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MICROBIAL SPECIES DIVERSITY, COMMUNITY DYNAMICS, SUBSTRATE CONSUMPTION, AND METABOLITE PRODUCTION DURING WATER KEFIR FERMENTATION

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INTRODUCTION

Many fermented beverages exist since ancient times, but only a few of them are widely available commercially. Originally, all fermented beverages were produced through a spontaneous or backslopped natural fermentation process, but nowadays most of the well-known fermented beverages are produced through a starter culture-initiated fermentation process. The latter is preferred for industrial fermentation processes, as it results in a faster process and safer and more consistent end-products. Nevertheless, many fermented beverages continue to be produced through a natural fermentation process at household level or small industrial scale. A starter culture-initiated fermentation process involves only one (or a few) microorganism(s) that is (are) deliberately added to start the fermentation, whereas spontaneous or backslopped natural fermentation processes usually involve many different microorganisms, some of which may be health-promoting. In Chapter 1, four different naturally fermented beverages, namely Belgian-style acidic ales, kombucha, milk kefir, and water kefir are described, and the metabolic and health potential of the microorganisms occurring in these fermented beverages are dealt with in detail.

Natural fermentation processes are often complex and not well understood, and the tools and methods available to study these ecosystems are still evolving, remain time-consuming, and have many limitations. However, to be able to optimize a naturally fermented beverage, a thorough understanding of its fermentation process is indispensible. Water kefir is one of the naturally fermented beverages that might have the potential to be optimized as a tasty and healthy naturally fermented beverage. It is produced by an anaerobic fermentation process, which is started by adding a water kefir grain inoculum to a mixture of water, sugar, and dried figs. In this doctoral thesis, the water kefir fermentation process was investigated in detail. The aims of the experimental work are outlined in Chapter 2.

In Chapter 3, the microbial species diversity, community dynamics, water kefir grain wet and dry mass, pH, and substrate consumption and metabolite production kinetics during a water kefir fermentation process were investigated to obtain a better understanding of the water kefir fermentation process. However, different water kefirs harbour different microbial species diversities and the influence of the water kefir grain inoculum on the fermentation process was investigated in Chapter 4 by comparing three fermentation processes started with different water kefir grain inocula. In Chapter 5, an industrial water kefir production process suffering from instability and low water kefir grain growth was characterized to gain more insight into the causes of these two common problems during water kefir fermentation. In several of the previous water kefir fermentation processes, a novel *Bifidobacterium* species was found. Therefore, in Chapter 6, a strain of this species was isolated from a water kefir fermentation process and characterized genotypically and phenotypically. The results in Chapter 4 suggested that acidic stress may influence the water kefir grain growth and a literature search suggested that the calcium concentration may also play a role in the water kefir grain growth. Therefore, the influence of the buffer capacity and calcium concentration of the water used for fermentation on the characteristics of the water kefir fermentation process was investigated in Chapter 7. Water kefir fermentation is usually performed anaerobically with dried figs as a source of nutrients. However, the influences of the presence of oxygen, nutrient concentration, and nutrient source during fermentation on the characteristics of the water kefir fermentation process needed more attention, which was investigated in Chapter 8. In Chapter 9, the kinetics of the water kefir fermentation processes differing in the presence of oxygen, and the type and concentration of the inoculum and substrate during fermentation were modelled and compared to investigate the influence of these factors on the water kefir fermentation process. In Chapter 10, the influence of the

backslopping time, rinsing of the water kefir grains before each backslopping step, and incubation temperature on the characteristics of the water kefir fermentation process were investigated.

CHAPTER 1

Exploring the potential of naturally fermented beverages

David Laureys and Luc De Vuyst

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1 Naturally fermented beverages in the human diet

1.1 Origins

People have been consuming fermented foods and beverages since ancient times (Hutkins, 2006). At first, this was probably unintentional, as fermentation of fresh raw food materials occurs spontaneously (Campbell-Platt, 1994). For example, milk ferments spontaneously into a sour yoghurt-like beverage. Similarly, grape juice or water sweetened with honey ferments spontaneously into a tasty and intoxicating wine-like beverage. Well-fermented foods and beverages can be stored for extended periods of time, and this property made fermentation of fresh raw materials an common preservation technique for years (Ross *et al.*, 2002). Later, other high-quality preservation techniques (such as pasteurization, sterilization, refrigeration, and freezing) were developed and displaced traditional fermentations. Nevertheless, fermented food products remained always widely available due to their unique organoleptic properties (Caplice & Fitzgerald, 1999).

Depending on the raw materials available and cultural habits applying, many different fermentation techniques were developed worldwide, whereby the fermented food products in China, Japan, and Korea were vastly different from those in the Middle East, the Mediterranean, and Europe (Hutkins, 2006; Tamang *et al.*, 2016). The outcome of a spontaneous food fermentation process depends on many factors, such as the status of the raw materials, the indigenous microorganisms they harbor, and the processing conditions (Leroy & De Vuyst, 2004). This can result not only in safe and tasty end-products but also in unsafe and putrid ones (Nout, 1994). Spontaneous fermentation processes are known to be slow, to result in end-products of variable quality, and to occasionally fail to produce well-fermented food products, as they depend on the microorganisms present in the raw materials. Several techniques were developed to circumvent these problems, such as backslopping practices or the use of starter cultures. In a backslopping practice, a part of a previously well-fermented food product (containing the desired microorganisms and metabolites) is added to a new batch of raw materials. This practice results in the dominance of the most adapted strains and makes the fermentation faster, safer, more uniform, and more reliable (Leroy & De Vuyst, 2004). Nevertheless, the composition of the backslopped microbiota may change or a backslopped fermentation process may become contaminated with undesired microorganisms, resulting in variability of the end-products as a function of time. Starter cultures are pure microbial strains or mixtures of pure strains (usually isolated from their niche of application) that are used to start a new fermentation process. They allow the industrial-scale production of consistent, uniform, high-quality fermented food products. The convenience of starter cultures is evident from their application in a wide variety of industrially fermented foods and beverages, such as beer, wine, yoghurt, cheese, bread, salami, and Bionade [a malt-based beverage fermented with acetic acid bacteria (AAB)].

1.2 Purposes

Despite the convenience of starter cultures, a whole range of fermented foods and beverages are still produced by either spontaneous fermentation (such as certain wines and beers) or backslopping (such as sourdoughs and sauerkraut). These naturally fermented foods and beverages are characterized by the presence of complex microbial communities, which encompass yeasts, lactic acid bacteria (LAB), AAB, bifidobacteria, staphylococci, bacilli, and/or filamentous fungi, and may result in a more desirable and complex flavor development compared to fermented foods and beverages obtained with starter cultures (Romano *et al.*, 2003; Camu *et al.*, 2007; Zhao *et al.*, 2011; Janssens *et al.*, 2012; Navarrete-Bolaños, 2012).

Many backslopped naturally fermented foods and beverages exist already for a long time and are often spread over the whole world by handing over the culture from person to person. Some of these products (such as kombucha and milk kefir) are already commercially available, whereas others (such as water kefir) are still predominantly produced at household level (Pothakos *et al.*, 2016).

Fermentation of raw materials can improve the shelf-life, taste and aroma, and texture of the end-products (Leroy & De Vuyst, 2004; Hutkins, 2006). Moreover, for a long time, fermented foods and beverages have been acknowledged for their positive influence on human health, which can be ascribed to the microorganisms they contain or their metabolites (Metchnikoff, 1908; Stanton *et al.*, 2005). A fermentation process can indeed enhance the nutritional value or health-promoting aspects of raw materials by the production of vitamins (Stanton *et al.*, 2005), bioactive peptides (Seppo *et al.*, 2003), antioxidants (Bernaert *et al.*, 2013), or compounds with certain therapeutic or prophylactic properties (Parvez *et al.*, 2006) by the microorganisms involved.

Exopolysaccharides (EPS) are carbohydrate polymers that can be classified into homo- or heteropolysaccharides, based on their composition and their production mechanism (De Vuyst & Degeest, 1999; Monsan *et al.*, 2001). Many microorganisms (and in particular LAB) present during food fermentations are able to produce certain exopolysaccharides, some of which may possess functional and/or prebiotic properties (De Vuyst *et al.*, 2001; Grosu-Tudor *et al.*, 2013; Salazar *et al.*, 2015; Leroy & De Vuyst, 2016). Prebiotics are selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, which confer a health benefit on the host (Gibson *et al.*, 2010). They are not digested nor absorbed in the human small intestine and thus reach the colon intact. Their selective fermentation by beneficial microorganisms in the colon, usually bifidobacteria and lactobacilli, separates them from common fibers (Slavin, 2013). Prebiotics are traditionally prepared from plant sources such as wheat, chicory, or potatoes (Fuentes-Zaragoza *et al.*, 2011; Apolinario *et al.*, 2014; Rivière *et al.*, 2016), but may also be produced *in situ* during a (food) fermentation process (Korakli *et al.*, 2002; De Vuyst *et al.*, 2003; Tieking *et al.*, 2003).

Also, certain live microorganisms can positively influence human health, which has resulted in the development of probiotics. Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill *et al.*, 2014). Probiotic microorganisms can be administered in the form of supplements (Pandey *et al.*, 2015) or (fermented) foods and beverages (Kumar *et al.*, 2015; Konar *et al.*, 2016). For example, the European Food Safety Authority has recognized that the consumption of yoghurt produced with and containing living cells of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strains, improves lactose digestion (EFSA, 2010). The combination of probiotics and prebiotics, also known as synbiotics, may have a synergistic effect (Schrezenmeir & de Vrese, 2001; Pandey *et al.*, 2015; Konar *et al.*, 2016).

Starter cultures for the industrial fermentation of foods and beverages are selected predominantly for their technological properties and not yet for their potential for health benefits (Heller, 2001). Alternatively, live probiotic bacteria are not easy to incorporate into food products (Saarela *et al.*, 2000). The isolation of microorganisms with probiotic potential from naturally fermented food products, such as fermented cereals (Lei & Jakobsen, 2004), fermented milk products (Thirabunyanon *et al.*, 2009), fermented meat (Pennacchia *et al.*, 2004), and fermented vegetables (Bautista-Gallego *et al.*, 2013), may result in the development of probiotic functional starter cultures for industrial food and beverage fermentations (Leroy & De Vuyst, 2004; De Vuyst *et al.*, 2008).

2 Naturally fermented beverages

Fermented beverages can be classified as alcoholic, low-alcoholic, and alcohol-free beverages (Belgisch Ministerie van Economische Zaken, 1993). Alcoholic beverages encompass beers, wines, and ciders [< 18 % (v v^{-1}) of alcohol], fortified wines [15-22 % (v v^{-1}) of alcohol], and distilled alcoholic beverages or spirits [> 35 % (v v^{-1}) of alcohol]. Low-alcoholic beverages (such as water kefir and milk kefir) may contain 0.5-1.2 % (v v^{-1}) of alcohol and alcohol-free beverages (such as kombucha) may contain < 0.5 % (v v^{-1}) of alcohol, but these limits vary depending on the country.

In this overview, the preparation, microbiology, and health benefits of four naturally fermented beverages (Belgian-style acidic ales, kombucha, milk kefir, and water kefir) will be discussed, and the metabolic, nutritional, prebiotic, and probiotic potential of the microorganisms involved will be explored in more detail.

2.1 Belgian-style acidic ales

Belgian-style acidic ales or lambic beers are spontaneously fermented beers made by pouring boiled wort into a coolship (a shallow open container) wherein it is cooled down overnight and becomes inoculated by the microorganisms in the air that is blown over the wort (Spitaels *et al.*, 2014; Spitaels *et al.*, 2015a). The wort is prepared with barley malt and at least 30 % of unmalted wheat (Belgisch Ministerie van Economische Zaken, 1993). The specific mashing process results in a high dextrinous wort (Van Oevelen *et al.*, 1976; Spitaels *et al.*, 2014). Over-aged hops are used for the preparation of the wort, because ageing decreases the bitterness of the fresh hops while retaining their antimicrobial activity (Spitaels *et al.*, 2015a). The cooled and inoculated wort is then transferred into horizontal wooden casks, which are completely filled to create anaerobic conditions. Fermentation and maturation takes place in the casks at cellar or ambient temperatures and can last for up to 36 months (Spitaels *et al.*, 2014). The resulting non-carbonated lambic beers can be used for the production of gueuze or fruit beers (*e.g.*, kriek). Gueuze beers are produced by preparing a mixture of young (< 12 months) and old (> 36 months) lambic beers, which are bottled and refermented in the bottle, resulting in an alcoholic, complex flavored, carbonated, and refreshing beer (Pothakos *et al.*, 2016). Acidic ales are probably the oldest known beers (Verachtert & Derdelinckx, 2014). Traditional Belgian lambic beers are brewed near the Senne valley during the coldest months of the year (from October till March) (Spitaels *et al.*, 2014). Recently, American craft breweries have developed similar types of beer, which are referred to as American coolship ales (Bokulich *et al.*, 2012).

The main carbohydrates during lambic beer fermentation are glucose, maltose, and maltooligosaccharides, which are converted into ethanol, lactic acid, and acetic acid (Verachtert & Derdelinckx, 2014; Spitaels *et al.*, 2015a). Furthermore, several aroma compounds such as isoamyl alcohol, ethyl acetate, ethyl lactate, isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate are formed during fermentation (Van Oevelen *et al.*, 1976; Spitaels *et al.*, 2015a). Overall, four different phases can be distinguished during the spontaneous lambic beer fermentation process (Spitaels *et al.*, 2014) (Figure 1). The first phase lasts for 7 days and is characterized by the presence of *Enterobacteriaceae*. This phase can be avoided by acidifying the wort with lactic acid until pH 4.0, as is the case in most lambic beer production processes (Spitaels *et al.*, 2015b). The second phase starts after 1 to 4 weeks and represents the main fermentation, which is carried out by *Saccharomyces cerevisiae* (producing mainly ethanol). The third phase starts after 1 to 4 months and is the acidification phase, which is characterized by the presence of *Pediococcus damnosus* (producing mainly lactic acid). The fourth phase starts after 4 to 8 months and is the

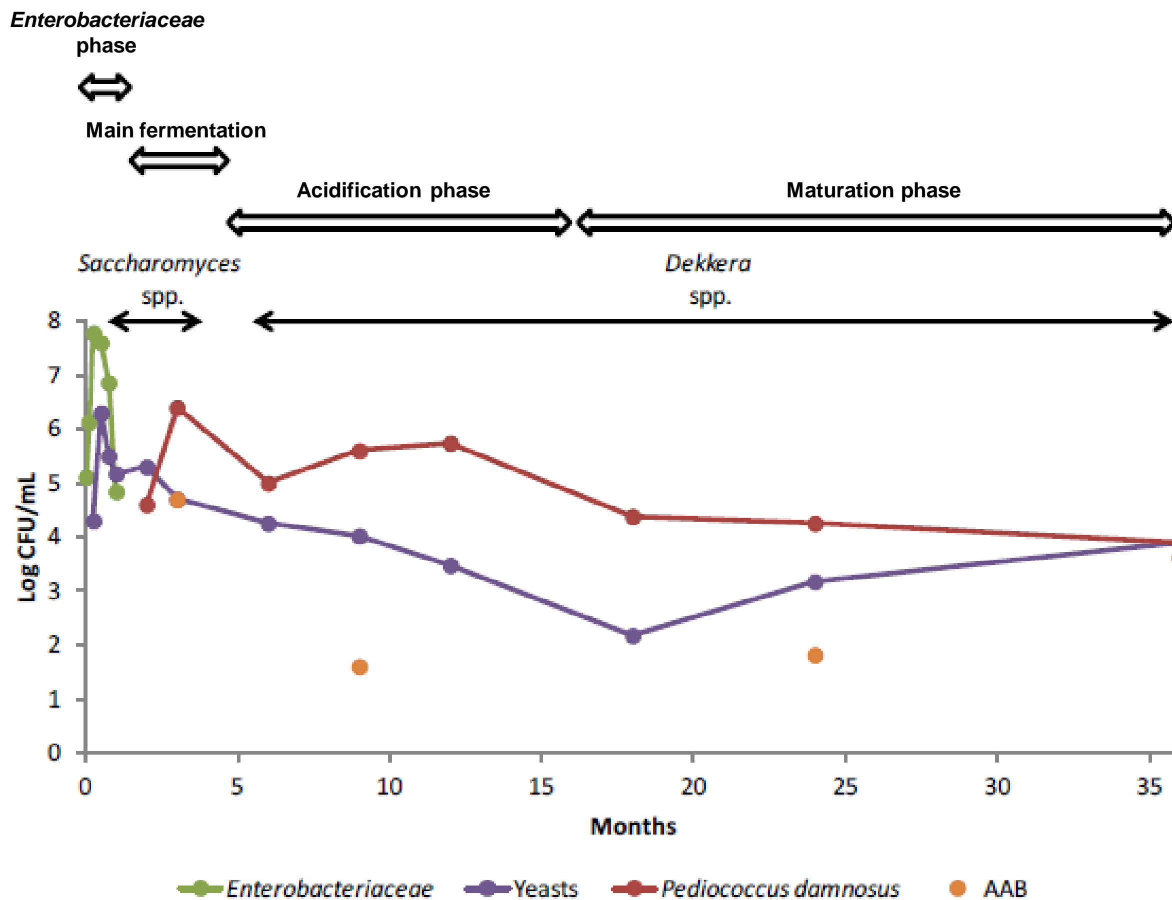

Figure 1. The viable counts of the different microorganisms during the main phases of the spontaneous fermentation process of Belgian-style acidic ales, adapted from Spitaels (2014). The viable counts are expressed as log colony forming units (cfu) per ml.

maturation phase, which is characterized by the presence of *Dekkera bruxellensis* (producing mainly ethanol) and a decreasing abundance of LAB. This last phase may last up to 2 to 3 years, during which the wort is further attenuated. AAB (producing mainly acetic acid) can be present during the entire process and include *Acetobacter lambici* and *Gluconobacter cerevisiae* (Pothakos *et al.*, 2016).

Only little information is available about the influence of these types of beer on health (Bamforth, 2002; Polak *et al.*, 2013).

2.2 Kombucha

Kombucha is a fermented tea made by adding a kombucha culture (the inoculum) to a tea infusion sweetened with sucrose (Dufresne & Farnworth, 2000). This mixture is fermented under atmospheric conditions at room temperature for 4 to 21 days, after which it is sieved to separate the liquor from the kombucha culture. The kombucha liquor is a mildly sour and sweet refreshing beverage, which develops into a sour beverage with a vinegar-like taste after prolonged fermentation (Jayabalan *et al.*, 2014). The high concentrations of organic acids may render the kombucha tea even harmful upon direct consumption. The fermentation is typically carried out in a wide mouth vessel covered with a muslin cloth to allow the presence of oxygen during the fermentation process. Usually, around 50-100 g l^{-1} of sucrose is added as

substrate for fermentation and 1-5 g l^{-1} of black or green tea is added as nutrient source (Reiß, 1993). The origin of kombucha is not known, but it occurs worldwide under a variety of different names such as tea fungus, tea kvass, and Kargasok tea, depending on the geographical location (Jayabalan *et al.*, 2014). In Japanese, kombucha is the name of a tea (*cha* in Japanese) made by water and the algae Dashi-kombu. The kombucha as referred to in this chapter is called *kohcha-kinoko* in Japanese, whereby *kohcha* is the word for the European-style of tea (English tea) and *kinoko* is the word for fungus (Pothakos *et al.*, 2016).

The kombucha culture (also called the "mother") contains cellulose [β-(1->4)-linked glucose polymer], is grayish and opaque, has a tough and gelatinous structure, is insoluble in water, and floats on top of the fermentation liquor. The kombucha microorganisms are attached to the cellulose-containing mass and detach partly into the liquor when the culture is added to the sweetened tea infusion. The main microorganisms occurring during kombucha fermentation are yeasts and AAB, whereas LAB can be present in lower abundances (Marsh *et al.*, 2014b). The most characteristic microorganism is the AAB species *Komagataeibacter xylinus*, formerly *Acetobacter xylinum* (Reva *et al.*, 2015). It converts part of the carbohydrates into cellulose, resulting in the formation of the characteristic kombucha culture during fermentation (Jayabalan *et al.*, 2014). The extra culture that is produced upon fermentation can be discarded, handed over to other persons, or used to scale up the fermentation process. A new kombucha culture also develops spontaneously in a sweetened tea that is inoculated with kombucha liquor containing live microorganisms. During fermentation, sucrose is converted into ethanol, acetic acid, and low concentrations of lactic acid. Glucose may be converted into gluconic acid and glucuronic acid by the AAB. Furthermore, nitrogen-fixating AAB species, such as *Acetobacter nitrogenifigens* and *Gluconobacter kombuchae*, have been found in kombucha tea (Dutta & Gachhui, 2006, 2007).

Kombucha tea has been claimed to possess many health benefits (Afsharmanesh & Sadaghi, 2013; Battikh *et al.*, 2013; Srihari *et al.*, 2013; Vīna *et al.*, 2013; Jayabalan *et al.*, 2014; Wang *et al.*, 2014).

2.3 Milk kefir

Milk kefir is a fermented milk beverage, which is made by adding milk kefir grains (the inoculum) to raw or heat-treated milk (Garofalo *et al.*, 2015). This mixture is fermented under anaerobic conditions at room temperature for one to two days, after which it is sieved to separate the milk kefir liquor from the milk kefir grains. The milk kefir liquor is a viscous, foaming beverage with an acidic and alcoholic taste and aroma. The milk kefir grains are reused as the inoculum for a next fermentation process through backslopping. The fermentation is usually carried out in a vessel with rubber sealing, such as a Weck jar. Traditionally, milk from cows, goats, or sheep is used, but milk substitutes (such as walnut, coconut, rice, peanut, and soy milks) have been used too (Liu & Lin, 2000; Liu *et al.*, 2002; Kesenkas *et al.*, 2011; Puerari *et al.*, 2012; Cui *et al.*, 2013; Nielsen *et al.*, 2014). The latter need to be supplemented with lactose, sucrose, glucose, or galactose to provide the microorganisms with a suitable substrate. The origin of milk kefir is thought to be the Caucasus (Leite *et al.*, 2013). However, it occurs worldwide under a variety of names, such as Tibetan kefir, kephir, kiaphur, keer, kepi, knapon, and kippi, depending on the geographical location. Most names are derived from the Turkish word *keyif*, which means good feeling (Ahmed *et al.*, 2013a).

