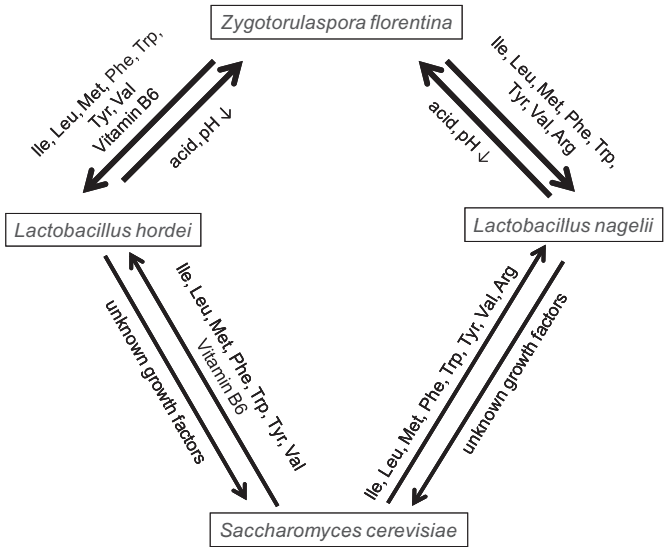


**Fig. 5.** Interaction overview of main representative cultivable water kefir isolates.

## 5. Conclusion

Water kefir medium is a demanding habitat for lactobacilli because of its low concentrations of essential amino acids and vitamins. Co-cultivation of predominant water kefir lactobacilli and yeasts in WKM results in support of growth of every interaction partner. This fact delineates the interaction between these water kefir isolates as mutualism. *Z. florentina* as well as *S. cerevisiae* can mutually provide *Lb. nagelii* with essential amino acids and *Lb. hordei* with vitamin $B_6$ in addition, whereas the impact of *Z. florentina* is greater than that of *S. cerevisiae*. We could demonstrate as first that the release of essential nutrients from yeasts for lactobacilli has to be induced by the lactobacilli, because stimulation only occurs in co-cultivation. The metabolic interaction for *Z. florentina* relies on the adjustment of the physiochemical environment by the production of organic acids by lactobacilli and the resulting acidification of the milieu.

