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Oxygen and diverse nutrients influence the water kefir fermentation process

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ABSTRACT

Eight water kefir fermentation series differing in the presence of oxygen, the nutrient concentration, and the nutrient source were studied during eight consecutive backslopping steps. The presence of oxygen allowed the proliferation of acetic acid bacteria, resulting in high concentrations of acetic acid, and decreased the relative abundance of *Bifidobacterium aquikefiri*. Low nutrient concentrations resulted in slow water kefir fermentation and high pH values, which allowed the growth of *Comamonas testosteroni*/*thiooxydans*. Further, low nutrient concentrations favored the growth of *Lactobacillus hilgardii* and *Dekkera bruxellensis*, whereas high nutrient concentrations favored the growth of *Lactobacillus nagelii* and *Saccharomyces cerevisiae*. Dried figs, dried apricots, and raisins resulted in stable water kefir fermentation. Water kefir fermentation with dried apricots resulted in the highest pH and water kefir grain growth, whereas that with raisins resulted in the lowest pH and water kefir grain growth. Further, water kefir fermentation with raisins resembled fermentations with low nutrient concentrations, that with dried apricots resembled fermentations with normal nutrient concentrations, and that with fresh figs or a mixture of yeast extract and peptone resembled fermentations with high nutrient concentrations.

1. Introduction

Water kefir is a traditional fermented beverage that is made by adding water kefir grains (the inoculum) to a mixture of water, (dried) fruits, and sugar (Gulitz et al., 2013; Laureys and De Vuyst, 2014; Laureys et al., 2017; Marsh et al., 2013; Stadie et al., 2013). Usually, this mixture is fermented at room temperature under anaerobic conditions for two to four days, after which it is sieved to separate the water kefir grains from the water kefir liquor. The water kefir liquor is a slightly sweet, acidic, alcoholic, and sparkling beverage that has a yellowish color and a fruity aroma. The water kefir grains consist of dextran exopolysaccharides (EPS), are translucent, have a brittle structure, and are insoluble in water (Laureys and De Vuyst, 2014; Waldherr et al., 2010). Many different microorganisms occur on the water kefir grains, whereby the key microorganisms of water kefir fermentation are the lactic acid bacteria (LAB) species *Lactobacillus hilgardii*, *Lactobacillus nagelii* and *Lactobacillus paracasei*; and the yeast species *Saccharomyces cerevisiae* (Laureys and De Vuyst, 2017). Sucrose is the main substrate for the water kefir microorganisms and is converted into water kefir grain EPS, ethanol, carbon dioxide, lactic acid, glycerol, acetic acid, mannitol, and a variety of aroma compounds (Laureys and De Vuyst, 2014; Martínez-Torres et al., 2017).

The contents of the vessel wherein water kefir fermentation takes place are usually separated from the atmosphere by a rubber sealing or water lock (Gulitz et al., 2011; Laureys and De Vuyst, 2014; Pidoux, 1989; Stadie et al., 2013). These configurations prevent the ingress of atmospheric oxygen, but allow the release of carbon dioxide, thus preventing excessive pressure build-up in the fermentation vessel. Consequently, the water kefir fermentation process starts aerobically and becomes gradually anaerobic, as oxygen is consumed and/or flushed out by the carbon dioxide produced by the yeasts. Oxygen can have an impact on the growth and metabolism of several of the water kefir microorganisms, such as on yeasts (Aceituno et al., 2012) and acetic acid bacteria (AAB) (Guillamón and Mas, 2009), suggesting that the presence of oxygen

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might influence the microbial species diversity and/or metabolite production during water kefir fermentation.

The water used for fermentation contains calcium ions and buffer compounds necessary for optimal water kefir grain growth (D. Laureys, M. Aerts, P. Vandamme, and L. De Vuyst, unpublished results). Other nutrients necessary for water kefir fermentation, such as amino acids, vitamins, and minerals are provided by the (dried) fruits added to the fermentation mixture. Although fruits are rich in such nutrients, the relatively small amount of (dried) fruits in the recipe makes the water kefir fermentation medium relatively poor in nutrients. As (dried) fruits are usually the sole source of a variety of important nutrients during water kefir fermentation, the amount and/or types of fruits used for fermentation might have an impact on the microbial species diversity, substrate consumption, and/or metabolite production during fermentation. Dried figs are the most common fruits used for water kefir fermentation (Gulitz et al., 2011; Laureys and De Vuyst, 2014; Pidoux, 1989; Stadie et al., 2013), but raisins, plums, or dates have also been used (Reiß, 1990).

This study aimed to investigate the influence of the presence of oxygen, the nutrient concentration, and the nutrient source on the microbial species diversity, water kefir grain growth, substrate consumption, and metabolite production during water kefir fermentation.