The milk kefir grains are composed of kefiran, a glucogalactan EPS, and are cauliflower-shaped, white to yellow in colour, opaque, have an elastic structure, are insoluble in water,

and sink to the bottom of the fermentation liquor. The milk kefir microorganisms are attached onto their surface and detach partly from the grains into the liquor when they are added to milk (Rea *et al.*, 1996; Magalhães *et al.*, 2011). The main microorganisms found in milk kefir are yeasts (Ahmed *et al.*, 2013a; Marsh *et al.*, 2013a; Nielsen *et al.*, 2014) and LAB (Chen *et al.*, 2008; Dobson *et al.*, 2011; Leite *et al.*, 2012; Marsh *et al.*, 2013a; Pogačić *et al.*, 2013; Ünal & Arslanoglu, 2013; Nielsen *et al.*, 2014), but AAB (Takizawa *et al.*, 1998; Witthuhn *et al.*, 2004; Dobson *et al.*, 2011; Leite *et al.*, 2012) and bifidobacteria (Dobson *et al.*, 2011; Leite *et al.*, 2012; Marsh *et al.*, 2013a) may also be present. Characteristic microorganisms are *Lactobacillus kefiri*, *Lactobacillus parakefiri*, and *Lactobacillus kefiranofaciens* (Chen *et al.*, 2008; Dobson *et al.*, 2011; Leite *et al.*, 2012). The milk kefir microorganisms convert lactose, the main carbohydrate in milk, into ethanol, carbon dioxide, lactic acid, glycerol, and acetic acid (Gul *et al.*, 2015). *Lactobacillus kefiranofaciens* is thought to be responsible for the production of kefiran (composing the milk kefir grains) during fermentation (Ahmed *et al.*, 2013b; Hamet *et al.*, 2013). Milk kefir liquor can also be produced using isolates from milk kefir as starter cultures (Ebner *et al.*, 2015), but milk kefir grains can only be produced from existing milk kefir grains.

Many health benefits have been ascribed to milk kefir (Alm, 1982; Kneifel & Mayer, 1991; Liu *et al.*, 2005a,b, 2006; Sarkar, 2007; Korhonen, 2009; Guzel-Seydim *et al.*, 2011; Ahmed *et al.*, 2013a; Zheng *et al.*, 2013; Diosma *et al.*, 2014; Jalali *et al.*, 2015; Prado *et al.*, 2015; Miao *et al.*, 2016).

2.4 Water kefir

2.4.1 Description

Water kefir is a fermented beverage made by adding water kefir grains (the inoculum) to a mixture of water, (dried) fruits, and sucrose (Ward, 1892; Kebler, 1921; Pidoux *et al.*, 1988, 1990; Pidoux, 1989; Neve & Heller, 2002; Magalhães *et al.*, 2010, 2011; Waldherr *et al.*, 2010; Gulitz *et al.*, 2011, 2013; Marsh *et al.*, 2013b; Stadie *et al.*, 2013; Laureys & De Vuyst, 2014). This mixture is fermented under anaerobic conditions at room temperature for two to four days, after which it is sieved to separate the water kefir liquor from the water kefir grains (Figure 2).

The water kefir liquor is a slightly sweet, acidic, alcoholic, sparkling beverage that has a yellowish color and a fruity aroma. The water kefir grains are white to yellow in color, translucent, have a brittle structure, are insoluble in water, and sink to the bottom of the fermentation liquor. They are used as the inoculum for a next fermentation process through backslopping. The fermentation is usually carried out in a vessel with a rubber sealing, such as a Weck jar. Traditionally, dried figs are used as fruit components, but other ingredients, such as lemon, other (dried) fruits, and herbs, can also be added (Reiß, 1990).

2.4.2 Origin

Water kefir occurs worldwide under a variety of names, such as ginger beer plants, tibicos, tibi grains, California bees, African bees, ale nuts, balm of Gilead, Japanese beer seeds, and sugary kefir grains, depending on the geographic location (Gulitz *et al.*, 2013). The origin of the water kefir grains has not been established yet, but one theory speculates that water kefir grains originate in Mexico, where they develop onto the leaves of the *Opuntia* cactus (Lutz, 1899). In America, water kefir grains are brought into sweetened water, which is fermented in a closed jar, but they are also used as starter for the production of moonshine whisky (Kebler, 1921). In the British Isles, ginger beer is made by adding water kefir grains to

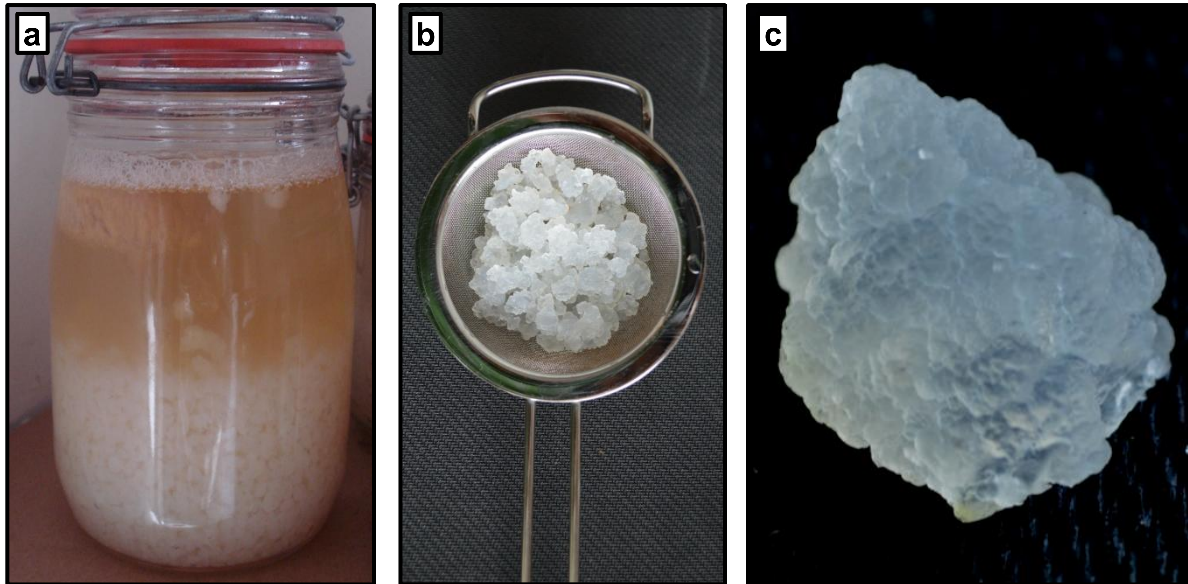

Figure 2. (a) A picture of a typical water kefir fermentation vessel. (b) A picture of sieved water kefir grains. (c) A close-up picture of a water kefir grain.

a 10-20 % sugar solution in tap water supplemented with ginger, lemon, and baking soda (Ward, 1892). After two to three days of fermentation in an open vessel, the liquor is bottled and refermented for three or more days. The water kefir grains grow during the fermentation, contain around 13.3 % (m m^{-1}) of EPS, and harbor yeasts and bacteria (Ward, 1892).

2.4.3 Microbial species diversity

Water kefir harbors yeasts and LAB, but AAB may also be present. The viable counts of the yeasts range from 5.9 to 8.3 log colony forming units (cfu) g^{-1} of water kefir grains and those of LAB from 6.0 to 9.2 log cfu g^{-1} (Pidoux, 1989; Magalhães *et al.*, 2010, 2011; Gulitz *et al.*, 2011), whereas those of AAB range from negligible to 8.5 log cfu g^{-1} of water kefir grains (Franzetti *et al.*, 1998; Gulitz *et al.*, 2011).

The microbial species diversity in water kefir has been investigated several times in the past with the techniques that were available at that moment. The microorganisms found in water kefir via culture-dependent or culture-independent species diversity analyses are summarized in Table 1. Most publications do not report other characteristics of the water kefir fermentation processes than their species diversities. The species diversities found in different water kefir samples vary widely, and do not allow to determine which are the key microorganisms of water kefir fermentation and which are present opportunistically. Recently, a novel AAB species, *Acetobacter sicerae*, was found in water kefir (Li *et al.*, 2014).

Table 1. Overview of genera and species of yeasts and bacteria found in water kefir via culture-dependent and -independent (grey) methods.

Reference	Yeasts	Bacteria
Ward (1892)	*Azymocandida mycoderma, Pichia membranifaciens, Rhodotorula glutinis, Saccharomyces cerevisiae*	*Acetobacteraceae, Lactobacillus hilgardii*
Horisberger (1969)	*S. cerevisiae*	*Lactobacillus brevis, Lactococcus lactis*
Pidoux *et al.* (1988)	*Candida fimetaria, Candida acidothermophilum, Cryptococcus vini, Torulaspora pretoriensis, Zygotorulaspora florentina*	*Lactobacillus casei, Lb. hilgardii, Lactobacillus plantarum, Lactobacillus rhamnosus, Lc. lactis, Leuconostoc mesenteroides*
Franzetti *et al.* (1998)	*S. cerevisiae, Hanseniaspora valbyensis*	*Lb. casei*
Waldherr *et al.* (2010)	*S. cerevisiae*	*Lb. hilgardii*
Magalhães *et al.* (2010)	*Kazachstania aerobia, Kluyveromyces lactis, Lachancea meyersii, S. cerevisiae*	*Acetobacter lovaniensis, Lactobacillus buchneri, Lactobacillus kefiri, Lactobacillus paracasei, Lactobacillus parabuchneri, Lc. lactis, Leuconostoc citreum*
Magalhães *et al.* (2011)	*Candida valdiviana, Meyerozyma caribbica, Pichia cecembensis, Pichia membranifaciens, S. cerevisiae, Torulaspora delbrueckii, Yarrowia lipolytica*	*Ac. lovaniensis, Bacillus cereus, Gluconobacter liquefaciens, Lb. buchneri, Lb. casei, Lactobacillus helveticus, Lb. kefiri, Lb. paracasei, Lactobacillus satsumensis, Lactobacillus sunkii*
Gulitz *et al.* (2011)	*H. valbyensis, Lachancea fermentati, S. cerevisiae, Z. florentina*	*Acetobacter fabarum, Acetobacter orientalis, Lb. casei, Lb. hilgardii, Lactobacillus hordei, Lactobacillus nagelii, Leuc. citreum, Leuc. mesenteroides*
Hsieh *et al.* (2012)	*Dekkera bruxellensis, S. cerevisiae, T. delbrueckii*	*Lb. hordei, Lactobacillus mali, Leuc. mesenteroides* *Pseudomonas, Sporolactobacillus, Zymomonas mobilis*
Gulitz *et al.* (2013)		*Acetobacter, Bifidobacterium, Clostridium, Gluconacetobacter, Gluconobacter, Lactobacillus, Leuconostoc*
Marsh *et al.* (2013b)	*Dekkera anomala, D. bruxellensis, H. valbyensis, Hanseniaspora vinae, La. fermentati, Me. caribbica, S. cerevisiae, Torulaspora, Zygosaccharomyces lentus*	*Acetobacter, Bifidobacterium, Gluconacetobacter, Gluconobacter, Lactobacillus, Leuconostoc, Zymomonas*
Diosma *et al.* (2014)	*Pichia occidentalis, S. cerevisiae*	

2.4.4 Water kefir grains

The examination of the structural organization of the water kefir grains with light, scanning, and transmission electron microscopy has indicated that the surface of the water kefir grains is more densely populated with microorganisms than their inside, which is composed of dextran (Moinas *et al.*, 1980).

The water kefir grain dextran is composed of an *α*-(1->6)-linked glucose backbone with *α*-(1->3)-linked branches, whereby the ratio of *α*-(1->3)- to *α*-(1->6)-linked glucose units is around 0.11 (Horisberger, 1969). The dextran in the water kefir grains is probably produced by *Lactobacillus hilgardii*, as this microorganism is found on the water kefir grains and produces a gelling polysaccharide when cultivated in Mayeux-Sandine-Elliker medium and a non-gelling polysaccharide when cultivated in de Man-Rogosa-Sharpe (MRS) medium supplemented with 60 $g\ l^{-1}$ of sucrose (Pidoux *et al.*, 1988). The ratios of *α*-(1->3)- to *α*-(1->6)-linked glucose units in the water kefir grain EPS and in the gelling EPS of *Lb. hilgardii* are 0.19 and 0.14, respectively, whereas this ratio is only 0.07 in the non-gelling EPS (Pidoux *et al.*, 1988). A pure culture of *Lb. hilgardii* produces gelling EPS in sucrose-yeast extract medium, which more or less resemble the water kefir grains (Pidoux, 1989). The production of EPS by *Lb. hilgardii* increases when the pH increases and does not require calcium. The presence of *Zygotorulaspora florentina* or *Torulaspora pretoriensis* during water kefir fermentation results in the degeneration of the water kefir grains, as these yeasts may compete with *Lb. hilgardii* for sucrose. However, these yeasts are easily removed from the culture by rinsing the water kefir grains with water. The glucansucrase of *Lb. hilgardii* has been isolated and characterized, revealing that its optimal pH range is 4.3-4.6 and its optimal temperature is around 40 °C (Waldherr *et al.*, 2010). Nevertheless, *Lactobacillus casei*, *Lactobacillus hordei*, *Lactobacillus nagelii*, and *Leuconostoc mesenteroides* have been reported to produce homopolysaccharides too (Gulitz *et al.*, 2011).

2.4.5 Ingredients

One study reported that dried figs may contain growth-promoting factors that can be extracted with water, and which are not present in other dried fruits such as raisins, dates, bananas, plums, or apricots (Reiß, 1990). During aerobic water kefir fermentation in the presence of dried figs, the concentration of ethanol reaches 3.6 $g\ l^{-1}$ after 6 d, that of lactic acid 2.8 $g\ l^{-1}$ after 18 d (whereby the majority is produced between 14 and 18 d), and that of acetic acid 1.1 $g\ l^{-1}$ after 6 d. When no dried figs are added, the concentration of ethanol reaches only 0.7 $g\ l^{-1}$ after 12 d, that of lactic acid 0.6 $g\ l^{-1}$ after 14 d, and that of acetic acid 7.0 $g\ l^{-1}$ after 18 d of aerobic fermentation. The concentrations of ethanol, lactic acid, and acetic acid are around 1.3, 2.0, and 1.4 $g\ l^{-1}$, respectively, after 24 h of aerobic fermentation when no (dried) fruits are added to the fermentation (Magalhães *et al.*, 2010).

2.4.6 Interactions between microorganisms

The water kefir fermentation medium is usually poor in nutrients, and part of the nutrients required by LAB may be provided by certain yeasts present during water kefir fermentation, such as *Z. florentina* or *S. cerevisiae* (Leroi & Pidoux, 1993; Stadie *et al.*, 2013). Indeed, *Z. florentina* can stimulate the growth and metabolism of *Lb. hilgardii* by the production of carbon dioxide, pyruvate, propionate, acetate, and/or succinate, but not by the production of ethanol, fumarate, vitamins, or amino acids (Leroi & Pidoux, 1993). The presence of *Z. florentina* and *S. cerevisiae* also stimulates the growth of *Lb. hordei* by the release of arginine and vitamin B6, and that of *Lb. nagelii* by the release of arginine (Stadie *et al.*, 2013). This release of nutrients by yeasts occurs only during coculture fermentation and is thought to be due to the autolysis of yeasts, which might be stimulated by LAB (Stadie *et al.*, 2013).

Alternatively, LAB also stimulate the growth of *Z. florentina* by the production of organic acids, as this yeast species grows faster at pH 4.0 than at higher pH values (Stadie *et al.*, 2013). The growth and metabolism of *Lb. hilgardii* is faster with fructose as substrate than with sucrose or glucose, whereas the growth and metabolism of *Z. florentina* is faster with sucrose or glucose as substrate than with fructose (Leroi & Pidoux, 1993).

2.4.7 Health

Water kefir is reported to possess several health benefits (Rodrigues *et al.*, 2005a,b; Moreira *et al.*, 2008; Silva *et al.*, 2009).

3 Microorganisms of relevance in naturally fermented beverages

3.1 Yeasts

3.1.1 Description

Yeasts are unicellular eukaryotic microorganisms that belong to the fungi (Neiman, 2005; Kurtzman *et al.*, 2011). They usually have a spherical, oval, or cylindrical form, and measure around 8 µm in diameter. Yeasts usually reproduce asexually via budding, whereby a new cell forms as a small outgrowth of the mother cell, gradually enlarges, and eventually separates from the mother cell. Some yeasts can also reproduce sexually via mating, whereby two haploid cells of a different mating type (a and *α*) fuse to form a diploid zygote (a/*α*), from which an ascus and eventually haploid ascospores (a or *α*) can form. In general, haploid cells will either express general stress responses or die under stress conditions, such as nutrient starvation, whereas diploid cells will undergo sporulation, forming a variety of haploid ascospores, which can undergo germination, followed by budding or mating. The haploid state of yeasts is called the anamorph and the diploid state is called the teleomorph, and both states of the same yeast species used to have a different valid name. Nowadays, the name of the teleomorph is preferred for identification, but the name of the anamorph is still in use when the teleomorph is unknown, or when the name of the anamorph is more widespread in publications (as is the case for *Brettanomyces bruxellensis*, which is the anamorph of the teleomorph *D. bruxellensis*).

Most yeasts grow optimally around 20-30 °C, many grow at 2-10 °C, and few species grow at 40-45 °C (Kurtzman *et al.*, 2011). This indicates that certain yeasts (including *Saccharomyces* and *Zygosaccharomyces* species) can cause spoilage of refrigerated foods. Budding yeast cells are rather quickly inactivated at 60-65 °C, but their ascospores can be 100 times more heat-resistant than the budding cells, as is the case for *S. cerevisiae*, which is considered to be one of the most heat-resistant yeast species. Yeasts usually prefer acidic environments of pH 4.5-7.0, and many still grow at pH 2.5. Most yeast species can tolerate a sucrose concentration of up to 50 % (m v^{-1}), but *Zygosaccharomyces* species are known to be extremely osmotolerant, able to tolerate more than 70 % (m v^{-1}) of sucrose. Yeasts that are exposed to certain stress conditions can quickly develop enhanced tolerance to these conditions by modifying their gene expression patterns (Guerzoni *et al.*, 2013). They are unable to grow under completely anaerobic conditions because they need oxygen for certain growth-maintaining hydroxylations, such as the biosynthesis of sterols and unsaturated fatty acids.

3.1.2 Metabolism

Yeasts metabolize glucose via the Embden-Meyerhof-Parnas (EMP) pathway in the cytosol, whereby glucose is converted into pyruvate, thereby generating energy (ATP) and reducing equivalents (NADH + H^+) (Figure 3). Under anaerobic conditions, alcoholic fermentation takes place, whereby pyruvate is decarboxylated into acetaldehyde, which is reduced into ethanol for the reoxidation of NADH + H^+ into NAD^+. Overall, 1 mole of glucose is (theoretically) converted into 2 moles of ethanol, 2 moles of carbon dioxide, and 2 moles of ATP.

Under aerobic conditions, pyruvate can be converted into carbon dioxide, NADH + H^+, and acetyl-CoA, whereby the latter can be completely degraded into carbon dioxide and water, thereby generating energy (ATP) via the Krebs cycle in the mitochondria (Figure 3). Aerobic respiration in the mitochondria reoxidizes the produced NADH + H^+ into NAD^+, energy (ATP), and water. Overall, 1 mole of glucose and 6 moles of oxygen are (theoretically) converted into 6 moles of carbon dioxide, 6 moles of water, and 38 moles of ATP. Depending on their glucose metabolism, yeasts can be classified as respirative, facultatively fermentative, or fermentative (Rodrigues *et al.*, 2006; Merico *et al.*, 2007). Respirative yeasts (such as *Kluyveromyces lactis* and *Yarrowia lipolytica*) can only perform aerobic respiration for their energy production, whereas fermentative yeasts (such as *Kazachstania telluris*) can only perform fermentation. Most yeasts (such as *S. cerevisiae*, *D. bruxellensis*, and *Kluyveromyces marxianus*) are classified as facultatively fermentative and can use both pathways for their energy production depending on the environmental conditions.

Many yeasts perform aerobic respiration in the presence of oxygen, as this generates more energy (ATP) from glucose than alcoholic fermentation (Pfeiffer & Morley, 2014). This metabolism creates a high intracellular ATP concentration, which allosterically inhibits the enzyme phosphofructokinase, resulting in a slower glycolysis. This decrease of the glucose consumption rate in the presence of oxygen is referred to as the Pasteur effect. Some yeasts continue to perform alcoholic fermentation even in the presence of oxygen, which is referred to as the Crabtree effect. In Crabtree-positive yeasts, such as *S. cerevisiae* and *D. bruxellensis*, respiration is repressed by high carbohydrate concentrations as part of a make-accumulate-consume strategy, whereby the available carbohydrates are quickly converted into ethanol via alcoholic fermentation during the make-accumulate phase (Rozpędowska *et al.*, 2011). This impairs the growth of competing microorganisms by fast consumption of the available substrates and the production of high ethanol concentrations. After exhaustion of the carbohydrates, the yeasts can metabolize the accumulated ethanol via aerobic respiration, and thus remain dominant in that niche during the consume phase.