## References

Abbas, C.A., 2006. Production of antioxidants, aromas, colours, flavours, and vitamins by yeasts. In: Querol, A., Fleet, G.H. (Eds.), 2006. Yeasts in Food and Beverages, vol. 184. Springer, Berlin, pp. 285–334.
Babayan, T.L., Bezrukov, M.G., 1985. Autolysis in yeasts. Acta Biotechnologica 5 (2), 129–136.
Challinor, S., Rose, A., 1954. Interrelationships between a yeast and a bacterium when growing together in defined medium. Nature (London) 174, 877–878.
Cheirsilp, B., Shimizu, H., Shioya, S., 2003a. Enhanced kefiran production by mixed culture of Lactobacillus kefiranofaciens and Saccharomyces cerevisiae. Journal of Biotechnology 100 (1), 43–53.
Cheirsilp, B., Shoji, H., Shimizu, H., Shioya, S., 2003b. Interactions between *Lactobacillus kefiranofaciens* and *Saccharomyces cerevisiae* in mixed culture for kefiran production. Journal of Bioscience and Bioengineering 96 (3), 279–284.
Damiani, P., Gobbetti, M., Cossignani, L., 1996. The sourdough microflora. Characterization of hetero-and homofermentative lactic acid bacteria, yeasts and their interactions on the basis of the volatile compounds. LWT-Food Science and Technology 70, 63–70.
De Bary, H.A., 1879. Die Erscheinung der Symbiose, vol. 8. Karl J. Trubner, Strasbourg.
De Vuyst, L., Neysens, P., 2005. The sourdough microflora: biodiversity and metabolic interactions. Trends in Food Science & Technology 16 (1–3), 43–56.
Farnworth, E., 2005. Kefir – a complex probiotic. Food Science and Technology 2, 1–17.
Frey-Klett, P., Burlinson, P., Deveau, A., Barret, M., Tarkka, M., Sarniguet, A., 2011. Bacterial-fungal interactions: hyphens between agricultural, clinical, environmental, and food microbiologists. Microbiology and Molecular Biology Reviews: MMBR 75 (4), 583–609.
Gadaga, T.H., Mutukumira, A.N., Narvhus, J.A., 2001. The growth and interaction of yeasts and lactic acid bacteria isolated from Zimbabwean naturally fermented milk in UHT milk. International Journal of Food Microbiology 68 (1–2), 21–32.
Gobbetti, M., Corsetti, A., Rossi, J., 1994a. The sourdough microflora. Interactions between lactic acid bacteria and yeasts: metabolism of amino acids. World Journal of Microbiology and Biotechnology 10, 275–279.
Gobbetti, M., Corsetti, A., Rossi, J., 1994b. The sourdough microflora. Interactions between lactic acid bacteria and yeasts: metabolism of carbohydrates. Applied Microbiology and Biotechnology, 456–460.
Gulitz, A., Stadie, J., Ehrmann, M.A., Ludwig, W., Vogel, R.F., 2013. Comparative phylobiomic analysis of the bacterial community of water kefir by 16S rRNA gene amplicon sequencing and ARDRA analysis. Journal of Applied Microbiology, 1–10.
Gulitz, A., Stadie, J., Wenning, M., Ehrmann, M.A., Vogel, R.F., 2011. The microbial diversity of water kefir. International Journal of Food Microbiology 151 (3), 284–288.
Hebert, E.M., Raya, R.R., De Giori, G.S., 2000. Nutritional requirements and nitrogen-dependent regulation of proteinase activity of Lactobacillus helveticus CRL 1062. Applied and Environmental Microbiology 66 (12), 5316–5321.
Horisberger, M., 1969. Structure of the dextran of the tibi grain. Carbohydrate Research 10, 379–385.
Kebler, L., 1921. California bees. Journal of the Amercian Pharmaceutical Association 10, 939–943.
Kurtzman, C., 2003. Phylogenetic circumscription of, and other members of the Saccharomycetaceae, and the proposal of the new genera Lachancea,

Nakaseomyces, Naumovia, Vanderwaltozyma and Zygotorulaspora. FEMS Yeast Research 4 (3), 233–245.
Leroi, F., Pidoux, M., 1993a. Detection of interactions between yeasts and lactic acid bacteria isolated from sugary kefir grains. Journal of Applied Microbiology 74 (1), 48–53.
Leroi, F., Pidoux, M., 1993b. Characterization of interactions between *Lactobacillus hilgardii* and *Saccharomyces florentinus* isolated from sugary kefir grains. Journal of Applied Microbiology 74 (1), 54–60.
Lewis, M.J., Stephanopoulos, D., 1967. Glucose-induced release of amino acids from Saccharomyces carlsbergensis by action on the cytoplasmic membrane. Journal of Bacteriology 93, 976–984.
Pidoux, M., Brillouet, J., Quemener, B., 1988. Characterization of the polysaccharides from a *Lactobacillus brevis* and from sugary kefir grains. Biotechnology Letters 10 (6), 415–420.
Reiß, J., 1990. Metabolic activity of Tibi grains. Zeitschrift für Lebensmitteluntersuchung und Forschung 191 (6), 462–465.
Rice, K.C., Bayles, K.W., 2008. Molecular control of bacterial death and lysis. Microbiology and Molecular Biology Reviews 72 (1), 85–109 (table of contents).
Sadykov, M.R., Bayles, K.W., 2012. The control of death and lysis in staphylococcal biofilms: a coordination of physiological signals. Current Opinion in Microbiology 15 (2), 211–215.
Vosti, D.C., Joslyn, M.A., 1954. Autolysis of several pure culture yeasts. Applied and Environmental Microbiology 1, 79–84.
Ward, M., 1892. The ginger-beer plant, and the organisms composing it: a contribution to the study of fermentation yeasts and bacteria. Philosophical Transactions of the Royal Society of London 183, 125–187.
Zourari, A., Accolas, J., Desmazeaud, M., 1992. Metabolism and biochemical characteristics of yogurt bacteria. A review. Le Lait, 1–34.