2. Materials and methods

2.1. Water kefir grain inoculum and prefermentations

A water kefir grain inoculum was obtained from the household water kefir fermentation process of a private person (Ghent, Belgium), as described before (Laureys and De Vuyst, 2014). To obtain the necessary amount of water kefir grains, the inoculum was cultivated through a series of consecutive prefermentations through backslopping until >1300 g of water kefir grain wet mass was produced. The prefermentations were performed in glass Schott bottles (1, 2, and 5 l) equipped with a polytetrafluoroethylene (PTFE) water lock. They were started by adding 10 g of sugar (Candico Bio, Merksem, Belgium), 5 g of dried figs (King Brand, Naziili, Turkey), and 160 ml of tap water (Brussels, Belgium) per 50 g of water kefir grains. The bottles were incubated in a water bath at 21 °C. Every 3 d, the backslopping practice was applied, whereby the water kefir grains were separated from the water kefir liquors by sieving, and then recultivated in fresh medium under the same conditions as described above.

2.2. Fermentations

The water kefir grain mass, obtained through the series of prefermentations described above, was rinsed and used to start eight series of water kefir fermentations differing in the presence of oxygen, the nutrient concentration, and the nutrient source for fermentation. Rinsing of the grains was performed with 2 l of tap water (Brussels, Belgium) per 50 g of water kefir grains. Each fermentation series was performed in independent biological triplicates. All fermentations were carried out in 250-ml Schott bottles. They were started with 10 g of sugar (Candico Bio), 160 ml of tap water (Brussels, Belgium), and 50 g of rinsed water kefir grains. To study the influence of oxygen, the fermentation mixtures were supplemented with 5 g of dried figs and incubated under anaerobic (fermentation series 1DF-An) or aerobic conditions (1DF-Ae). To study the influence of the nutrient concentration under anaerobic conditions, the fermentation mixtures were supplemented with 0 (0DF-An), 5 (1DF-An), or 10 g of dried figs (2DF-An). To study the influence of the nutrient source under anaerobic conditions, the fermentation mixtures were supplemented with 5 g of dried figs (1DF-An), 5 g of dried apricots (1DA-An), 5 g of dried raisins (1DR-An), 17 g of fresh figs (1FF-An), or 1 ml of autoclaved yeast extract-peptone (YP) solution (YP-An). The YP solution was prepared by adding 125 g l^{-1} of yeast extract (Merck, Darmstadt, Germany) and 125 g l^{-1} of bacteriological peptone (Oxoid, Basingstoke, UK) to ultrapure water (gradient A10 Milli-Q water purification system; EMD Millipore, Billerica, MA, USA), after which this mixture was sterilized by autoclaving (121 °C, 2.1 bar, 20 min). The Schott bottles were equipped with a PTFE water lock for fermentations under anaerobic conditions (0DF-An, 1DF-An, 2DF-An, 1DA-An, 1DR-An, 1FF-An, and YP-An) or were covered with a muslin cloth for fermentations under aerobic conditions (1DF-Ae). All bottles were incubated in a water bath at 21 °C. The contents of the fermentation bottles were mixed by gently turning the bottles at the start and at the end of each backslopping step. Every 3 d, the backslopping practice was applied for each fermentation bottle, whereby the water kefir grains were separated from the water kefir liquors by sieving, rinsed, after which 50 g of water kefir grains were recultivated in fresh medium with the same composition and under the same conditions as before. This practice was continued for eight backslopping steps.

2.3. Analyses

The pH and the water kefir grain wet mass were determined at the end of each backslopping step. The water kefir grain dry mass was determined at the end of backslopping step 8. The viable counts of the LAB, yeasts, and AAB were determined for the non-rinsed water kefir grains of the inoculum and the eight fermentation series at the end of backslopping step 8. The culture-dependent microbial species diversity of the LAB, yeasts, and AAB was determined for the non-rinsed water kefir grains of the inoculum and the eight fermentation series at the end of backslopping step 8. The culture-independent microbial species diversity was determined for the water kefir liquors and the non-rinsed water kefir grains of the inoculum and the eight water kefir fermentation series at the end of backslopping step 8. The substrate and metabolite concentrations were determined for the water kefir liquors of the eight fermentation series at the end of backslopping steps 1 and 8. At the end of backslopping step 8, the water kefir grains were assessed visually.

The results are presented as the mean ± standard deviation of the three independent biological replicates performed for each fermentation series.

2.4. pH, water kefir grain wet and dry mass, and water kefir grain growth determinations

The pH, the water kefir grain wet mass, the water kefir grain growth, and the water kefir grain dry mass were determined as described before (Laureys and De Vuyst, 2014), except for the fact that the water kefir grains were not rinsed with saline.

2.5. Microbial enumerations

The viable counts of the presumptive LAB and AAB were determined as described before (Laureys and De Vuyst, 2014), except for the fact that an additional antibiotic, amphotericin B (final concentration of 0.0025 g l^{-1}; Sigma-Aldrich, Saint Louis, MO, USA), was added to the de Man-Rogosa-Sharpe (MRS) and modified deoxycholate-mannitol-sorbitol (mDMS) agar media. The viable counts of the presumptive yeasts were determined on yeast extract-peptone-dextrose (YPD) agar medium supplemented with chloramphenicol (final concentration of 0.1 g l^{-1}; Sigma-Aldrich),