Besides ethanol and carbon dioxide, yeasts can produce a variety of other metabolic products such as glycerol, acetic acid, succinic acid, higher alcohols, and esters (Figures 3 and 4). Yeast cells produce glycerol as an osmoprotectant in response to high external sugar and salt concentrations, as is the case for *Zygosaccharomyces bailii* and *S. cerevisiae* (Pigeau *et al.*, 2007). Yeast cells can also produce glycerol from dihydroxyacetone phosphate to reoxidise an excess of NADH + H^+ into NAD^+, but this is energetically expensive, as it requires ATP for the glycolysis without concomitant energy production. Yeast cells can also produce acetic acid in response to high osmotic stress, which is often found during the fermentation of ice wines, containing carbohydrate concentrations above 35 % (m v^{-1}) (Pigeau *et al.*, 2007). Acetic acid can also be produced to reduce an excess of NAD^+ into NADH + H^+, which occurs mostly under aerobic conditions (Aguilar Uscanga *et al.*, 2003), or in response

Figure 3. Schematic representation of the main metabolic pathways in yeasts, based on Nevoigt & Stahl (1997) and Kurtzman *et al.* (2011): 1, glucokinase; 2, phosphoglucose isomerase; 3, phosphofructokinase; 4, aldolase; 5, triosephosphate isomerase; 6, glyceraldehyde 3-phosphate dehydrogenase; 7, 3-phosphoglycerate kinase; 8, phosphoglycerate mutase; 9, enolase; 10, pyruvate kinase; 11, pyruvate decarboxylase; 12, alcohol dehydrogenase; 13, aldehyde dehydrogenase; 14, NAD^+-dependent glycerol 3-phosphate dehydrogenase; 15, glycerol 3-phosphatase; 16, glycerol dehydrogenase; 17, dihydroxyacetone kinase; 18: glycerol kinase; 19, FAD-dependent glycerol 3-phosphate dehydrogenase; 20, acetyl-CoA synthethase; 21, pyruvate dehydrogenase complex; 22, citrate synthase; 23, aconitase; 24, isocitrate dehydrogenase; 25, α-ketoglutarate dehydrogenase; 26, succinyl-CoA synthetase; 27, succinate dehydrogenase; 28, fumarase; 29, malate dehydrogenase; 30, isocitrate lyase; 31, malate synthase; 32, lactate dehydrogenase; 33, fatty acid biosynthesis; and 34, respiration.

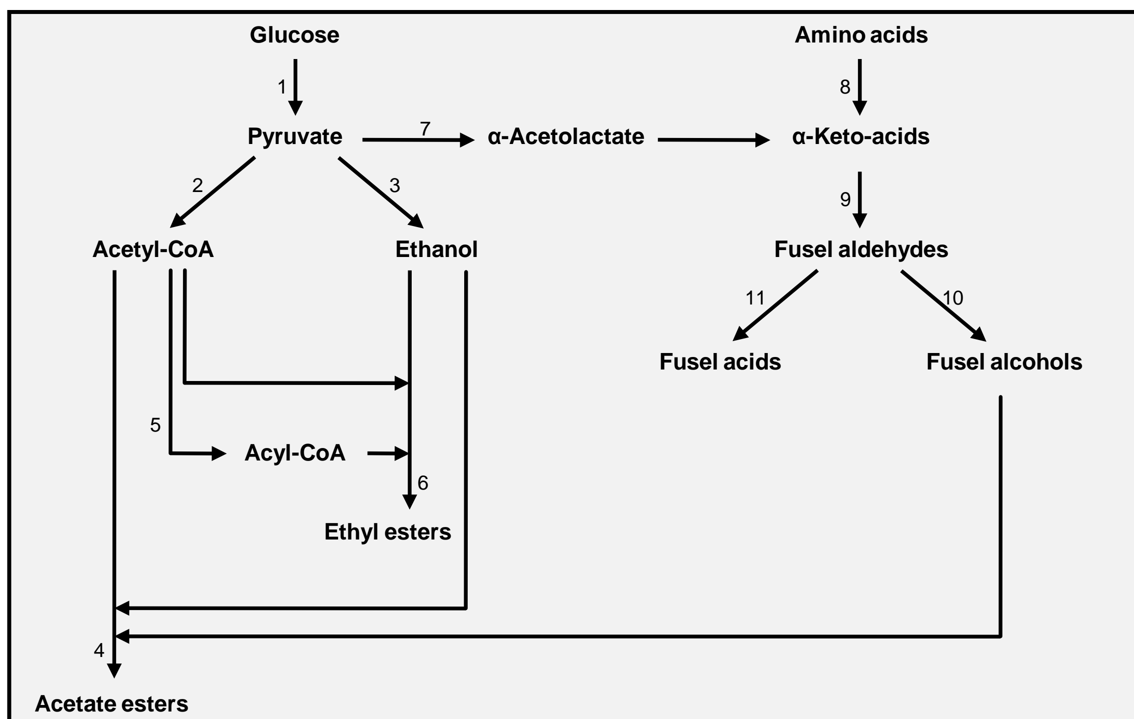

Figure 4. The anabolic and catabolic (Ehrlich pathway) production of aroma volatile compounds by yeasts, based on Pires *et al.* (2014) and Ravasio *et al.* (2014): 1, carbohydrate metabolism; 2, pyruvate dehydrogenase complex; 3, pyruvate decarboxylase and alcohol dehydrogenase; 4, alcohol acetyl esterase; 5, fatty acid biosynthesis; 6, ethanol acyl esterase; 7, *α*-acetolactate synthase; 8, transaminase; 9, decarboxylase; 10, reduction by dehydrogenase; and 11, oxidation by dehydrogenase.

to a nutrient deficiency, as acetic acid production is higher when unsaturated fatty acids are not present in the medium (Moruno *et al.*, 1993). Acetic acid concentrations above 1-2 g l^{-1} have a detrimental effect on the sensory quality of wines and can be produced by *Cryptococcus vini*, *Wickerhamomyces anomalus*, *Candida acidothermophilum*, and *Dekkera* species (Lambrechts & Pretorius, 2000). The production of glycerol and acetic acid is species- and strain-dependent, but *S. cerevisiae* seems to produce more glycerol than *D. bruxellensis* (Blomqvist *et al.*, 2010), whereas *Dekkera* seems to produce more acetic acid than *Saccharomyces* (Freer, 2002). Yeasts can produce succinic acid via the Krebs cycle (Arikawa *et al.*, 1999), especially when oxygen is present, as is the case during the fermentation of Japanese sake (Motomura *et al.*, 2012). Its taste has been described as unpleasant, acidic, salty, and bitter, and is thought to be of influence in Japanese sake (Arikawa *et al.*, 1999). However, the taste of succinic acid is not found in wines with high succinic acid concentrations. Ethyl esters of succinic acid are formed during the ageing process. They have a pleasant, mild, and fruity aroma, and are present in high concentrations in sherry wines (Zea *et al.*, 2012). Methyl esters of succinic acid are thought to contribute to the characteristic aroma of Muscadine wines (Lamikanra *et al.*, 1996).

Apart from glucose, other monosaccharides and disaccharides such as fructose, galactose, maltose, and sucrose can be used as substrate by many yeasts via the same metabolic pathways as described above (Walker, 2000). Sucrose is usually hydrolyzed into glucose and fructose by an extracellular invertase, whereas maltose is usually transported into the cell via a maltose permease, after which it is hydrolyzed into glucose by an intracellular maltase. The

consumption of maltotriose and higher maltooligosaccharides is species- and strain-dependent. For example, *S. cerevisiae* can use glucose, maltose, and part of the maltotriose, whereas *D. bruxellensis* can use higher maltooligosaccharides (Steensels *et al.*, 2015).

Consumption of less energetic substrates by yeasts is usually inhibited as long as glucose is present, but many yeasts can consume ethanol, acetic acid, lactic acid, and glycerol in the presence of oxygen (Ronne, 1995). Acetic acid is converted into acetyl-CoA and enters the Krebs cycle in cofermentation with glucose, as in *Z. bailii* (Rodrigues *et al.*, 2012), or can be consumed as the sole energy source via the glyoxylate pathway, which is repressed by glucose, as in *S. cerevisiae* (Casal *et al.*, 1996) (Figure 3). Lactic acid can be converted by lactic acid dehydrogenases into pyruvate, as is the case in *S. cerevisiae*, *Debaryomyces hansenii*, *K. marxianus*, and *Y. lipolytica* (Leclercq-Perlat *et al.*, 2004; Mansour *et al.*, 2008; Mourier *et al.*, 2008). Glycerol can be first phosphorylated into glycerol phosphate, which is further dehydrogenated into dihydroxyacetone phosphate, or can be first oxidized into dihydroxyacetone, which is then phosphorylated into dihydroxyacetone phosphate (Nevoigt & Stahl, 1997).

Yeasts do not fix atmospheric nitrogen and the majority of yeast strains do not produce extracellular proteolytic enzymes, so the major sources of nitrogen are free amino acids and inorganic ammonium compounds (Kurtzman *et al.*, 2011). Nevertheless, some strains of the species *Saccharomyces*, *Hanseniaspora*, and *Candida* can produce proteases. A shortage in amino acids can cause sluggish or stuck wine fermentations, resulting in an incomplete fermentation with high residual carbohydrate concentrations (Maisonnave *et al.*, 2013). Free amino acids can be assimilated via the Ehrlich pathway, resulting in the production of higher alcohols or organic acids (Figure 4). The most prevalent higher alcohols are isoamyl alcohol (from leucine), 2-methyl-1-propanol (valine), and 2-phenylethanol (phenylalanine) (Hazelwood *et al.*, 2008). They may also be produced from pyruvate during amino acid biosynthesis. Low concentrations of higher alcohols may contribute to the perception of body in wine, whereas higher concentrations may cause a rather undesirable solvent-like aroma (Lambrechts & Pretorius, 2000). Yeasts possess esterases and lipases, which catalyze the esterification of alcohols (such as ethanol and higher alcohols) with organic acids (such as acetic acid, lactic acid, and medium- to long-chain fatty acids) (Figure 4). The medium- and long-chain fatty acids are synthesized *de novo* from acetyl-CoA (Tehlivets *et al.*, 2007). The most prevalent esters are acetate esters (ethyl acetate, isoamyl acetate, and 2-phenylethyl acetate) and ethyl esters (ethyl hexanoate, ethyl octanoate, ethyl decanoate, and ethyl lactate). These esters contribute usually desirable fruity and floral aromas to fermented alcoholic beverages (Lambrechts & Pretorius, 2000).

Dekkera bruxellensis is a key microorganism for the fermentation of Belgian-style acidic ales (lambic, gueuze, kriek) and the Belgian trappist beer Orval, and degrades several maltooligosaccharides (Phillips, 1955). However, it is often regarded as a spoilage yeast in wine and beer, as it can produce off-flavors (Schifferdecker *et al.*, 2014). This yeast species can convert lysine and ornithine into acetyltetrahydropyridines, which contribute mousy odors and taints to fermented alcoholic beverages (Grbin & Henschke, 2000). Also, it can convert *p*-coumaric acid and ferulic acid (present in plant cell walls) into 4-ethyl- and 4-vinylphenols and -guaiacols, which contribute a phenolic odour (wet horse aroma) to the fermented beverage (Vanbeneden *et al.*, 2008; Godoy *et al.*, 2009).

Many yeast species, including *K. marxianus*, *S. cerevisiae*, and *W. anomalus*, can also degrade pectin, which may result in an undesirable loss of structure, texture, and viscosity of the end-products, as can occur during vegetable fermentations (Jayani *et al.*, 2005). Yeasts, including *S. cerevisiae*, may exhibit pectin methylesterase activity, which degrades pectin into pectinate and methanol. In the human body, methanol is converted into formic acid, which is

further converted into carbon dioxide and water. However, the latter process is very slow, resulting in the accumulation of formic acid in the body, which causes visual blurring, metabolic acidosis, and eventually death (Rathi *et al.*, 2006). However, methanol is not considered a health risk in small concentrations, and is produced during most fermentation processes, ripening of fruits and vegetables (Frenkel *et al.*, 1998), in the human colon (Siragusa *et al.*, 1988), and after consuming beverages sweetened with aspartame (Španěl *et al.*, 2015).

When yeasts die, the cells start a process of self-degradation or autolysis, whereby intracellular compounds such as nucleic acids, proteins, lipids, and polysaccharides are extensively degraded by endogenous enzymes. This can occur during maturation of beer and wine, and results in a wide diversity of compounds, which may affect the flavor of the end-products (Masschelein, 1986; Alexandre & Guilloux-Benatier, 2006). Additionally, the released nutrients may serve as nutrients for the growth of other microorganisms, such as LAB.

In response to certain environmental factors or quorum-sensing stimuli, some yeasts (such as *Candida albicans* and *D. bruxellensis*) can produce EPS and form biofilms, which may hamper the clarification of fermented alcoholic beverages (Chandra *et al.*, 2001; Joseph *et al.*, 2007). In addition to biofilms, yeast cells can adhere to abiotic surfaces, cells, and tissues via the production of adhesins (Bruckner & Mosch, 2012). When the fermentable carbohydrate and/or nitrogen sources are depleted at the end of a brewing process, yeast cells normally start to flocculate, resulting in the formation of macroscopic flocs, which sink to the bottom (in the case of lager strains) or float to the surface (ale strains) of the fermentation tank, which facilitates their removal (Sampermans *et al.*, 2005; Verstrepen & Klis, 2006).

3.1.3 Occurrence and health

Yeasts are only rarely associated with outbreaks of food-borne gastroenteritis or other food-borne infections or intoxications (Kurtzman *et al.*, 2011). They are not very infectious but some can exploit local or systemic weaknesses in the host defense mechanisms. For example, *C. albicans* exists in a commensal relationship with humans as a normal resident of mucocutaneous tissues, the gastrointestinal tract, and the skin, but can cause endogenous infections in immunocompromised people. Also yeasts commonly found in foods have been recognized as opportunistic pathogens, as is the case for *S. cerevisiae*, *C. acidothermophilum*, *W. anomalus*, *Y. lipolytica*, and *K. marxianus* (Enache-Angoulvant & Hennequin, 2005; Munoz *et al.*, 2005; Fleet & Balia, 2006; Jacques & Casaregola, 2008; Daniel *et al.*, 2011). Consumption of yeast cells may in some individuals result in a broad range of allergic and hypersensitive reactions, for which the mechanisms are not clear and may include adverse reactions against yeast cell wall polysaccharides, cell proteins, or metabolites (Main *et al.*, 1988; Savolainen *et al.*, 1998).

Some yeasts are able to decarboxylate amino acids into biogenic amines, which are low-molecular-mass, heat-stable, biologically active amines. These compounds are frequently found in fermented foods and beverages, such as cheese, meat, fish products, beer, and wine (Önal, 2007). Biogenic amines can cause food poisoning, resulting in headaches, low blood pressure, heart palpitations, edema, vomiting, diarrhea, and others, especially in the presence of ethanol. The most prevalent biogenic amines in foods and beverages are histamine (from histidine), tyramine (tyrosine), 2-phenylethylamine (phenylalanine), tryptamine (tryptophane), putrescine (ornithine), and cadaverine (lysine). The production of biogenic amines by yeasts is strain-dependent and *D. bruxellensis* produces higher amounts than *S. cerevisiae* (Caruso *et al.*, 2002). Nevertheless, the production of biogenic amines is not common among wine and

beer yeasts, and the production of biogenic amines during wine fermentation is usually attributed to LAB rather than yeasts (Smit *et al.*, 2008, 2012).

Saccharomyces cerevisiae is generally considered as safe (GRAS) as a production microorganism for foods and beverages (Kurtzman *et al.*, 2011). Certain yeasts may even have therapeutic value, as is the case for *Saccharomyces boulardii* (originally isolated from a fruit in Indochina), which is used for the treatment of gastrointestinal diseases since 1950 (Edwards-Ingram *et al.*, 2007; Kelesidis & Pothoulakis, 2012). Yeast biomass is used for the production of yeast extract, which can be used as a flavor enhancer (Ferreira *et al.*, 2010). Further, yeast cell wall polysaccharides have been used in animal feed because they promote animal growth and health by various mechanisms, including immunomodulation, oxidative status, binding of toxins and pathogens, and interaction with gut constituents (Holck *et al.*, 2007; Kurtzman *et al.*, 2011). The ingestion of glycerol via fermented foods and beverages may offer protection against human intestinal pathogens such as *Salmonella enterica*, as glycerol can be converted by *Lactobacillus reuteri* into reuterin, an antimicrobial compound (De Weirdt *et al.*, 2012).

3.2 Lactic acid bacteria

3.2.1 Description

LAB are a group of Gram-positive bacteria that belong to the phylum of the Firmicutes and that are characterized by certain morphological, metabolic, and physiological characteristics (Axelsson, 2004). Although they comprise more than 20 genera, the most prevalent ones in fermented foods are *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, and *Weissella*. Their cells are rod-shaped (for example *Lactobacillus* and *Carnobacterium*) or spherical (for example *Lactococcus* and *Leuconostoc*), and occur singly, in tetrads, or in short or long chains. They are usually fastidious, not motile, and do not sporulate. LAB are usually aerotolerant but are fermentative. Their optimal growth temperature is usually 25-40 °C, but *Strep. thermophilus* grows until 52 °C and *Lactobacillus algidus* grows at 4 °C. They are usually acid-tolerant and prefer a pH around 4.0-6.0, but *Tetragenococcus* grows at pH 9.0 and *Oenococcus oeni* grows at pH 3.5.

3.2.2 Metabolism

LAB can be classified according to their metabolism as obligately homofermentative (*Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Streptococcus*, and *Tetragenococcus*), facultatively heterofermentative (*Lactobacillus*), and obligately heterofermentative (*Lactobacillus*, *Leuconostoc*, *Oenococcus*, and *Weissella*) (Axelsson, 2004). The first group metabolizes glucose via the EMP pathway (characterized by aldolase as key enzyme) and performs a homolactic fermentation with lactic acid as the main end-product, generating 2 moles of ATP per mole of glucose (Figure 5). Pyruvate is used as internal electron acceptor and reduced into lactate to reoxidize NADH + H^+ into NAD^+. The third group metabolizes glucose (and pentoses) via the pentose phosphate pathway (PPP) (characterized by phosphoketolase as key enzyme) and performs a heterolactic fermentation with lactic acid, carbon dioxide, and ethanol as the main end-products, generating 1 mole of ATP per mole of glucose. Pyruvate is used as internal electron acceptor and reduced into ethanol to reoxidize NADH + H^+ into NAD^+. The second group performs homolactic fermentation of glucose and heterolactic fermentation of gluconate and pentoses. The latter are first converted into xylulose 5-P, which enters the heterolactic fermentation pathway, producing equimolar amounts of lactic acid and ethanol.

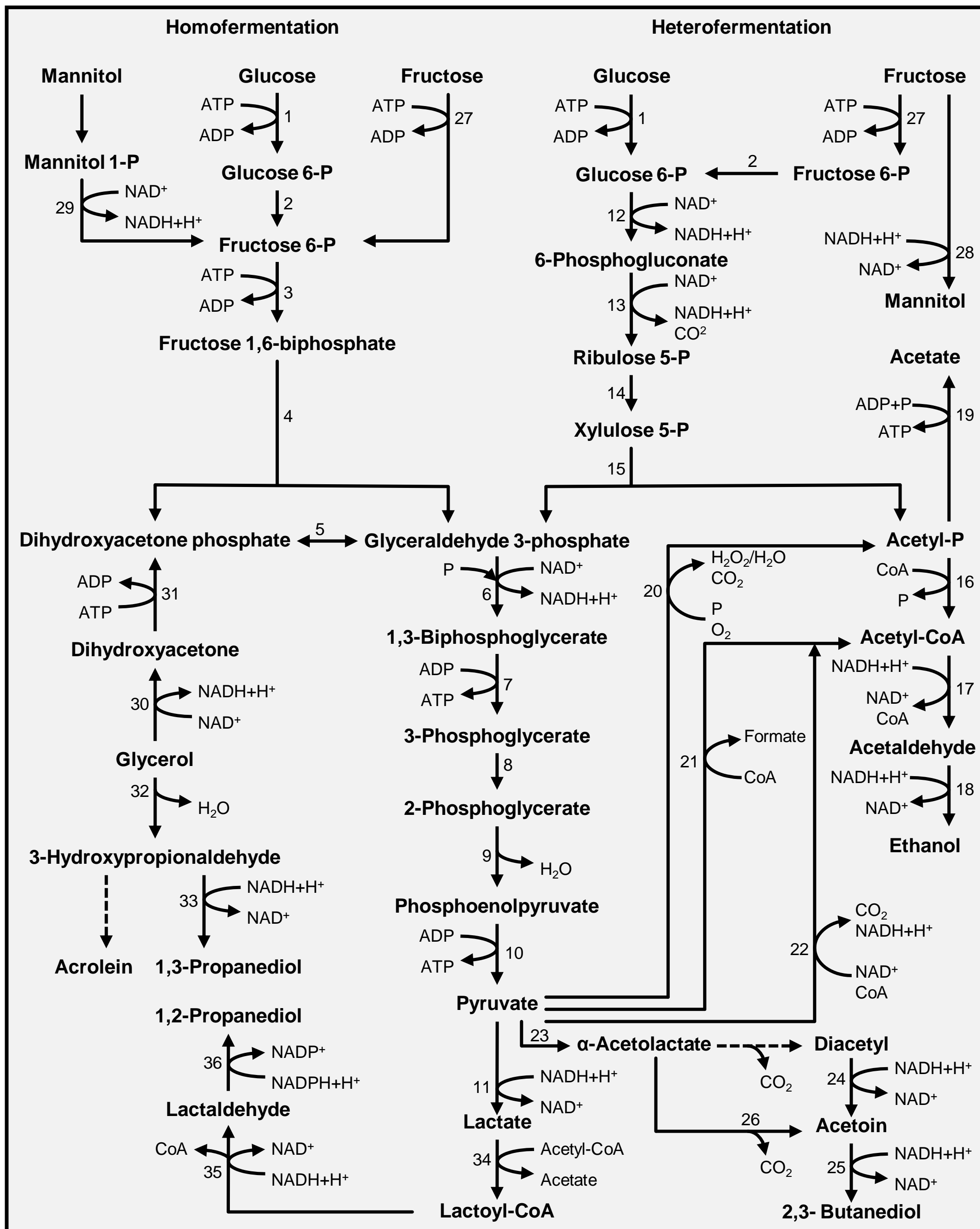

Figure 5. Schematic representation of the carbohydrate degradation pathways in lactic acid bacteria, based on Axelsson (2004), Zaunmüller *et al.* (2006), Pasteris & de Saad (2009), and Niu & Guo (2015): 1, glucokinase; 2, phosphoglucose isomerase; 3, phosphofructokinase; 4, aldolase; 5, triosephosphate isomerase; 6, glyceraldehyde 3-phosphate dehydrogenase; 7, 3-phosphoglycerate kinase; 8, phosphoglycerate mutase; 9, enolase; 10, pyruvate kinase; 11, lactate dehydrogenase; 12, glucose 6-phosphate dehydrogenase; 13, 6-phosphogluconate dehydrogenase; 14, ribulose 5-phosphate epimerase; 15, phosphoketolase; 16, phosphotransacetylase; 17, acetaldehyde dehydrogenase; 18, alcohol dehydrogenase; 19, acetate kinase; 20, pyruvate oxidase/peroxidase; 21, pyruvate-formate lyase; 22, pyruvate dehydrogenase; 23, *α*-acetolactate synthase; 24, diacetyl reductase; 25, acetoin reductase; 26, *α*-acetolactate decarboxylase; 27, fructokinase; 28, mannitol dehydrogenase; 29, mannitol 1-phosphate dehydrogenase; 30, glycerol dehydrogenase; 31, dihydroxyacetone kinase; 32, glycerol dehydratase; 33, 1,3-propanediol dehydrogenase; 34, lactoyl-CoA transferase; 35, CoA-dependent lactaldehyde dehydrogenase; and 36, lactaldehyde reductase. Dashed arrows represent non-enzymatic reactions.

The presence of external electron acceptors (such as citrate, oxygen, fructose, glycerol, and acetaldehyde) allows the regeneration of NADH + H^+ into NAD^+ without the reduction of pyruvate into lactic acid or ethanol. This allows acetyl-P to be used for the phosphorylation of ADP into ATP, whereby acetate is produced instead of ethanol. This increases the energy efficiency of the obligately heterofermentative LAB metabolism to 2 moles of ATP per mole of glucose. Oxygen allows the reoxidation of NADH + H^+ via NADH oxidase or NADH peroxidase (producing either water or H_2O_2) and increases the cell yield of certain *Leuconostoc* species. Many obligately heterofermentative LAB, such as *Leuc. mesenteroides* and *O. oeni* can reduce large amounts of fructose into mannitol, whereas homofermentative LAB species only produce minor amounts of mannitol (Zaunmüller *et al.*, 2006; Van der Meulen *et al.*, 2007).

Pyruvate is not only converted into D(-)- or L(+)-lactic acid, or a racemic mixture of both, depending on the lactate dehydrogenases and/or racemase present, but can also be converted into other products, especially when pyruvate is present in excess, which may be the case when external electron acceptors such as citrate are present (Axelsson, 2004). Citrate is present in milk and can be converted into pyruvate and oxaloacetate by citrate lyase. Excess pyruvate can be converted via α-acetolactate into diacetyl (which has a butter aroma), acetoin (weak butter aroma), and/or 2,3-butanediol (no aroma), as is the case for *Lc. lactis* subsp *lactis* biovar *diacetylactis* and *Leuconostoc mesenteroides* subsp. *cremoris*. Excess pyruvate can also be converted into acetyl-CoA, which may be used for lipid biosynthesis or for the production of acetic acid under aerobic conditions. Under anaerobic conditions, pyruvate-formate lyase can convert pyruvate into acetyl-CoA and formic acid, as in *Lc. lactis* and *Lb. casei*. However, this enzyme is sensitive to oxygen, and under aerobic conditions, pyruvate dehydrogenase converts pyruvate into acetyl-CoA, carbon dioxide, and NADH + H^+. Aerobic conditions also allow the decarboxylation of pyruvate by a pyruvate oxidase into acetyl-P, carbon dioxide, and hydrogen peroxide (as found in *Lb. plantarum*), and allow the consumption of glycerol by *Pediococcus pentosaceus*, of mannitol by *Lb. casei*, and of lactic acid by *Lb. plantarum*.

Several heterofermentative LAB species, such as *Lb. reuteri*, *Lb. hilgardii*, and *Lb. brevis*, can use glycerol as external electron acceptor in anaerobic cofermentation with glucose, which increases the energy efficiency of heterofermentation and increases the ratio of acetic acid to lactic acid (Axelsson, 2004). Hereto, glycerol is first dehydrated into 3-hydroxypropionaldehyde (3-HPA), also known as reuterin, which can be further reduced into 1,3-propanediol (Figure 5). Reuterin is soluble in water in dynamic equilibrium with its hydrate and its dimer, and is resistant to heat, stable over a wide pH range, and has potent antimicrobial properties against Gram-negative (*Escherichia coli*, *Salmonella enterica*, *Campylobacter jejuni*, and *Yersinia enterocolitica*) and Gram-positive (*Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium perfringens*) bacteria, yeasts (*S. cerevisiae*), and even protozoa (*Trypanosoma cruzi*), whereas its activity towards *Lactobacillus* species is low (Montiel *et al.*, 2014). A spontaneous chemical intramolecular dehydration converts 3-HPA into 2-propenal (acrolein), which is toxic and imparts a bitter taste to wine and cider (Garai-Ibabe *et al.*, 2008; Bauer *et al.*, 2010a).

Lactobacillus buchneri and *Lactobacillus parabuchneri* can convert lactic acid under anoxic conditions into equimolar amounts of acetic acid and 1,2-propanediol (Oude Elferink *et al.*, 2001). The latter can be converted further into 1-propanol or propionic acid by other LAB such as *Lactobacillus diolivorans* and *Lb. reuteri* (Krooneman *et al.*, 2002; Zhang *et al.*, 2010). This conversion of lactic acid can occur in silage and sourdough, where propionic acid is thought to contribute to its preservation (Gänzle *et al.*, 2009; Zhang *et al.*, 2010). Malate can be used as the sole energy source by *Enterococcus faecalis* and *Lb. casei*, whereby it is

first decarboxylated into carbon dioxide and pyruvate, whereafter pyruvate is converted into carbon dioxide and acetate or ethanol (Axelsson, 2004). In cofermentation with carbohydrates, malate can also be converted into carbon dioxide and lactic acid by the malolactic enzyme. This malolactic fermentation (MLF) can be performed by many LAB species, such as *Lb. plantarum* and *O. oeni*, during fermentation of vegetables and fruits (Axelsson, 2004; Hutkins, 2006). This process occurs during wine fermentation, where it may or may not be desired, depending on the type of wine. Wine MLF is usually performed by *O. oeni* during a secondary fermentation, which takes place during or at the end of the alcoholic fermentation. The MLF decreases the acidity, because malic acid is a diprotic acid with a low pK_a (pK_{a1}, 3.40; pK_{a2}, 5.20) and a harsh acidic taste, whereas lactic acid is a monoprotic acid with a high pK_a (pK_a, 3.86) and a soft acidic taste.

The main carbohydrate in milk is lactose, which can be hydrolyzed intracellularly into glucose and galactose 6-P [when taken up by a phosphotransferase system (PTS) and hydrolyzed by a 6-P-*β*-galactosidase, as is the case for *Lc. lactis* and *Lb. casei*], or glucose and galactose (when taken up by a permease and hydrolyzed by a *β*-galactosidase, as is the case for *Strep. thermophilus* and *Lb. delbrueckii*). Galactose 6-P can be metabolized through the tagatose-6-phosphate pathway, as is the case for *Lc. lactis*. Galactose can be excreted via a lactose-galactose antiporter (as is the case for *Strep. thermophilus*, *Lb. delbrueckii*, and *Lactobacillus acidophilus*) or can be converted into glucose 6-P via the Leloir pathway (as is the case for *Lb. casei*, *Strep. thermophilus*, and *Lactobacillus helveticus*) (Grossiord *et al.*, 1998; de Vin *et al.*, 2005). Milk also contains 2-10 % *β*-galacto-oligosaccharides [Gal-*β*-(1->4)-Glu], which can enter the cytoplasm via a PTS (whereafter they can be hydrolyzed by a P-*β*-galactosidase), or via a lactose permease (whereafter they can be hydrolyzed by a *β*-galactosidase) (Gänzle & Follador, 2012).

Maltose is one of the main carbohydrates during the fermentation processes of beer and sourdough, and can enter the cytoplasm via a maltose-H^+ symport system, after which it is hydrolyzed by a highly specific maltose phosphorylase into glucose and glucose 1-P, as is found in LAB species such as *Lactobacillus sanfranciscensis* (Gänzle & Follador, 2012; De Vuyst *et al.*, 2014b). Dextrins with a higher degree of polymerization and starch can be hydrolyzed by extracellular amylases (found in *Lactobacillus fermentum*, *Lb. plantarum*, and *Lactobacillus amylovorus*) or may be hydrolyzed by glycogen phosphorylases (found in *Lb. casei*, *Lb. reuteri*, and *Lb. rhamnosus*) (Gänzle & Follador, 2012).

Sucrose is one of the main carbohydrates during the fermentation processes of water kefir and kombucha and is also present in for instance sourdough. It can be metabolized by three different pathways in many LAB species. Extracellular glucan- or fructansucrases can convert sucrose into free fructose and glucans or free glucose and levans, respectively, as is the case for *Lb. hilgardii* (Waldherr *et al.*, 2010) and *Lb. sanfranciscensis* (Tieking *et al.*, 2005). Sucrose can also enter the cell via a PTS as sucrose 6-P, which is hydrolyzed into fructose and glucose 6-P by a phospho-fructofuranosidase, as is the case for *Lb. plantarum* and *Lb. acidophilus* (Gänzle & Follador, 2012). Furthermore, sucrose can also be transported, and phosphorylated and hydrolyzed by a sucrose phosphorylase into glucose 1-P and fructose, as is the case in *Lb. reuteri*. Inulin-type fructooligosaccharides can be transported into the cytoplasm, after which fructose is liberated by a terminal *β*-fructofuranosidase, as is the case in *Lb. acidophilus* (Gänzle & Follador, 2012).

Certain LAB may also produce mousy taints in fermented beverages, as they can produce acetyltetrahydropyridines from lysine, similar to certain *Dekkera* yeast species (Heresztyn, 1986). Some LAB species (*Lc. lactis* subsp. *cremoris*) also possess pectin methylesterases, which release methanol from pectin (Jayani *et al.*, 2005).

3.2.3 Exopolysaccharides

LAB are known to produce a wide variety of EPS, which can be classified as hetero- (HePS) or homopolysaccharides (HoPS) (De Vuyst & Degeest, 1999; De Vuyst *et al.*, 2001; Monsan *et al.*, 2001; De Vuyst & De Vin, 2007). Heteropolysaccharides are composed of repeating units of two to eight saccharides (often containing glucose, galactose, and rhamnose) and are usually produced in low concentrations (0.05-0.50 g l^{-1}). The repeating units are synthesized intracellularly, translocated over the membrane, and polymerized extracellularly. This is also the case for kefiran, the EPS composing milk kefir grains and produced by *Lb. kefiranofaciens* (Kooiman, 1968; Mukai *et al.*, 1990). Kefiran is polymerized from hexa- or heptasaccharide units composed of more or less equal proportions of glucose and galactose. It can be produced from lactose, sucrose, and glucose (Yokoi & Watanabe, 1992), whereby the structure of the repeating unit depends on the carbohydrate source in the fermentation medium (Wang & Bi, 2008). *Pediococcus damnosus* can produce EPS from glucose, which are composed of a repeating unit of three glucose monosaccharides [β-(1->3)] (Dueñas-Chasco *et al.*, 1997).

Homopolysaccharides are composed of only one monosaccharide and can be produced in large quantities (> 10 g l^{-1}) (De Vuyst & Degeest, 1999; Monsan *et al.*, 2001). They are usually synthesized from sucrose by extracellular transglycosylases (such as glucan- and fructansucrases). These enzymes transfer one monosaccharide of the glycosidic donor to an acceptor molecule (a growing dextran or levan molecule) and release the other monosaccharide into the medium (De Vuyst & Degeest, 1999; Torino *et al.*, 2015). In the presence of acceptor molecules such as maltose, oligosaccharides are produced. Glucansucrases release fructose from sucrose and polymerize glucose into dextran [α-(1->6)] (*Leuc. mesenteroides* and *Lb. hilgardii*), mutan [α-(1->3)] (*Leuc. mesenteroides* and *Streptococcus mutans*), reuteran [α-(1->4)] (*Lb. reuteri*), and alternan [alternating α-(1->6) and α-(1->3)] (*Leuc. mesenteroides*). Fructansucrases release glucose from sucrose and polymerize fructose into levan-type fructans [β-(2->6)] (as found for *Leuc. mesenteroides*, *Lb. reuteri*, *Streptococcus salivarius*, and *Strep. mutans*) or inulin-type fructans [β-(2->1)] (as found for *Lactobacillus johnsonii* and *Strep. mutans*).

The specific properties of EPS depend on their monomer composition, backbone and branching glycosidic linkages, degree of polymerization, and possible secondary structures (De Vuyst & Degeest, 1999; De Vuyst *et al.*, 2001; Monsan *et al.*, 2001; De Vuyst & De Vin, 2007). EPS may contribute to the formation of a biofilm, wherein microorganisms are protected against phages, antibiotics, dessication, detachment, and other physical and chemical stressors. The production of EPS may be desirable in certain fermented food products, as they contribute to the production of smooth and soft cheeses, decrease syneresis in yoghurt because of their water-binding capacity, and improve the texture of sourdough breads. However, EPS can also be undesirable in certain fermented food products, as they increase the viscosity of wine, beer, and cider, which is unappealing in these products.

EPS have many commercial applications as emulsifier, carrier, and stabilizer in the food, beverage, pharmaceutical, chemical, and other industries (De Vuyst & Vaningelgem, 2003; Patel *et al.*, 2012; Torino *et al.*, 2015). They can be applied as additives or can be produced *in situ*, as is possible for yoghurt and sourdough.

3.2.4 Occurrence and health

LAB are widespread in nature and occur on plants (e.g., *Lb. plantarum* and *Leuc. mesenteroides*), in the oral cavity (*Lb. casei* and *Strep. mutans*), the gastrointestinal tract (*E. faecalis*, *E. faecium*, and *Lb. reuteri*), the female genital tract (*Lb. acidophilus*), and in

fermented foods and beverages (Axelsson, 2004). Some LAB are found in many habitats, as is the case for *Lb. plantarum* (vegetable fermentation, gastrointestinal tract, oral cavity, sourdough) (Siezen *et al.*, 2010) and *Lb. casei* (fermented milk products, gastrointestinal tract, silage, wine) (Broadbent *et al.*, 2012), whereas others occur only in very specific niches, as is the case for *Lb. sanfranciscensis* (sourdough) (De Vuyst *et al.*, 2014b) and *O. oeni* (wine) (Campbell-Sills *et al.*, 2015).

Many LAB have already been exploited as starter cultures for industrial food fermentation processes (Leroy & De Vuyst, 2004). For example, *Lb. delbrueckii* subsp. *bulgaricus* and *Strep. thermophilus* are used for the production of yoghurt, *Lc. lactis* and *Lb. casei* are used for the production of cheese, and *Lb. sakei* is used for the production of fermented sausages. However, LAB can also cause spoilage of foods and beverages. For example, *P. damnosus* is known to cause ropy beer due to its EPS production (Snauwaert *et al.*, 2015), growth of *Leuc. mesenteroides*, *Lb. sakei*, and *Lb. plantarum* causes spoilage of vacuum-packaged cooked meat products (Chenoll *et al.*, 2007), and the psychrotrophic *Leuconostoc gelidum* contributes to spoilage of refrigerated ready-to-eat vegetable salads (Pothakos *et al.*, 2014).

Many *Streptococcus* species have been implicated in human and animal diseases, as is the case for *Strep. mutans*, *Streptococcus pneumoniae*, *Streptococcus suis*, *Streptococcus agalacticae*, and *Streptococcus pyogenes* (Mitchell, 2003). Alternatively, milk fermented with *Strep. thermophilus* possesses many health benefits (Nagpal *et al.*, 2012). Also, *Streptococcus macedonicus* is a promising functional starter culture for dairy products, as it can produce anti-clostridial bacteriocins (De Vuyst & Tsakalidou, 2008). The association of *Enterococcus* species with the human gastrointestinal tract has resulted in their use as indicator for human fecal contamination (Layton *et al.*, 2010). In general, *Enterococcus* strains from the gastrointestinal tract of healthy humans are not virulent or pathogenic, but some strains are resistant to many antibiotics and are a common cause of hospital-acquired infections (Vancanneyt *et al.*, 2002; De Angelis *et al.*, 2014). Nevertheless, *Enterococcus* species develop during the spontaneous fermentation of vegetables, sausages, and cheeses, and contribute to flavor formation (Foulquié Moreno *et al.*, 2006). Strains that do not possess haemolytic activity or antibiotic resistance genes may be regarded as safe and can be used as starter cultures, for example for the production of cheese (Izquierdo *et al.*, 2009) or silage (Ellis *et al.*, 2016). Some *Enterococcus* strains are even used as probiotics, as is the case with certain strains of *E. faecium* and *E. faecalis* (Franz *et al.*, 2011; Bourdichon *et al.*, 2012). Other LAB are rarely pathogenic but may occur as opportunistic pathogens in immunocompromised persons, as has been the case for certain strains of *Pediococcus*, *Lactobacillus*, *Carnobacterium*, *Lactococcus*, and *Leuconostoc* species (Wessels *et al.*, 2004).

Food-associated LAB are generally recognized as safe (GRAS), and the genus *Lactobacillus* is among the bacteria with the lowest risk to humans (Wessels *et al.*, 2004; Bourdichon *et al.*, 2012). They develop during many spontaneous food fermentations and are frequently used as starter cultures for these fermentations. Foods and beverages fermented with LAB may possess health-promoting properties, as LAB can produce vitamins such as vitamins B2 and B12, and bioactive peptides (possessing anti-hypertensive, cholesterol-lowering, or other effects) from proteins present in the raw materials (Stanton *et al.*, 2005). Several *Lactobacillus* strains have been used as probiotics, for example certain strains of *Lb. rhamnosus* and *Lb. paracasei* can be of therapeutic value against diarrhea, irritable bowel disorders, allergies, and lactose intolerance (Hungin *et al.*, 2013). Furthermore, the probiotic potential of LAB strains from different niches, such as wine (*P. pentosaceus*) and milk kefir (*Lb. kefiranofaciens*), has been tested as well (Chen *et al.*, 2012; García-Ruiz *et al.*, 2014).

EPS produced by LAB species may possess prebiotic properties (Salazar *et al.*, 2015). Indeed, homopolysaccharides produced by *Lc. lactis*, *Leuc. citreum*, and *Weissella confusa*/*cibaria* could be degraded by beneficial bacteria (Hongpattarakere *et al.*, 2012; Grosu-Tudor *et al.*, 2013). In particular, the prebiotic properties of dextran oligosaccharides have been investigated in more detail (Sarbini *et al.*, 2014). This may allow the selection of functional starter cultures that produce prebiotic EPS *in situ* during the fermentation process of sourdough, milk kefir, water kefir, or cheese. Furthermore, some EPS possess additional health benefits, as is the case for kefiran, which has antimicrobial activity against several pathogenic bacteria (such as *Strep. pyogenes* and *Staphylococcus aureus*) and yeasts (*C. albicans*) (Rodrigues *et al.*, 2005a), has cicatrizing (wound-healing) activity *in vivo* in rats (Rodrigues *et al.*, 2005a), and possesses antitumor activity (Shiomi *et al.*, 1982; Murofushi *et al.*, 1983). Consumption of kefiran can lower blood pressure and serum cholesterol levels (Maeda *et al.*, 2004; Furuno & Nakanishi, 2012), can improve constipation (Maeda *et al.*, 2004), may offer protection against the cytotoxic effects of pathogenic bacteria such as *Bacillus cereus* (Medrano *et al.*, 2009), and can reduce atherosclerosis in rats (Uchida *et al.*, 2010). However, pathogenic bacteria can also produce EPS, which can form biofilms, enhancing their ability to adhere and attach to the infection site, as is the case for *Sal. enterica* serovar Typhimurium in the gastrointestinal tract and *Strep. mutans* in dental caries (Ledeboer & Jones, 2005). These EPS may even mimic the host cell surface components to avoid a reaction of the immune system, as is the case for *Strep. pyogenes* (Cress *et al.*, 2014).

Humans convert L-lactic acid via L-lactic acid dehydrogenase into pyruvate, which can be further converted into acetyl-CoA and oxidized in the mitochondria (Ewaschuk *et al.*, 2005). However, mammals do not possess an efficient D-lactic acid dehydrogenase and excessive amounts of D-lactic acid can accumulate and result in D-lactic acidosis, which is characterized by neurological manifestations such as ataxia, confusion, lethargy, and coma (Kang *et al.*, 2006). D-Lactic acidosis typically results from small intestinal bowel overgrowth by LAB rather than from the consumption of foods or beverages rich in D-lactic acid (Dahlqvist *et al.*, 2013). LAB can also produce low concentrations of formic acid, which is toxic for humans, as described above. LAB are known to be able to produce biogenic amines, in particular histamine, tyramine, phenylethylamine, and putrescine (Lonvaud-Funel, 2001; Landete *et al.*, 2007). The production of biogenic amines is strain-dependent and the best way to control the production of biogenic amines during a fermentation process is through the use of selected starter cultures (Latorre-Moratalla *et al.*, 2012).

3.3 Acetic acid bacteria

3.3.1 Description

AAB are Gram-negative (or Gram-variable) bacteria belonging to the class of the *α*-Proteobacteria and the family *Acetobacteraceae* (Sievers & Swings, 2005; Kersters *et al.*, 2006). At the time of writing, there were 17 genera of AAB, among which *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, and *Komagataeibacter* are the most prevalent in foods and beverages (Wang *et al.*, 2015). AAB can be motile, do not sporulate, are around 0.5 µm wide and 1-4 µm long, and form ellipsoidal to rod-shaped cells, occurring singly, in pairs, or in short chains. AAB are obligately aerobic, but they can survive for extended periods under low oxygen conditions, as is the case for strains occurring in bottled wine (Bartowsky & Henschke, 2008) and during the cocoa bean fermentation process (Papalexandratou *et al.*, 2011a,c). Low oxygen availability may result in a viable but non-culturable (VBNC) state in AAB, decreasing their recovery via culture-dependent methods (Millet & Lonvaud-Funel,

2000). AAB grow optimal at 25-30 °C and at pH 5.0-6.5, but many grow also at pH 3.0-4.0 (Sievers & Swings, 2005).

3.3.2 Metabolism

The most characteristic trait of AAB is the periplasmic oxidation of alcohols, monosaccharides, and sugar alcohols by pyrroloquinoline quinone (PQQ)- or flavin adenine dinucleotide (FAD)-dependent membrane-bound enzymes (Prust *et al.*, 2005). Ethanol can be oxidized by alcohol dehydrogenase (ADH) into acetaldehyde, which is further oxidized by aldehyde dehydrogenase (ALDH) into acetic acid. D-Glucose can be oxidized into D-gluconic acid, 2-keto-D-gluconic acid, 5-keto-D-gluconic acid, and/or 2,5-diketo-D-gluconic acid, depending on the AAB species. D-fructose can be oxidized into 5-keto-D-fructose. D-mannitol, D-sorbitol, and D-arabitol can be oxidized into D-fructose, L-sorbose, and D-xylulose, respectively. In general, *Acetobacter* and *Gluconacetobacter* species prefer the oxidation of ethanol over glucose, whereas *Gluconobacter* species prefer the oxidation of glucose over ethanol (Mamlouk & Gullo, 2013).

When ethanol is depleted, AAB can oxidize glycerol, a common side-product of alcoholic fermentation, into dihydroxyacetone (Mamlouk & Gullo, 2013). Dihydroxyacetone is known to react with the amino groups of amino acids and proteins to form a brown-colored complex. Dihydroxyacetone can also enter the EMP pathway and the gluconeogenesis, resulting in the production of cellulose, acetic acid, and carbon dioxide. However, AAB do not possess a complete functional EMP pathway, because they lack the enzyme phosphofructokinase, as was the case for *G. oxydans* and *A. pasteurianus* (Prust *et al.*, 2005; Illeghems *et al.*, 2013), and usually metabolize glucose via the PPP. Additionally, some AAB possess a functional Entner-Doudoroff (ED) pathway, which is usually less active than the PPP.

Acetobacter, *Gluconacetobacter*, and *Komagataeibacter* species possess a functional TCA cycle associated with the cytoplasmic membrane, which allows them to completely oxidize (overoxidize) organic acids, such as acetic acid, lactic acid, pyruvic acid, malic acid, succinic acid, and citric acid into carbon dioxide and water (Mamlouk & Gullo, 2013). Hereto, acetic acid and lactic acid are converted in the cytoplasm into acetyl-CoA and pyruvate, respectively. *Gluconobacter* species lack the enzymes α-ketoglutarate dehydrogenase and succinate dehydrogenase and therefore do not overoxidize acetic acid or other carboxylic acids (Matsushita *et al.*, 2004). Many AAB can convert lactic acid into acetoin (weak butter aroma), which may be undesirable in wine, beer, and vinegar (Akasaka *et al.*, 2013; Moens *et al.*, 2014). AAB also possess intracellular esterases, catalyzing the condensation of acetic acid and ethanol into ethyl acetate (Kashima *et al.*, 1998). *Acetobacter japonicus* is able to degrade pectin into pectinate and methanol (Jayani *et al.*, 2005), and *Acetobacter pasteurianus* may be able to degrade pectin via an endopolygalacturonase (Illeghems *et al.*, 2013).

Many strains of the species *Acetobacter*, *Komagataeibacter*, *Gluconobacter*, and *Gluconacetobacter* produce water-insoluble cellulose homopolysaccharides [β-(1->4) glucans] from glucose and fructose (Valera *et al.*, 2015). This is visible as a pellicle that develops on the surface of the fermentation liquor, as is the case during vinegar and kombucha fermentations (Jayabalan *et al.*, 2014; Yetiman & Kesmen, 2015). *Gluconobacter frateurii*, *Gluconobacter cerinus*, *Gluconobacter nephelii*, and *Kom. xylinus* can produce water-soluble levan homopolysaccharides [probably β-(2->6) fructans] from sucrose (Tajima *et al.*, 1997; Jakob *et al.*, 2013; Semjonovs *et al.*, 2016). *Komagataeibacter xylinus* can also produce a water-soluble acetan heteropolysaccharide, which consists of glucose, mannose, glucuronic acid, and rhamnose (Jansson *et al.*, 1993). *Acetobacter capsulatus* and *Acetobacter viscosus* can cause ropiness in beer by the production of water-soluble dextran

homopolysaccharides [α-(1->6) glucans with α-(1->4) branches) from maltooligosaccharides (Yamamoto *et al.*, 1993).

3.3.3 Occurrence and health

AAB are found during the fermentation process of vinegar, wine, beer, water kefir, milk kefir, kombucha, and cocoa beans, and on fruits, flowers, honey bees, soft drinks, and fruit juices (Kersters *et al.*, 2006; Cleenwerck & De Vos, 2008). *Gluconobacter* species are typically found in sugar-rich environments, whereas *Acetobacter* and *Gluconacetobacter* species are typically found in alcohol-rich environments (Sievers & Swings, 2005; Bartowsky & Henschke, 2008). Some strains of *Acetobacter*, *Gluconacetobacter*, and *Gluconobacter* species have been associated with the gut of insects that have a sugar-based diet (nectar, fruit sugars, or phloem juice), such as fruit flies and honey bees (Crotti *et al.*, 2010). Several species of *Acetobacter* and *Gluconacetobacter* can fix atmospheric nitrogen, such as *Gluconacetobacter diazotrophicus* (associated with sugar cane, coffee, tea, and sweet potato plants), *Acetobacter peroxydans* (associated with rice plants), and *A. nitrogenifigens* (associated with kombucha) (Pedraza, 2016).

AAB are generally not considered to be pathogenic towards humans or animals, and they do not produce toxic compounds or biogenic amines (Landete *et al.*, 2007). Nevertheless, *Acetobacter cibinongensis* (Gouby *et al.*, 2007), *Acetobacter indonesiensis* (Bittar *et al.*, 2008), and *Gluconobacter japonicus* (Alauzet *et al.*, 2010) may cause infections in immunocompromised patients. Some *Gluconobacter* species can also cause bacterial rot of apples and pears, which is accompanied by various shades of browning (Sievers & Swings, 2005).

3.4 Bifidobacteria

3.4.1 Description

Bifidobacteria are Gram-positive bacteria and belong to phylum of the Actinobacteria. They are not motile, do not sporulate, are around 0.7 μm wide and 0.7-6.0 μm long, and usually form irregular club-shaped rods with occasional bifurcations (Ballongue, 2004; Biavati, 2012). Bifidobacteria are obligately anaerobic, but their tolerance to oxygen varies and some species can grow under aerobic conditions. The optimal growth temperature for bifidobacteria is around 30-40 °C but some species can also grow at 4 °C. The optimal pH for growth is around 6.0 to 7.0, but some species also grow at pH 4.0.

3.4.2 Metabolism

Bifidobacteria possess the enzyme fructose 6-phosphate phosphoketolase (FPPK) and degrade glucose via the fructose 6-phosphate phosphoketolase pathway (known as the bifid shunt) into acetic acid and lactic acid in a molar ratio of 3 to 2, whereby 2.5 moles of ATP are generated per mole of glucose (De Vuyst & Leroy, 2011; Pokusaeva *et al.*, 2011; De Vuyst *et al.*, 2016). Apart from acetic acid and lactic acid, bifidobacteria can convert pyruvate into formic acid and acetic acid (yielding an extra mole of ATP per mole of glucose), or formic acid and ethanol (reoxidizing 2 moles of NADH + H^+ into 2 moles of NAD^+ per mole of glucose) (Van der Meulen *et al.*, 2006). Additionally, low concentrations of succinate may be produced by bifidobacteria. Low pH values and slow carbohydrate consumption increases the production of acetic acid, formic acid, and ethanol, and decreases the production of lactic acid (Van der Meulen *et al.*, 2006; Sánchez *et al.*, 2007). Pentoses are converted into xylulose 5-P and are metabolized into equimolar amounts of acetate and lactate, yielding 2 moles of ATP per mole of pentose (Pokusaeva *et al.*, 2011).

Bifidobacteria are saccharolytic and possess many genes encoding glycoside hydrolases (which degrade polysaccharides and oligosaccharides into fermentable monosaccharides) and transport mechanisms for oligosaccharides (van den Broek *et al.*, 2008; Ventura *et al.*, 2014; De Vuyst *et al.*, 2016). They possess *β*-fructofuranosidases, which can hydrolyze sucrose and fructooligosaccharides into glucose and fructose. *Bifidobacterium dentium* can degrade dextran (Kaster & Brown, 1983), and many *Bifidobacterium* species can hydrolyze the *α*-(1->6) and *α*-(1->4) glucosidic bonds in starch, amylopectin, and pullulan (Ryan *et al.*, 2006). Several bifidobacteria such as *Bifidobacterium animalis*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium adolescentis*, and *Bifidobacterium pseudocatenulatum* are capable of heteropolysaccharide production, which may improve commensal-host interactions, offer protection against pathogens, and serve as fermentable substrates for neighboring microorganisms. The production of homopolysaccharides has not yet been described for bifidobacteria (Salazar *et al.*, 2015).

3.4.3 Occurrence and health

Bifidobacteria are usually associated with the human colon microbiota (*Bifidobacterium bifidum*, *B. longum*, *B. adolescentis*), where they play a role in the degradation of complex polysaccharides (Falony *et al.*, 2009; De Vuyst *et al.*, 2014a; Rivière *et al.*, 2014; De Vuyst *et al.*, 2016). However, they are also associated with the human genital tract (*B. bifidum*, *B. catenulatum*, and *B. breve*), human milk (*B. breve*, *B. adolescentis*, and *B. bifidum*), dental caries and abscecces (*B. dentium*), and are found in the bovine rumen (*B. adolescentis*), the digestive tract of bumblebees (*B. asteroides*), pigs (*B. minimum*), and chickens (*B. gallinarum*), and in sewage (*B. minimum*, *B. angulatum*, and *B. breve*) (Biavati, 2012). Finally, bifidobacteria have been found in fermented foods such as raw milk cheese (*B. crudilactis*), fermented milk (*B. mongoliense*), milk kefir (non-identified *Bifidobacterium* species), and water kefir (*B. aquikefiri*) (Delcenserie *et al.*, 2007; Watanabe *et al.*, 2009; Dobson *et al.*, 2011; Laureys *et al.*, 2016).

Bifidobacteria do not produce substantial amounts of harmful compounds, but can produce small amounts of biogenic amines (Lorencová *et al.*, 2012) and formic acid (Liesivuori & Savolainen, 1991). Furthermore, bifidobacteria are considered to be non-pathogenic, although several species can occur as opportunistic pathogens (Wessels *et al.*, 2004; Sanders *et al.*, 2010). For example, *B. dentium* has been associated with dental caries and tooth decay, *B. scardovii* has been found in human blood and urinary tract infections, *B. longum* has been isolated from blood, and *B. breve* may cause neonatal meningitis. Infections with bifidobacteria may actually be underreported because they are difficult to cultivate and identify and belong to the commensal gut microbiota.

Nevertheless, bifidobacteria have a function in the human colon ecosystem (De Vuyst *et al.*, 2014a, 2016). Indeed, a decrease of the relative abundances of *Bifidobacterium* species in the human colon has been associated with several gastrointestinal disorders, such as antibiotic-associated diarrhea, irritable bowel syndrome, inflammatory bowel disease, obesity, allergies, and regressive autism. Many strains of *B. breve*, *B. bifidum*, *B. animalis*, *B. longum*, and *B. infantis* have already been used in probiotics, as their consumption is beneficial for many digestive disorders such as lactose intolerance, constipation, and irritable bowel syndrome; the immune response such as inflammation, resistance to infections, and allergies; and even psychiatric disorders such as anxiety and depression (Mulle *et al.*, 2013; Zhang *et al.*, 2015).

3.5 Other microorganisms

3.5.1 Enterobacteriaceae

Enterobacteriaceae are Gram-negative bacteria belonging to the *γ*-Proteobacteria (Brenner & Farmer, 2005). Many spontaneous fermentation processes (such as fermented vegetables and Belgian-style acidic ales) start with an *Enterobacteriaceae* phase, which lasts several days, after which LAB, yeasts, AAB, and/or other microorganisms continue the fermentation. They are rod-shaped, around 1-5 μm in length, do not form spores, and are usually motile. *Enterobacteriaceae* are facultatively anaerobic bacteria with low nutrient requirements, and metabolize carbohydrates via the EMP pathway and mixed-acid fermentation, resulting in the production of lactic acid, acetic acid, succinic acid, formic acid, carbon dioxide, and ethanol. However, a large variety of other end-products may be formed depending on the strain and the fermentation conditions. For example, butanediol can be produced by *Enterobacter*, *Erwinia*, and *Serratia* species.

Enterobacteriaceae are part of the normal gut microbiota of humans and animals, but some are well-known gastrointestinal pathogens such as *Enterobacter*, *Escherichia*, *Klebsiella*, *Salmonella*, *Serratia*, *Shigella*, and *Yersinia*. An certain (artificially constructed) group within the *Enterobacteriaceae* is referred to as the coliforms, which constitute around 10 % of the intestinal microbiota of warm-blooded animals and encompasses the species *Enterobacter*, *Escherichia*, *Citrobacter*, *Hafnia*, and *Klebsiella*. Therefore, the presence of coliforms (as well as *Escherichia coli* alone) has been used as an indicator for fecal contamination of water. However, not all coliforms are of intestinal origin and some occur naturally in soil, vegetation, and aquatic environments. Their presence on fresh vegetable products explains their presence during the first part of certain spontaneous fermentation processes. *Enterobacteriaceae* are considered as spoilage microorganisms, as they can contribute to the biogenic amine content of fermented foods and beverages, for example cadaverine in cheese (Marino *et al.*, 2000).

Species of *Bacillus*, *Enterobacter*, *Pantoea*, *Pseudomonas*, and *Serratia* are found during the first days of spontaneous vegetable fermentations (Heperkan, 2013; Jeong *et al.*, 2013; Wouters *et al.*, 2013a), and species of *Enterobacter*, *Hafnia*, and *Klebsiella* species are found during the first month of spontaneous beer fermentations (Martens *et al.*, 1991; Spitaels *et al.*, 2014; Spitaels *et al.*, 2015a).

3.5.2 Zymomonas

Zymomonas species are Gram-negative bacteria belonging to the *α*-Proteobacteria (Sprenger & Swings, 2005). They are usually not motile, are 2.0-6.0 μm long and 1.0-1.4 μm wide, and form rod-shaped cells with rounded ends. They are usually facultatively anaerobic, but some strains are obligately anaerobic. Their optimal growth temperature and pH are around 25-30 °C and 5.0-7.5, respectively, but some species can grow at 4 °C and pH 3.5. *Zymomonas* species use the ED pathway for glucose metabolism, producing ethanol and carbon dioxide. *Zymomonas* species are found in several naturally fermented beverages such as water kefir (Marsh *et al.*, 2013b), beer (Dadds *et al.*, 1971; Jespersen & Jakobsen, 1996), cider (Carr & Passmore, 1971), and pulque (Escalante *et al.*, 2008). The growth of *Zymomonas anaerobia* is characterized by high concentrations of acetaldehyde (apple aroma) and hydrogen sulfide (rotten egg aroma) (Dadds *et al.*, 1971). This fault, known as *framboisé*, can be described as rotten lemon skin or grassy, and can be prevented by acidification below pH 3.7 (Coton & Coton, 2003; Coton *et al.*, 2006). Some species can produce levan EPS, which causes undesirable turbidity in fermented beverages (Coton *et al.*, 2006). Alternatively,

the EPS from *Zymomonas* can be used as thickening agent and may possess antitumor activity (Calazans *et al.*, 2000; Yoo *et al.*, 2004).

4 Conclusion

Naturally fermented beverages possess extensive metabolic, nutritional, prebiotic, and probiotic potential, but at this moment, most of this potential remains untapped and unavailable to end-consumer. In particular, Belgian-style acidic ales, kombucha, and water kefir may offer interesting alternatives to the milk-based fermented products and probiotics, which are unsuitable for people with a milk allergy. Furthermore, specific strains of microorganisms from the wide diversity of microorganisms found in naturally fermented beverages may be selected for the development of novel probiotic and/or functional starter cultures.

Recently, consumers have become more interested in food and health, resulting in an increased demand for healthy foods and beverages (Frost & Sullivan, 2008). In the past, healthy products were considered to be those with reduced fat, salt, sugar, or cholesterol, but this changed in favor of products with added nutritional and functional value, such as the incorporation of vitamins, prebiotics, or probiotics (Frost & Sullivan, 2007, 2010). Furthermore, a clear trend towards natural products without additives or preservatives is observed (Frost & Sullivan, 2008). The unique organoleptic properties of naturally fermented beverages can result in the creation of innovative water kefir-based fermented vegetable juices (Corona *et al.*, 2016), beers (Rodrigues *et al.*, 2016), and fermented fruit juices (Randazzo *et al.*, 2016). Restaurants are already exploring the unique complex organoleptic properties of naturally fermented foods and beverages to be able to offer novel experiences to their customers (Verhaeghe, 2015). These trends indicate that there may be a market opportunity for the commercial exploitation of naturally fermented beverages such as water kefir.

However, the properties and compositions of these naturally fermented beverages vary enormously, depending on the fermentation conditions and practices. Given their complexity, their successful commercial exploitation will require an elaborate investigation and optimization process, as contemporary beverage consumers demand healthy, tasty, high-quality, as well as highly convenient beverages.

CHAPTER 2

Aims and objectives

David Laureys and Luc De Vuyst

At the start of the present study, only little scientific information was available about the water kefir fermentation process, and the majority of this information concerned the microbial species diversity and the composition of the water kefir grain exopolysaccharides (EPS). Therefore, not only the microbial species diversity but also the community dynamics, the water kefir grain growth, the substrate consumption, and the metabolite production during a water kefir fermentation process needed to be elucidated in more detail.

However, from the literature data, it was clear that different water kefirs harbour different microbial species diversities. To investigate the impact of the water kefir grain inoculum on the microbial species diversity, community dynamics, and substrate consumption and metabolite production kinetics of the water kefir fermentation process, first three water kefir fermentation processes were started with different water kefir grain inocula, followed as a function of time, and compared with each other. As a low waterkefir grain growth is a common problem during water kefir fermentation, the EPS production capacity of the lactic acid bacteria (LAB) isolated was determined to investigate the relationship between EPS-producing LAB species and the water kefir grain growth in more detail. Furthermore, the microbial species diversities in the three water kefir fermentation processes were compared with those reported in the literature to be able to select the key microorganisms of water kefir fermentation from the wide microbial species diversity reported in the literature.

Only few companies produce water kefir on a commercial scale. Two problems that often occur are low water kefir grain growth during fermentation and instability of the production process. The former may prevent upscaling of the production process and the latter may result in variable end-products. To find their possible causes, the water kefir fermentation process of a small Belgian company suffering from these problems was characterized in more detail and compared with the laboratory water kefir fermentation processes carried out during the present study.

In several water kefir fermentation processes studied, a non-identified *Bifidobacterium* species was detected. Therefore, a strain of this *Bifidobacterium* species was isolated from a water kefir fermentation process and characterized genotypically and phenotypically.

The causes behind low water kefir grain growth during water kefir fermentation were not yet completely elucidated. Results obtained during the present study suggested that the pH during fermentation might influence the water kefir grain growth and the literature suggested that the calcium concentration in the fermentation medium might affect the water kefir grain growth. Therefore, the effect of the buffer capacity and the calcium concentration of the water used for fermentation on the characteristics of the water kefir fermentation process (and in particular the water kefir grain growth) were investigated in detail.

Water kefir fermentation is usually carried out under anaerobic conditions with dried figs as the source of nutrients, but might also be performed under aerobic conditions (as was the case for the industrial water kefir fermentation that was investigated) or with other (dried) fruits and/or herbs as nutrient sources (as reported in the literature). However, the impact of these factors needed to be investigated in more detail, as they may have a pronounced influence on the microbial species diversity and metabolism.

During water kefir fermentation, sucrose is usually (partially) converted into water kefir grain wet mass, which is not always desirable, as water kefir fermentation is usually carried out to obtain the water kefir liquor for its use as a beverage. Substitution of sucrose by glucose and/or fructose may decrease the water kefir grain growth, as sucrose is necessary for the production of water kefir grain EPS. However, the effect of glucose and/or fructose on the kinetics of the water kefir fermentation process was not known. Results obtained during the present study indicated that the majority of the water kefir microorganisms were associated with the grains, suggesting that the majority of the metabolic activity was also associated with the grains. This indicated that the amount of grain inoculum added may influence the water kefir fermentation rate. Nevertheless, the water kefir liquors contained a substantial amount of microorganisms, with a diversity similar to that on the water kefir grains. Hence, water kefir liquor might be used as an innovative inoculation strategy, whereby no water kefir grain wet mass is needed or produced, as water kefir grain wet mass might be considered as a waste stream. Sometimes, the production of water kefir grain wet mass might be desirable, for example to scale up a fermentation process. The literature suggested that the production of EPS from sucrose by glucansucrases might suffer from substrate inhibition, whereby the concentration of sucrose may impact the production of water kefir grain wet mass. Therefore, the influences of the type and concentration of the inoculum and the substrate on the kinetics of the water kefir fermentation process were investigated. Mathemathical models were fitted to the experimental data and the biokinetic parameters of the different proceses were compared. Additionally, the density of the water kefir grains was determined, as this might be useful for the development of certain industrial production processes, and the microbial colonization of the grains by the water kefir microorganisms was visualized with a state-of-the-art scanning electron microscope, as outdated results needed to be reassessed.

At the start of the present study, the water kefir fermentation process was still difficult to control, which hampered the further industrial exploitation of water kefir. To achieve greater control over the water kefir fermentation process, the influence of several process conditions needed to be known. For instance, increasing backslopping times might result in more excessive acidic stress, which may impact the composition of the water kefir microorganisms and/or cause a low water kefir grain growth. Water kefir grains are usually rinsed before each backslopping step, but it was not known how this practice influences the fermentation process. Rinsing of the grains may remove residual substrates and metabolites and/or microorganisms from the water kefir grains. The former might reduce the residual substrate and metabolite concentrations, whereas the latter might decrease the water kefir fermentation rate. Furthermore, the incubation temperature is known to have a profound influence on a fermentation process. Increasing temperatures might increase the water kefir fermentation rate, but might also impact the composition of the water kefir microorganisms. The influence of the incubation temperature should therefore be investigated in more detail. These investigations will provide more insight into the water kefir fermentation process and will allow greater control over this process, which will be of value for both artisan and industrial production of water kefir.

CHAPTER 3

Microbial species diversity, community dynamics, substrate consumption, and metabolite production during water kefir fermentation: first insights

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SUMMARY

Water kefir is a slightly sweet, acidic, alcoholic, and fruity naturally fermented beverage. The water kefir fermentation process is started with water kefir grains, which are composed of glucan exopolysaccharides and contain the microorganisms responsible for water kefir fermentation. In this study, the species diversity, community dynamics, substrate consumption, and metabolite production during a water kefir fermentation process were investigated as a function of time. The most prevalent microbial species present were *Lactobacillus paracasei*, *Lactobacillus harbinensis*, *Lactobacillus hilgardii*, a non-identified *Bifidobacterium* species, *Saccharomyces cerevisiae*, and *Dekkera bruxellensis*. This microbial species diversity was similar in the water kefir liquor and on the water kefir grains, and remained stable during the entire fermentation process. The majority of the water kefir microorganisms was associated with the water kefir grains. Sucrose was the main substrate and was completely converted after 24 h of fermentation, whereby water kefir grain exopolysaccharide was produced as long as sucrose was present. The main metabolites produced during the fermentation process were ethanol, lactic acid, glycerol, acetic acid, and mannitol. The main aroma compounds produced during fermentation were ethyl acetate, isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate. The major part of these metabolites was produced during the first 72 h of fermentation, during which the pH decreased from 4.3 to 3.5.

1 Introduction

Water kefir is a fermented beverage that is made by adding water kefir grains, which are polysaccharide grains that serve as the inoculum, to a mixture of water, sugar (sucrose), (dried) fruits (usually dried figs), and possibly other ingredients (such as lemon), depending on the recipe (Pidoux, 1989; Gulitz *et al.*, 2011, 2013; Hsieh *et al.*, 2012; Stadie *et al.*, 2013). After 2 to 4 days of anaerobic fermentation at room temperature, a yellowish, sparkling, fermented beverage is obtained, that has a slightly sweet, acidic, alcoholic, and fruity taste and aroma. Water kefir grains occur worldwide under a variety of names, such as 'ginger beer plants', 'Tibicos', 'Tibi grains', 'California bees', 'African bees', 'ale nuts', 'balm of Gilead', 'Bèbées', 'Japanese beer seeds', and 'sugary kefir grains' (Ward, 1892; Lutz, 1899; Kebler, 1921; Pidoux *et al.*, 1988; Pidoux, 1989; Gulitz *et al.*, 2013). Their origin is still unknown, but it has been postulated that water kefir grains originate from the leaves of the *Opuntia* cactus (Lutz, 1899).

Currently, research on water kefir is still limited and most of the scientific information available deals with its species diversity (Ward, 1892; Kebler, 1921; Moinas *et al.*, 1980; Pidoux, 1989; Galli *et al.*, 1995; Franzetti *et al.*, 1998; Neve & Heller, 2002; Magalhães *et al.*, 2010, 2011; Gulitz *et al.*, 2011, 2013; Miguel *et al.*, 2011). It is known that the microbial species diversity of water kefir consists mainly of lactic acid bacteria (LAB), yeasts, and acetic acid bacteria (AAB), as shown by both culture-dependent and culture-independent techniques (Waldherr *et al.*, 2010; Gulitz *et al.*, 2011, 2013; Marsh *et al.*, 2013b). Recently, *Bifidobacterium psychraerophilum/crudilactis* was found in water kefir via culture-independent techniques (Gulitz *et al.*, 2013). It became clear, however, that different water kefirs display different species diversities. Hence, a systematic approach for the study of the microbiology of water kefir fermentations is necessary. Also, the chemical and structural composition of the water kefir grain polysaccharide has been studied (Horisberger, 1969; Moinas *et al.*, 1980; Pidoux, 1989; Waldherr *et al.*, 2010). It is known that the water kefir grains are composed of dextran exopolysaccharides (EPS), which are α-(1−>6)-linked glucose homopolymers, produced by certain *Lactobacillus* and/or *Leuconostoc* species. However, until now no thorough metabolite analysis has been performed on a water kefir fermentation process.

This chapter aimed to investigate the microbial species diversity, community dynamics, substrate consumption profile, and metabolite production course of a water kefir fermentation process, to obtain a deeper understanding of this process.

2 Materials and methods

2.1 Water kefir grain inoculum and prefermentations

To prepare an inoculum, approximately 100 g of water kefir grains was obtained from a private person, who maintains a household water kefir fermentation process (Ghent, Belgium). To obtain the necessary amount of water kefir grains, the inoculum was cultivated through a series of consecutive prefermentations through backslopping until > 600 g of water kefir grain wet mass was produced. The prefermentations were performed in glass bottles (1, 2, and 5 l) equipped with a polytetrafluoroethylene (PTFE) water lock. They were started by adding 6 g of unrefined cane sugar (Candico Bio, Merksem, Belgium), 85 ml of tap water (Brussels, Belgium), and 5 g of dried figs (King Brand, Naziili, Turkey) per 15 g of water kefir grains. The bottles were incubated in a water bath at 21 °C. Every 3 days, the backslopping practice was applied, whereby the water kefir grains were separated from the

water kefir liquor by sieving, and recultivated in fresh medium and under the same conditions as described above.

2.2 Fermentations

The water kefir grain wet mass, obtained through the series of prefermentations mentioned above, was used to start the water kefir fermentation processes. The fermentations were performed in 100-ml glass bottles (12 bottles per fermentation) equipped with a PTFE water lock. They were started by adding 15 g of water kefir grain inoculum to 85 ml of autoclaved (121 °C, 2.1 bar, 21 min) water kefir simulation medium (WKSM). The WKSM contained 6 g of unrefined cane sugar (Candico Bio), 65 ml of tap water (Brussels, Belgium), and 20 ml of fig extract. Fig extract was prepared by mixing 5 g of dried figs (King Brand) with 20 ml of distilled water, after which the suspension was centrifuged (7200 x *g*, 20 min, 4 °C). The supernatant was filtered through a coffee filter. The 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 of the fermentation processes and before their sampling.

2.3 Analyses

After 0, 3, 6, 12, 18, 24, 36, 48, 72, 96, 144, and 192 h of fermentation, three fermentation bottles (representing three independent biological replicates) were removed and their contents were analyzed. The pH, the water kefir grain wet mass, the water kefir grain dry mass, the viable counts of the LAB, AAB, and yeasts in the water kefir liquors and on the rinsed water kefir grains, and the concentrations of the substrates and metabolites were determined at each sampling point. The viable counts of the *Enterobacteriaceae* and the enterococci plus streptococci in the water kefir liquors and on the rinsed water kefir grains were determined after 0 and 72 h of fermentation. The culture-dependent microbial species diversity and community dynamics of the LAB, AAB, and yeasts in the water kefir liquors and on the rinsed water kefir grains were determined after 0, 24, 48, 72, and 192 h. The culture-independent microbial species diversity and community dynamics in the water kefir liquors and on the rinsed water kefir grains were determined after 0, 24, 72, and 192 h. The results are presented as the mean ± standard deviation of the three independent biological replicates performed for each sampling point.

2.4 pH and water kefir grain wet and dry mass determinations

The pH of the water kefir liquor was determined with a SenTix 41 glass electrode (WTW, Weilheim, Germany), immediately after a fermentation bottle was opened. Then, the contents of the fermentation bottles were sieved to separate the water kefir grains from the water kefir liquors. The sieved water kefir grain wet mass was rinsed with 200 ml of sterile saline [8.5 g l^{-1} of NaCl (Merck KGaA, Darmstadt, Germany)] and weighed. The water kefir grain growth was defined as the increase of the water kefir grain wet mass at the time of sampling (compared with that at the start of the fermentation) divided by the water kefir grain wet mass at the start of the fermentation, and expressed as % (m m^{-1}). To determine the water kefir grain dry mass, approximately 5 g of rinsed water kefir grain wet mass was transferred into an aluminium recipient and dried at 105 °C for 48 h. The water kefir grain dry mass was defined as the mass after drying divided by the mass before drying, and expressed as % (m m^{-1}).

2.5 Microbial enumerations

The viable counts of the microorganisms in the water kefir liquors and on the water kefir grains were determined by preparing appropriate decimal dilutions of water kefir liquors and water kefir grain suspensions in sterile saline, and plating them on selective agar media. To prepare the water kefir grain suspensions, 5.0 g of rinsed water kefir grains were brought into a sterile stomacher bag, crushed by rolling a glass bottle over the outside of the bag, after which 45 ml of sterile maximum recovery diluent [8.5 g l^{-1} of NaCl (Merck) and 1 g l^{-1} of bacteriological peptone (Oxoid, Basingstoke, UK)] were added. This mixture was homogenized for 15 min at high speed in a Stomacher 400 apparatus (Seward, Worthington, UK).

The viable counts of the presumptive LAB were determined on de Man-Rogosa-Sharpe (MRS) agar medium (Oxoid), supplemented with cycloheximide (final concentration of 0.1 g l^{-1}; Sigma-Aldrich, Saint Louis, MO, USA); those of the presumptive AAB on modified deoxycholate-mannitol-sorbitol (mDMS) agar medium, supplemented with cycloheximide (final concentration of 0.1 g l^{-1}; Sigma-Aldrich) (Papalexandratou *et al.*, 2011b, 2013); those of the presumptive yeasts on yeast extract-glucose (YG) agar medium, supplemented with chloramphenicol (final concentration of 0.1 g l^{-1}; Sigma-Aldrich); those of the presumptive enterococci plus streptococci on kanamycin-aesculin-azide (KAA) agar medium (Oxoid); and those of the presumptive *Enterobacteriaceae* on violet-red-bile-glucose (VRBG) agar medium (Oxoid). MRS, mDMS, and YG agar media were incubated at 30 °C for 2 to 4 days, and KAA and VRBG agar media were incubated at 42 °C for 24 h. The viable counts were expressed as log cfu (colony forming units) per ml of water kefir liquor or per g of water kefir grains.

2.6 Culture-dependent microbial species diversity and community dynamics analysis

The culture-dependent microbial species diversity and community dynamics analyses of the LAB (based on isolates from MRS agar medium), AAB (based on isolates from mDMS agar medium), and yeasts (based on isolates from YG agar medium) were determined by randomly picking up 10 to 20 % of the total number of colonies from the respective agar media with 30 to 300 colonies. The microbial species diversity on the agar media of the water kefir grains or liquors was assumed to reflect the real microbial species diversity of the targeted group of microorganisms (LAB, AAB, or yeasts) in the water kefir grains or liquors. Bacteria were subcultivated in MRS medium (30 °C, 24 h) and yeasts in yeast extract-peptone-dextrose (YPD) medium (30 °C, 24 h). These cultures were supplemented with glycerol [final concentration of 25 % (v v^{-1})] and stored at -80 °C. In parallel, 2 ml of these cultures were centrifuged (21,000 x *g*, 5 min, 4 °C) and the cell pellets obtained were stored at -20 °C for rep-PCR fingerprinting analysis.

Thawed cell pellets were resuspended in 1 ml of TES buffer [6.7 % (m v^{-1}) sucrose, 50 mM Tris-base, 1 mM EDTA, pH 8.0], after which the suspensions were centrifuged (21,000 x *g*, 20 min, 4 °C) and the supernatants were discarded. Bacterial cell pellets were resuspended in 180 µl of TET buffer [20 mM Tris-HCl, 2 mM EDTA, 1.0 % (v v^{-1}) of Triton X-100, pH 8.0] supplemented with 12.5 U of mutanolysin (Sigma-Aldrich) and 4 mg of lysozyme (Merck), and incubated at 37 °C for 1 h. These suspensions were supplemented with 25 µl of proteinase K solution (NucleoSpin® 96 tissue kit; Macherey-Nagel, Düren, Germany) and incubated at 56 °C for 2 h. Yeast cell pellets were resuspended in 600 µl of sorbitol buffer [1.2 M sorbitol, 50 mM Tris-base, pH 7.5] supplemented with 30 mM 2-mercaptoethanol and 200 U of lyticase (Sigma-Aldrich), and incubated at 30 °C for 1 h, after which the suspensions were centrifuged (10,000 x *g*, 10 min) and the supernatants were discarded. These pellets

were resuspended in 180 µl of T1 buffer (Macherey-Nagel) and 25 µl of proteinase K solution (Macherey-Nagel), and incubated at 56 °C for 2 h. The DNA obtained from the bacterial and yeast cell pellets was purified with the NucleoSpin® 96 tissue kit (Macherey-Nagel), according to the instructions of the manufacturer. Bacterial DNA was diluted to approximately 50 ng $µl^{-1}$ and used for $(GTG)_5$-PCR fingerprinting with the $(GTG)_5$ primer (5'-GTGGTGGTGGTGGTG-3') (Wouters *et al.*, 2013b). Briefly, 1.00 µl of diluted DNA solution was added to 24 µl of PCR assay mixture, consisting of 13.45 µl of ultrapure water, 5.00 µl of 5 x Gitschier buffer [83 mM $(NH_4)_2SO_4$, 335 mM Tris-HCl, 33.5 mM $MgCl_2$, 33.5 µM EDTA, 150 mM *β*-mercaptoethanol, pH 8.8], 2.50 µl of 100 % dimethylsulfoxide (VWR International, Darmstadt, Germany), 1.25 µl of a solution containing 25 mM of each of the deoxynucleotide triphosphates (dNTPs; Sigma-Aldrich), 1.00 µl of 0.3 µg $µl^{-1}$ $(GTG)_5$ primer (Integrated DNA Technologies, Leuven, Belgium), 0.40 µl of 10 mg ml^{-1} bovine serum albumine (BSA; Acros Organics, Geel, Belgium), and 0.40 µl of 5 U $µl^{-1}$ of *Taq* DNA polymerase (Roche Diagnostics, Brussels, Belgium). The thermal cycling reaction consisted of an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 40 °C for 1 min, and elongation at 65 °C for 8 min, and was finalized with an elongation at 65 °C for 16 min. The PCR amplicons were separated by electrophoresis in a 1.5 % (m v^{-1}) agarose gel, in 1 x Tris-acetate-EDTA buffer (TAE; Bio-Rad, Hercules, CA, USA) at 4 °C and 55 V for 16 h. For alignment of the fingerprints, a DNA ladder (Gene ruler DNA ladder mix, 0.1 µg $µl^{-1}$; Sigma-Aldrich) was used. The agarose gels were stained in 1 liter of 1 x TAE buffer (Bio-Rad) with 2 drops of 10 mg ml^{-1} of ethydium bromide (Bio-Rad) and visualized under UV light in a Proxima imaging platform (Isogen Life Sciences, De Meern, The Netherlands) with the Proxima AQ-4 software (Isogen Life Sciences). Yeast DNA was diluted to approximately 20 ng $µl^{-1}$ and used for M13-PCR fingerprinting using the M13 primer (5'-GAGGGTGGCGGTTCT-3') (Daniel *et al.*, 2009). Briefly, 1.00 µl of diluted DNA solution was added to 24 µl of PCR assay mixture, consisting of 9.85 µl of ultrapure water, 2.50 µl of 10 x PCR buffer (Roche Diagnostics), 9.00 µl of 25 mM $MgCl_2$, 1.25 µl of a solution containing 25 mM of each of the dNTPs (Sigma-Aldrich), 1.00 µl of 0.3 µg $µl^{-1}$ M13 primer (Integrated DNA Technologies), and 0.40 µl of 5 U $µl^{-1}$ of *Taq* DNA polymerase (Roche Diagnostics). The thermal cycling reaction consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and elongation at 65 °C for 6 min, and was finalized with an elongation at 65 °C for 16 min. The PCR amplicons were separated by electrophoresis in an agarose gel and visualized as described above.

The fingerprint patterns obtained were clustered numerically into similarity trees using the Pearson correlation coefficient (PCC) and the unweighted pair group method with arithmetic mean (UPGMA) algorithm with the BioNumerics software version 5.10 (Applied maths, Sint-Martens-Latem, Belgium). Representative bacterial isolates within each cluster were identified by amplifying and sequencing part of their 16S rRNA gene from genomic DNA with primer pair pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCCGCA-3') (Edwards *et al.*, 1989). Briefly, 1.00 µl of diluted DNA solution was added to 49 µl of PCR reaction mixture, consisting of 35.75 µl of ultrapure water, 6 µl of 10 x PCR buffer (Roche Diagnostics), 2.5 µl of 0.1 mg ml^{-1} BSA (Acros Organics), 2 µl of each of the two 5 µM primer solutions (Integrated DNA Technologies), 0.5 µl of a solution containing 5 mM of each of the dNTPs (Sigma-Aldrich), and 0.25 µl of 5 U $µl^{-1}$ of *Taq* DNA polymerase (Roche Diagnostics). The thermal cycling reactions consisted of an initial denaturation at 95 °C for 5 min, followed by 3 cycles of denaturation at 95 °C for 45 s, annealing at 55 °C for 2 min, and elongation at 72 °C for 1 min; and 30 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 1 min and elongation at 72 °C for 1 min, and was finalized with an elongation at 72 °C for 7 min. Representative yeast isolates within

each cluster were identified by amplifying and sequencing part of their 26S large subunit (LSU) rRNA gene from genomic DNA with primer pair LR0R (5'-ACCCGCTGAACTTAAGC-3') and LR3 (5'-CCGTGTTTCAAGACGGG-3') (Vilgalys & Hester, 1990) and their internal transcribed spacer (ITS) region with primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). Briefly, 1.00 µl of diluted DNA solution was added to 49 µl of PCR reaction mixture as described for primer pair pA/pH, but with primer pairs LR03/LR3 (Integrated DNA Technologies) or ITS1/ITS4 (Integrated DNA Technologies). The thermal cycling reactions consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 1 min, and elongation at 72 °C for 2 min, and was finalized with an elongation at 72 °C for 7 min. PCR amplicons were purified with a Wizard Plus SV Mini-preps DNA purification system (Promega, Madison, WI, USA). The type strains with sequences most similar to the sequenced fragments (expressed as % identity) were determined with the BLAST algorithm (Altschul *et al.*, 1990) and the GenBank database (http://blast.ncbi.nlm.nih.gov/). The accession numbers of their sequences are reported.

2.7 Culture-independent microbial species diversity and community dynamics analysis

The culture-independent microbial species diversity and community dynamics in the water kefir liquors and on the water kefir grains were determined after preparing total DNA extracts from the cell pellets of the water kefir liquors and water kefir grain suspensions, respectively. These cell pellets were obtained by centrifuging (7,200 x *g*, 20 min, 4 °C) of 40 ml of water kefir liquors and 10 ml of water kefir grain suspensions, and discarding the supernatants.

The cell pellets were resuspended in 1 ml of TES buffer, after which the suspensions were centrifuged (21,000 x *g*, 20 min, 4 °C) and the supernatants were discarded. The resulting cell pellets were resuspended in 600 µl of sorbitol buffer supplemented with 30 mM *β*-mercaptoethanol and 200 U of lyticase (Sigma-Aldrich), and incubated at 30 °C for 1 h, after which the suspensions were centrifuged (10,000 x *g*, 10 min) and the supernatants were discarded. Then, the cell pellets were resuspended in 400 µl of STET buffer [8.0 % (m v^{-1}) sucrose, 50 mM Tris-base, 50 mM EDTA, 5.0 % (v v^{-1}) Triton X-100, pH 8.0] supplemented with 12.5 U of mutanolysin (Sigma-Aldrich) and 20 mg ml^{-1} of lysozyme (Merck), and incubated at 37 °C for 1 h. These suspensions were supplemented with 100 µl of 1 mg ml^{-1} of proteinase K solution (Merck) and incubated at 56 °C for 2 h. A pinch of acid washed glass beads (Sigma-Aldrich) and 40 µl of 20 % (m v^{-1}) sodium dodecyl sulfate (SDS) were added, after which the suspensions were vortexed for 60 s, and incubated at 65 °C for 10 min. Finally, the suspensions were supplemented with 515 µl of chloroform:phenol:isoamylalcohol (49.5:49.5:1.0), vortexed, and centrifuged (13,000 x *g*, 5 min). The total DNA extracts obtained were further purified with the Nucleospin® food kit (Macherey-Nagel), according to the instructions of the manufacturer, and the purified total DNA extracts were diluted to approximately 50 ng $µl^{-1}$.

The culture-independent microbial community profiles were obtained by amplifying selected genomic fragments in the total DNA with the universal prokaryotic primer pair 357f-GC (5'-CCTACGGGAGGCAGCAG-3') and 518r (5'-ATTACCGCGGCTGCTGG-3') (V3) (Ercolini *et al.*, 2001), the LAB-specific primer pair LAC1 (5'-AGCAGTAGGGAATCTTCCA-3') and LAC2-GC (5'-ATTYCACCGCTACACATG-3') (LAC) (Walter *et al.*, 2001), the *Bifidobacterium*-specific primer pair bif164f (5'-

GGGTGGTAATGCCGGATG-3’) bif662r-GC (5’-CCACCGTTACACCGGGAA-3’) (Bif) (Satokari *et al.*, 2001), and the universal eukaryotic primer pair NL1-GC (5’-GCCATATCAATAAGCGGAGGAAAAG-3’) and LS2 (5’-ATTCCCAAACAACTCGACTC-3’) (Yeast) (Cocolin *et al.*, 2000). A GC clamp (5’-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3’) was attached to the 5’ end of one primer of each primer pair, as indicated, to ensure incomplete dissociation of the amplified fragments during denaturing gradient gel electrophoresis (DGGE). For the PCR assays, 1.00 µl of diluted DNA solution was added to 49 µl of PCR assay mixture as described for primer pair pA/pH but with the primer pairs V3, LAC, Bif, and Yeast (Integrated DNA Technologies). The thermal cycling reactions consisted of an initial denaturation at 95 °C for 5 min, followed by 30 (V3, LAC, and Bif primer pairs) or 35 (Yeast primer pair) cycles of denaturation at 95 °C for 20 s (V3, LAC, and Yeast primer pairs) or 1 min (Bif primer pair), annealing at 55 °C (V3 primer pair), 61 °C (LAC and Yeast primer pairs), or 58 °C (Bif primer pair) for 45 s, and elongation at 72 °C for 1 min, and was finalized with an elongation at 72 °C for 7 min.

The PCR amplicons were separated in a 6 % (v v^{-1}) polyacrylamide gel, as described previously (Garcia-Armisen *et al.*, 2010; Papalexandratou *et al.*, 2011b). The denaturing gradients of the gels were, from top to bottom, 45-60 % for the V3 and the Yeast primer pairs, 40-55 % for the LAC primer pair, and 45-55 % for the Bif primer pair. Selected bands of the community profiles were cut from the gels, and amplified and sequenced with their respective primer pairs without GC clamps, as described previously (Garcia-Armisen *et al.*, 2010; Papalexandratou *et al.*, 2011b). The type strains with sequences most similar to the sequenced fragments (expressed as % identity) were determined as described above. The accession numbers of their sequences are reported. Finally, although it is not straightforward to correlate band intensities with species abundances, relative comparisons often indicate certain trends.

2.8 Substrate and metabolite concentration determinations

The concentrations of the substrates and metabolites in the water kefir liquors were determined after centrifugation (7,200 x *g*, 20 min, 4 °C) of the sieved water kefir liquors to obtain cell-free supernatants. Quantifications were performed with external calibration curves with standards prepared in the same way as the samples.

Concentrations of sucrose, glucose, and fructose were determined through high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) with a Dionex ICS3000 chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Dionex Carbopac^{TM} PA10 column (Thermo Fisher Scientific) coupled to a Dionex pulsed amperometric detector (Thermo Fisher Scientific). Hereto, 50 µl of cell-free supernatant was added to 950 µl of ultrapure water, and 50 µl of this dilution was added to 950 µl of deproteinization solution [500 µl of acetonitrile (Sigma-Aldrich), 449.5 µl of ultrapure water, and 0.5 µl of 50 g l^{-1} of rhamnose (internal standard; Sigma-Aldrich)]. All samples and standards were vortexed, centrifuged (21,000 x *g*, 20 min, 4 °C), and filtered (0.2-µm pore-size Whatman filters; GE Healthcare Life Sciences, Bucks, UK); after which they were injected (10 µl) into the column and eluted at 1 ml min^{-1} as described before (Janssens *et al.*, 2012). Briefly, the mobile phase consisted of ultrapure water (eluent A), 167 mM NaOH (eluent B), and 500 mM NaOH (eluent C), with the following gradient: 0-18 min, 87 % A, 13 % B, and 0 % C; 18-19 min, linear gradient until 0 % A, 0 % B, and 100 % C; 19-23 min, 0 % A, 0 % B, and 100 % C; 23-24 min, linear gradient until 87 % A, 13 % B, and 0 % C; and 24-28 min, 87 % A, 13% B, and 0 % C.

Concentrations of glycerol and mannitol were determined through HPAEC-PAD with the same Dionex chromatograph and pulsed amperometric detector (Thermo Fisher Scientific) as described above, but equipped with a Dionex Carbopac^{TM} MA1 column (Thermo Fisher Scientific). Hereto, 100 µl of cell-free supernatant was added to 400 µl of ultrapure water, and 100 µl of this dilution was added to 900 µl of deproteinization solution. All samples and standards were vortexed, centrifuged, and filtered as described above, after which they were injected (10 µl) into the column and eluted at 0.4 ml min^{-1} as described before (Wouters *et al.*, 2013a). Briefly, the mobile phase consisted of ultrapure water (eluent A) and 500 mM NaOH (eluent B), with the following gradient: 0-8 min, 50 % A and 50 % B; 8-22 min, linear gradient until 0 % A and 100 % B; 22-39 min, 0 % A and 100 % B; 39-40 min, linear gradient until 50 % A and 50 % B; and 40-50 min, 50 % A and 50 % B.

Concentrations of acetic acid and D- and L-lactic acid were determined through high-performance liquid chromatography with ultraviolet detection (HPLC-UV) with a Waters chromatograph (Waters Corporation, Milford, MA, USA) equipped with a Shodex ORpak CRX-853 column (Showa Denko K.K., Tokyo, Japan) coupled to a UV-detector operating at 253 nm (Waters). Therefore, 250 µl of cell-free supernatant was added to a mixture of 500 µl of acetonitrile and 250 µl of ultrapure water. All samples and standards were vortexed, centrifuged, and filtered as described above; after which they were injected (30 µl) into the column and eluted at 1 ml min^{-1} with 10 % acetonitrile and 90 % 1 mM $CuSO_4$.

Concentrations of ethanol were determined through gas chromatography with flame ionization detection (GC-FID) with a Focus gas chromatograph (Thermo Fisher Scientific) equipped with a Stabilwax-DA column (Restek, Bellefonte, PA, USA) coupled to a flame ionization detector (Thermo Fisher Scientific). Therefore, 100 µl of cell-free supernatant was added to 1100 µl of deproteinization solution [720 µl of acetonitrile, 367.7 µl of ultrapure water, 12 µl of formate, and 0.2 µl of 4-methyl-2-pentanol (internal standard; Sigma-Aldrich)]. All samples and standards were vortexed, centrifuged, and filtered as described above; after which they were injected (1 µl) into the column with a split ratio of 20:1, and eluted at 1 ml min^{-1} as described before (Rimaux *et al.*, 2011). Briefly, hydrogen gas was used as a carrier gas and nitrogen gas was used as a make-up gas, and the injector and detector temperatures were set at 240 °C and 250 °C, respectively. The following temperature gradient was used: 0.0-10.0 min, linear gradient at 10 °C min^{-1} until 140 °C; 10.0-11.8 min, linear gradient at 50 °C min^{-1} until 230 °C; and 11.8-21.8 min, 230 °C.

Concentrations of the aroma compounds in the water kefir liquors were determined through static headspace gas chromatography with mass spectrometry detection (SH-GC-MS) with a 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a DB-WAXetr column (Agilent Technologies) and coupled to a 5973N mass spectrometer (Agilent Technologies). Hereto, 5 ml of cell-free supernatant was brought into a 20-ml glass headspace vial (Gerstel, Mülheim-an-der-Ruhr, Germany) and closed with a magnetic screw cap (18 mm diameter) with a silicon/PTFE septum (Gerstel). Before analysis, 1.5 g of NaCl and 100 µl of internal standard solution [0.5 ml l^{-1} of 4-methyl-2-pentanol (Sigma-Aldrich)] were added. The headspace vials were equilibrated at 40 °C for 30 min at 400 rpm in a MPS2 Gerstel autosampler, after which 1.0 ml of headspace was injected into the column with a split ratio of 5:1 and eluted at 1 ml min^{-1} as described before (Wouters *et al.*, 2013b). Briefly, the needle was kept at 90 °C, the temperature of the transfer tube was kept at 280 °C, and helium gas was used as a carrier gas. The following temperature gradient was used: 0.0-5.0 min, 40 °C; 5.0-9.0 min, linear gradient at 20 °C min^{-1} until 120 °C; 9.0-19.5 min, linear gradient at 10 °C min^{-1} until 225 °C; 19.5-24.5 min, 225 °C. The compounds were identified by comparison of the mass spectra with library data (NIST 08 database, http://www.nist.gov) and of the retention times with those of the reference compounds (if available). All volatile aroma

compounds found in the water kefir fermentation samples taken after 72 h of fermentation were compared with their threshold values as reported in the literature.

2.9 Carbon recovery

The carbon recovery was calculated as the total amount of carbon recovered at a certain sampling time divided by the total amount of carbon recovered at 0 h, and expressed as % (mol mol^{-1}). The total amount of recovered carbon was calculated as the sum of the amount of carbon in the water kefir liquor plus that in the water kefir grains plus that produced as carbon dioxide. The calculation of the carbon recovery was based on the measurements of the water kefir grain wet mass, the water kefir grain dry mass, and the concentrations of sucrose, glucose, fructose, ethanol, glycerol, lactic acid, acetic acid, and mannitol. It was assumed that the water kefir grain density was 1 g cm^{-3}, that the water kefir grain dry mass consisted of pure glucan homopolysaccharides, that the ethanol and acetic acid present in the water kefir grain matrix evaporated during the water kefir grain dry mass determinations, that the ethanol and acetic acid concentrations in the water kefir grain matrix were the same as those in the water kefir liquor, and that the production of ethanol and acetic acid released equimolar amounts of carbon dioxide.

3 Results

3.1 Water kefir grain wet mass, water kefir grain dry mass, and pH

The water kefir grain wet mass increased from 16.4 ± 0.5 to 28.6 ± 0.6 g during the first 24 h of the water kefir fermentation process, which corresponded with a water kefir grain growth of approximately 105 % (Figure 1A). Afterwards, the water kefir grain wet mass remained constant. The water kefir dry mass initially increased from 13.8 ± 0.1 % (m m^{-1}) at 0 h (inoculum not yet added to the WKSM) to 16.7 ± 0.2 % (m m^{-1}) after 3 h of fermentation. Thereafter, the dry mass decreased until it remained stable at 13 to 14 % (m m^{-1}).

The pH of the WKSM was 4.85 ± 0.01, and dropped to 4.26 ± 0.03 after the addition of the water kefir grains at 0 h. After 72 h of fermentation, the pH reached 3.45 ± 0.01, whereafter the pH continued to decrease slowly to reach 3.35 ± 0.01 after 192 h of fermentation (Figure 1B).

3.2 Microbial enumerations

Immediately after the water kefir grain inoculum was added to the WKSM and the bottles were turned gently, the viable counts of the LAB and yeasts in the water kefir liquors and on the water kefir grains plateaued at a certain level and remained constant during the entire fermentation process (Figure 2). The average viable counts of the yeasts in the water kefir liquors and on the water kefir grains were 6.3 ± 0.2 log cfu ml^{-1} of water kefir liquor and 7.4 ± 0.1 log cfu g^{-1} of water kefir grains, respectively, during the entire water kefir fermentation process, and those of the LAB were 6.9 ± 0.1 log cfu ml^{-1} of water kefir liquor and 8.2 ± 0.1 log cfu g^{-1} of water kefir grains, respectively. The viable counts of the AAB could only be quantified (> 30 colonies on the agar medium with the lowest dilution) in the water kefir liquors after 144 and 192 h and on the grains after 192 h of fermentation (Figure 2). No colonies were found on the KAA and VRBG agar media, indicating the absence of enterococci plus streptococci and of *Enterobacteriaceae*, respectively.

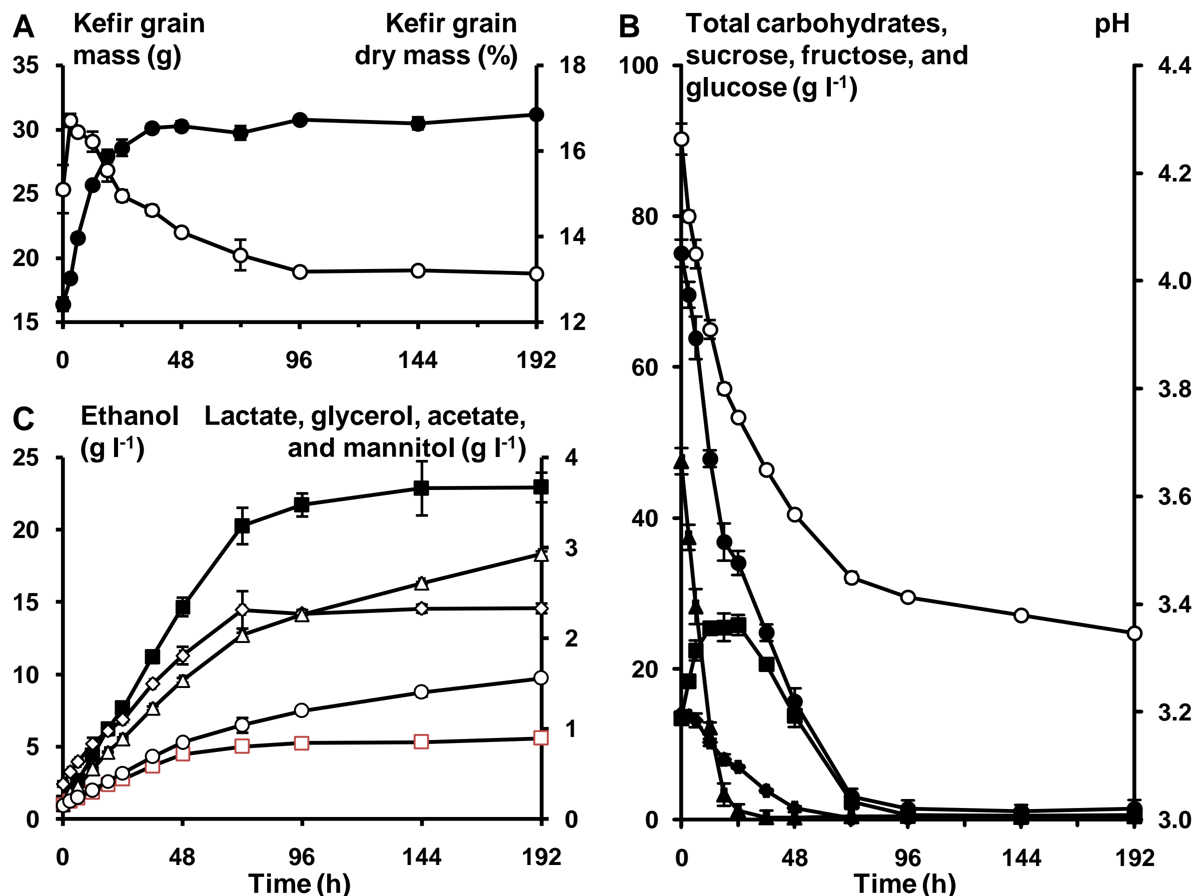

Figure 1. (A) The water kefir grain wet mass (●) and the water kefir grain dry mass (○) as a function of time. (B) The pH (○), and the concentrations of sucrose (▲), fructose (■), glucose (♦), and total carbohydrates (●) as a function of time. (C) The concentrations of ethanol (■), lactate (Δ), glycerol (◊), acetate (○), and mannitol (□) as a function of time (h).

The ratios of the viable counts of the LAB to those of the yeasts remained relatively constant, with averages of approximately 4 and 6 in the water kefir liquors and on the water kefir grains, respectively, during the entire fermentation process. Thus, there were always approximately 2 to 10 LAB cells for each yeast cell, both in the water kefir liquors and on the water kefir grains. The ratios of the viable counts of the LAB and yeasts on the water kefir grains (cfu g^{-1}) to those in the water kefir liquors (cfu ml^{-1}) remained relatively constant too, with averages of approximately 20 and 15, respectively, during the entire fermentation process. Hence, the cell density was 10 to 30 times higher on the water kefir grains than in the water kefir liquors. If the amounts of the water kefir grains and water kefir liquors during the fermentation process were taken into account, the ratios of the total amounts of cells on the water kefir grains (cfu) to those in the water kefir liquors (cfu) remained relatively constant as well, with averages of approximately 9 and 7 for the LAB and the yeasts, respectively, during the entire fermentation process. There were thus 4 to 10 times more microorganisms on the water kefir grains than in the water kefir liquors. However, because the water kefir grain mass, with higher viable counts than the water kefir liquor, increased in mass as a function of time, there was an overall increase of the total cell counts during the first 48 h of the fermentation process.

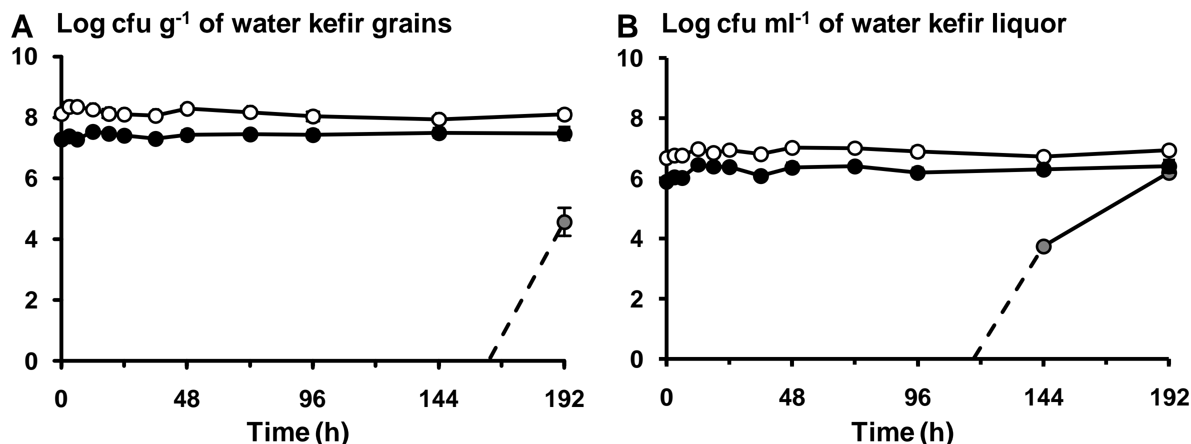

Figure 2. Viable counts of the lactic acid bacteria (○), yeasts (●), and acetic acid bacteria (●) on the water kefir grains (A) and in the water kefir liquors (B) as a function of time (h).

3.3 Culture-dependent microbial species diversity and community dynamics

The culture-dependent microbial species diversity and community dynamics of the LAB and yeasts in the water kefir liquors were more or less similar to those on the water kefir grains (Figure 3). Furthermore, they remained more or less stable during the entire water kefir fermentation process (data not shown).

The main LAB species were *Lactobacillus paracasei*, *Lactobacillus hilgardii*, *Lactobacillus harbinensis*, *Lactobacillus nagelii*, and *Lactobacillus mali*, of which the first three were the most abundant (Figure 3). The relative abundances of *Lb. hilgardii* were higher on the water kefir grains than in the water kefir liquors, and those of *Lb. nagelii* were higher in the liquors than on the grains. All AAB isolates picked up after 192 h of fermentation were identified as *A. fabarum*. The main yeast species were *Saccharomyces cerevisiae* and *Dekkera bruxellensis*, whereby the relative abundance of *D. bruxellensis* was higher in the water kefir liquors than on the water kefir grains.

3.4 Culture-independent microbial species diversity and community dynamics

The rRNA-PCR-DGGE community profiles, obtained with the four different primer pairs used (V3, LAC, Bif, and Yeast), of the water kefir liquors and grains for the three biological replicates after 0, 24, 72, and 192 h of fermentation were similar (data not shown). Furthermore, these community profiles remained more or less stable as a function of time during the entire course of the water kefir fermentation process (Figure 4).

The main bands in the community profiles obtained with the V3 primer pair were attributed to *Lb. paracasei/casei/zeae/rhamnosus*, *Lb. hilgardii/diolivorans*, *Lb. nagelii/ghanensis*, *B. psychraerophilum/crudilactis*, *Lb. mali/hordei*, and *Lb. harbinensis* (Figure 4). A band attributed to the taxon *Acetobacteraceae* was found in the community profiles of the water kefir liquors after 192 h of fermentation (for the three replicates), but not in those of the water kefir grains at that time. The relative intensities of the bands attributed to *Lb. hilgardii/diolivorans* were always higher for the water kefir grains than for the liquors,

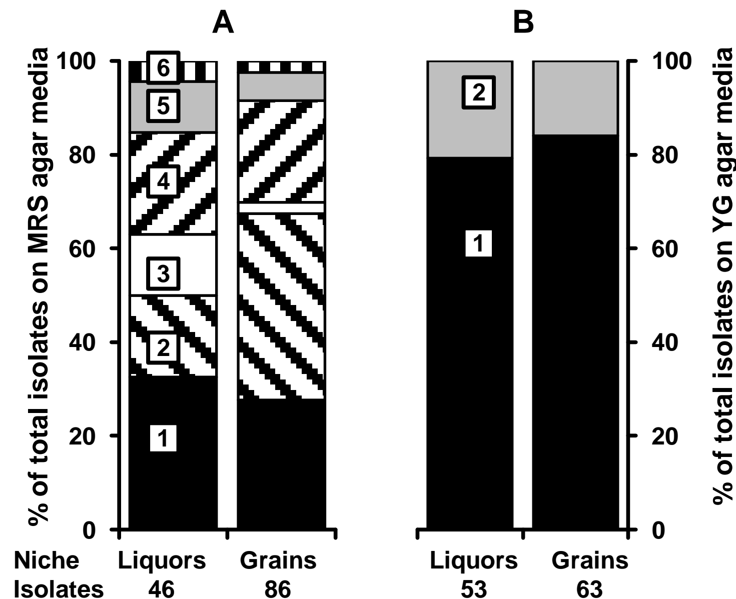

Figure 3. Culture-dependent microbial species diversities and community dynamics of the bacteria and the yeasts in the water kefir liquors and on the water kefir grains, obtained by pooling the samples from the different sampling points. The closest known type strains of the sequenced fragments are given. (A) Isolates on MRS agar media: 1, *Lactobacillus paracasei* (100 % identity, GenBank accession no. AP012541); 2, *Lactobacillus hilgardii* (99 % identity, accession no. LC064898); 3, *Lactobacillus nagelii* (99 % identity, accession no. NR112754); 4, *Lactobacillus harbinensis* (100 % identity, accession no. NR028658); 5, *Acetobacter fabarum* (100 % identity; accession no. NR113556); and 6, *Lactobacillus mali* (99 % identity; accession no. LC064888). (B) Isolates on YG agar media: 1, *Saccharomyces cerevisiae* [large subunit rRNA gene (LSU) (99 % identity, accession no. KC881066) and the internal transcribed spacer (ITS) region (99 % identity, accession no. KC881067)]; and 2, *Dekkera bruxellensis* [LSU (100 % identity, accession no. AY969049) and ITS (100 % identity, accession no. NR111030)]. LSU, 26S large subunit rRNA gene; ITS, internal transcribed spacer region.

and those of the bands attributed to *Lb. harbinensis* and *Lb. mali/hordei* were always higher for the water kefir liquors than for the grains.

The community profiles obtained with the LAC primer pair confirmed the presence of *Lb. paracasei/casei/zeae*, *Lb. hilgardii/diolivorans*, *Lb. nagelii*, *Lb. mali/hordei*, and *Lb. harbinensis*; and those obtained with the Bif primer pair confirmed the presence of *B. psychraerophilum* (98 % identity; Genbank accession no. NR029065) (Figure 4). Furthermore, the partial 16S rRNA gene sequence of this *Bifidobacterium* species in the community profiles obtained with the Bif primer pair was identical (100 % identity, accession no. HE804184) to the sequence of an uncultivated *Bifidobacterium* species found on water kefir grains in Germany (Gulitz *et al.*, 2013).

The main bands in the community profiles obtained with the Yeast primer pair were attributed to *S. cerevisiae* and *D. bruxellensis*, whereby the relative intensities of the bands attributed to *D. bruxellensis* were always higher for the water kefir liquors than for the water kefir grains (Figure 4). Furthermore, the relative intensities of the bands attributed to *D. bruxellensis* increased after 72 and 192 h of fermentation.

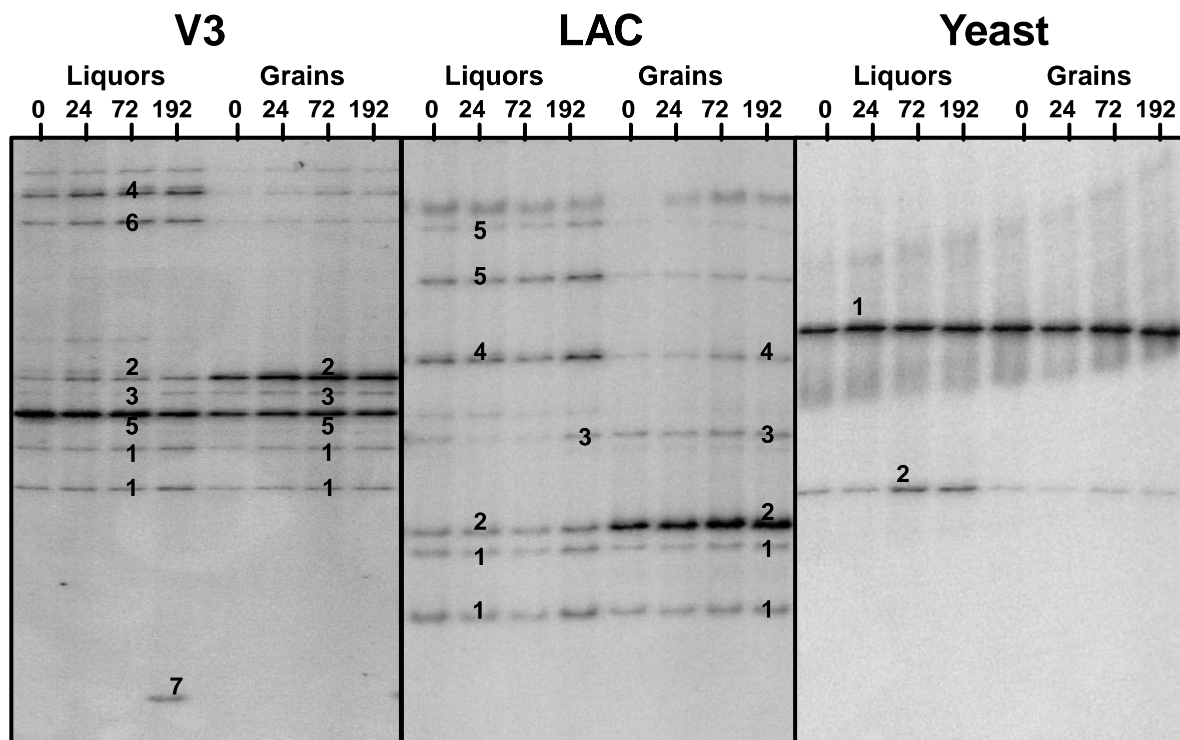

Figure 4. Culture-independent microbial community profiles for the water kefir liquors and the water kefir grains after 0, 24, 72, and 192 h of fermentation. The numbers indicate the bands that were sequenced and the closest known type strains of the sequenced fragments are given. With the V3 primer pair: 1, *Lactobacillus paracasei/casei/zeae/rhamnosus* (100 % identity for all species; GenBank accession no. AP012541/AP012544/NR037122/JQ580982); 2, *Lactobacillus hilgardii/diolivorans* (100 % identity; accession no. LC064898/NR037004); 3, *Lactobacillus nagelii/ghanensis* (99 % identity; accession no. NR112754/NR043896); 4, *Lactobacillus mali/hordei* (99 % identity; accession no. LC064888/NR044394); 5, *Bifidobacterium psychraerophilum/crudilactis* (98 % identity; accession no. NR029065/NR115342); 6, *Lactobacillus harbinensis* (100 % identity; accession no. NR113969). and 7, *Acetobacteriaceae* (100 % identity). With the LAC primer pair: 1, *Lb. paracasei/casei/zeae* (100 % identity; accession no. AP012541/AP012544/NR037122); 2, *Lb. hilgardii/diolivorans* (99 % identity; accession no. LC064898/NR037004); 3, *Lb. nagelii* (99 % identity; accession no. NR119275); 4, *Lb. mali/hordei* (99 % identity; accession no. LC064888/NR044394); and 5, *Lb. harbinensis* (100 % identity; accession no. NR113969). With the Yeast primer pair: 1, *Saccharomyces cerevisiae* (100 % identity; accession no. KC881066); and 2, *Dekkera bruxellensis* (100 % identity; accession no. AY969049).

3.5 Substrate consumption and metabolite production profiles

Sucrose was the main substrate present at the start of the fermentation (0 h), and its concentration decreased fast from 47.5 ± 1.7 g l^{-1} at 0 h to 1.2 ± 0.8 g l^{-1} after 24 h of fermentation (Figure 1). The concentrations of fructose increased due to the consumption of sucrose and reached a maximum after 24 h of fermentation. In contrast, the concentrations of glucose decreased continuously during the fermentation. After 72 h, most of the carbohydrates were consumed, with only 3.1 ± 1.0 g l^{-1} of total carbohydrates left of the initial 75.1 ± 2.1 g l^{-1}. The concentrations of ethanol, glycerol, lactic acid, acetic acid, and mannitol

increased linearly during the first 72 h of fermentation, and reached 20.3 ± 1.3, 2.31 ± 0.21, 2.03 ± 0.03, 1.0 ± 0.1, and 0.8 ± 0.1 g l^{-1}, respectively.

The main aroma compounds (besides acetic acid and ethanol) found in the static headspaces of the water kefir liquors were ethyl acetate, 2-methyl-1-propanol, isoamyl alcohol, isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate (Figure 5). Ethyl butanoate and ethyl 2-methyl-butanoate were also found, but their concentrations were below the limit of quantification. The major part of the aroma compounds was produced during the first 72 h of fermentation, but the production of ethyl acetate and ethyl decanoate continued until 192 h (Figure 5). Considering their threshold levels, the aroma compounds with the highest impact on the aroma of the water kefir liquors after 72 h of fermentation were probably the esters (Table 1). In particular, the concentration of ethyl octanoate was approximately 688 times its threshold concentration.

3.6 Carbon recovery

After 192 h of fermentation, the carbon recovery was approximately 101 %, indicating that all major substrates and metabolites were recovered from the water kefir fermentation process studied.

4 Discussion

The multiphasic microbial approach of the present study revealed that LAB, yeasts, and bifidobacteria were the main microorganisms present during the water kefir fermentation process. The LAB were present in higher numbers than the yeasts. As the viable counts of the AAB became only quantifiable after 144 h of fermentation, the AAB were thus not abundant during the water kefir fermentation process studied. The viable counts of AAB reported in

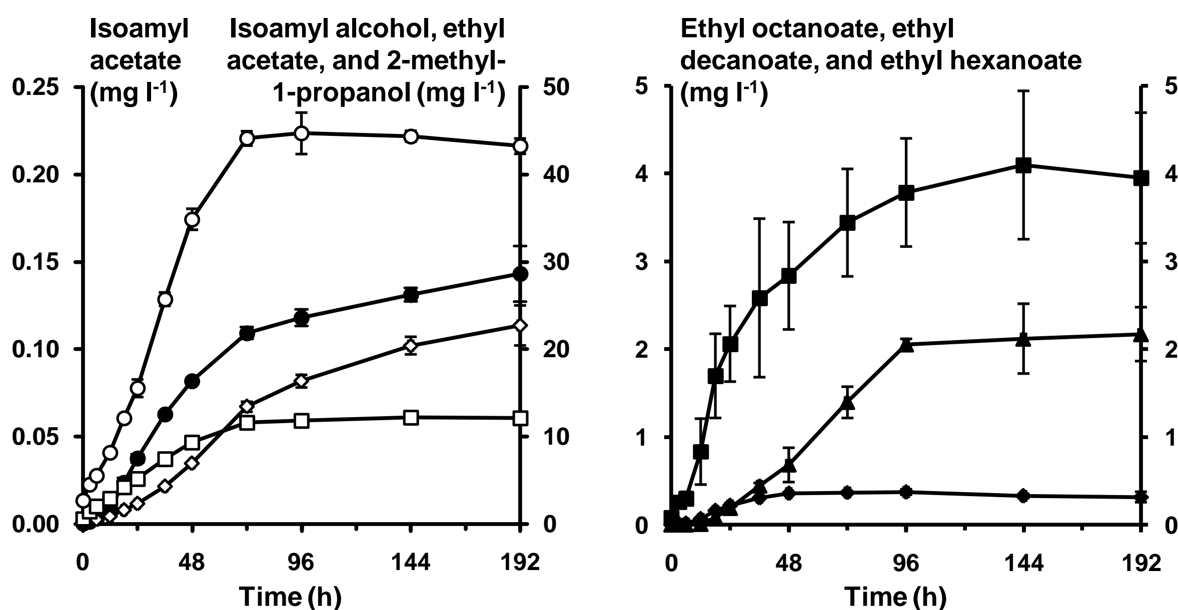

Figure 5. Concentrations of 2-methyl-1-propanol (□), isoamyl alcohol (○), ethyl acetate (◊), isoamyl acetate (●), ethyl hexanoate (♦), ethyl octanoate (■), and ethyl decanoate (▲) in the water kefir liquors as a function of time (h).

Table 1. The concentrations and Kovats indices (KI) of the aroma compounds found in the water kefir liquors after 72 h of fermentation. The threshold values and aroma descriptors are given for each compound (Corison *et al.*, 1979; Guth, 1997; Ferreira *et al.*, 2000; Lambrechts & Pretorius, 2000; Mamede *et al.*, 2005; Molina *et al.*, 2009).

Aroma compound	Concentration after 72 h (mg l^{-1})	KI	Threshold value (mg l^{-1})	Aroma description
2-Methyl-1-propanol	11.62 ± 0.05	1097	40	Spirituous, fuel
Isoamyl alcohol	44.13 ± 0.82	1222	30	Harsh, nail polish remover
Ethyl acetate	13.40 ± 0.58	831	7.5	Fruity
			> 150	Varnish, nail polish remover
Isoamyl acetate	0.11 ± 0.01	1141	0.03	Sweet, fruity, banana, pear
Ethyl hexanoate	0.37 ± 0.01	1250	0.014	Fruity, apple, banana, violets
Ethyl octanoate	3.44 ± 0.61	1450	0.005	Fruity, pineapple, pear
Ethyl decanoate	1.40 ± 0.18	1659	0.2	Floral

water kefir usually range from negligible (Franzetti *et al.*, 1998) to > 8 log cfu ml^{-1} (Gulitz *et al.*, 2011), and this wide variation is probably related with the presence or absence of oxygen during the water kefir fermentation process. The absence of enterococci plus streptococci, and *Enterobacteriaceae* was to be expected, considering the fast decrease of the pH during water kefir fermentation to < 3.5.

The density of the water kefir microorganisms was higher on the water kefir grains than in the water kefir liquors. In addition, the water kefir grains harbored the majority of the water kefir microorganisms during the entire water kefir fermentation process. The latter explained the absence of an increase in the viable counts of the water kefir microorganisms in the water kefir liquors or on the water kefir grains during the water kefir fermentation process.

The microbial species diversities in the water kefir liquors and on the water kefir grains were more or less similar and remained more or less stable during the entire water kefir fermentation process. The main LAB species (in decreasing order) in the water kefir fermentation process studied were *Lb. paracasei*, *Lb. hilgardii*, and *Lb. harbinensis*. *Lactobacillus paracasei* is a facultatively heterofermentative LAB species, which is frequently associated with water kefir fermentation (Waldherr *et al.*, 2010; Gulitz *et al.*, 2011; Marsh *et al.*, 2013b). It is also associated with the human oral ecosystem, the human intestinal tract, and raw and fermented dairy and vegetable products (Cai *et al.*, 2007). Some strains of *Lb. paracasei* show probiotic potential (Galdeano & Perdigon, 2006), which makes water kefir a possible source of novel probiotic *Lb. paracasei* strains. *Lactobacillus hilgardii* is an obligately heterofermentative LAB species, which is assumed to be responsible for the production of water kefir grain EPS during water kefir fermentation (Pidoux, 1989; Pidoux *et al.*, 1990; Leroi & Pidoux, 1993; Waldherr *et al.*, 2010). It is also regularly found in wine and cocoa fermentations (Rodriguez & Denadra, 1995; Ardhana & Fleet, 2003). However, not all *Lb. hilgardii* strains isolated from water kefir produce EPS (Gulitz *et al.*, 2011). Furthermore, other LAB species frequently isolated from water kefir can produce EPS, such as *Leuconostoc mesenteroides*, *Lactobacillus brevis*, *Lb. casei*, *Lb. nagelii*, and *Lb. hordei* (Pidoux *et al.*, 1988; Gulitz *et al.*, 2011). *Lactobacillus harbinensis* is a facultatively heterofermentative LAB species, and to our knowledge, this is the first time that this LAB species was found in water kefir. It was first isolated from a Chinese vegetable fermentation (Miyamoto *et al.*, 2005), and has since been found in French cow milk (Delavenne *et al.*, 2013), the human oral ecosystem (Lonnermark *et al.*, 2012), Parmigiano Reggiano cheese (Solieri *et al.*, 2012), and

sorghum sourdough fermentations (Sekwati-Monang *et al.*, 2012). It is worth to notice that *Lb. harbinensis* can produce antifungal compounds that inhibit yeasts (Delavenne *et al.*, 2013; Belguesmia *et al.*, 2014).

The water kefir of the present study also harbored *Lb. nagelii*, a homofermentative LAB species frequently found in water kefir (Gulitz *et al.*, 2011, 2013; Stadie *et al.*, 2013) and wine fermentations (Edwards *et al.*, 2000), and *Lb. mali*, a homofermentative LAB species frequently found in cider and apple juice (Carr & Davies, 1970). The detection of a *Bifidobacterium* species closely related to *B. psychraerophilum* confirms a recent finding that water kefir harbors bifidobacteria (Hsieh *et al.*, 2012; Gulitz *et al.*, 2013; Marsh *et al.*, 2013b). The latter species was first isolated from a porcine cecum (Simpson *et al.*, 2004). However, the *Bifidobacterium* species found in the water kefir fermentation process of the present study may represent a novel species, as its partial 16S rRNA gene sequence obtained was only 98 % identical to that of its closest known type strains, but it was 100 % identical to the 16S rRNA gene sequence of an uncultivated *Bifidobacterium* species found in water kefir grains in Germany (Gulitz *et al.*, 2013). Bifidobacteria usually produce more acetate than lactate, and the low acetate concentrations in the water kefir liquors indicated that their metabolic activity was probably limited during the water kefir fermentation process of the present study.

The most abundant yeast species was *S. cerevisiae*, which is frequently associated with water kefir (Franzetti *et al.*, 1998; Magalhães *et al.*, 2010; Waldherr *et al.*, 2010; Gulitz *et al.*, 2011; Miguel *et al.*, 2011; Hsieh *et al.*, 2012; Marsh *et al.*, 2013b; Diosma *et al.*, 2014) and with bread, beer, and wine fermentations (Lambrechts & Pretorius, 2000; Picinelli *et al.*, 2000). *Dekkera bruxellensis* (anamorph *Brettanomyces bruxellensis*) was less abundant during the water kefir fermentation process of the present study, and was only recently found in water kefir (Hsieh *et al.*, 2012; Marsh *et al.*, 2013b). This yeast species is a key microorganism during the spontaneous fermentation of typical Belgian-style acidic ales (Martens *et al.*, 1997; Spitaels *et al.*, 2014, 2015b), although it is usually associated with spoilage of beer and wine (Wedral *et al.*, 2010). Whether the presence of *D. bruxellensis* during water kefir fermentation has a positive or negative influence on the end-product is unclear at this moment.

The wide metabolite target analysis of the present study elucidated the substrate consumption and metabolite production profiles of the microbial consortium described above. Sucrose is necessary for the production of homopolysaccharides (Monsan *et al.*, 2001), and water kefir grain wet mass was indeed produced as long as sucrose was present. Further, the accumulation of fructose in the water kefir liquors indicated that the water kefir grains were composed of glucans, as has been found previously (Horisberger, 1969). The water kefir grain EPS did probably not serve as a reserve polymer, as the water kefir grain wet and dry masses did not decrease noticeably upon prolonged fermentation. Nevertheless, the concentrations of lactic acid and acetic acid continued to increase after all carbohydrates were exhausted.

Although there were always approximately 2 to 10 LAB cells for every yeast cell, the yeasts produced the majority of the metabolites during the water kefir fermentation process. The main end-products of the yeast metabolism were ethanol, glycerol, and carbon dioxide. Glycerol is a slightly sweet molecule that may slightly increase the viscosity of a fermented beverage, but does not seem to have a direct influence on the taste and aroma of fermented beverages (Picinelli *et al.*, 2000). The main metabolites of the LAB metabolism were lactic acid and acetic acid. Lactic acid contributes a mild and refreshing acidic taste, whereas acetic acid contributes a harsh acidic taste. Acetic acid is also produced during wine and beer fermentations and is undesirable at high concentrations (Lambrechts & Pretorius, 2000). Despite the high concentrations of fructose at the initial stage of the fermentation process, the production of mannitol from fructose by heterofermentative LAB species remained limited

(Zaunmüller *et al.*, 2006). Mannitol has a fresh sweet taste and possesses antioxidant activity (Shen *et al.*, 1997), and might thus be desirable in water kefir beverages. In addition, bifidobacteria and yeasts may contribute to acetate production.

All esters and higher alcohols found in the water kefir liquors are associated with yeast metabolism and are also found in wine and beer (Lambrechts & Pretorius, 2000). For instance, hexanoic acid, octanoic acid, and decanoic acid, necessary for the production of the corresponding ethyl esters, originate from the fatty acid biosynthesis pathway in yeasts. However, a direct comparison of water kefir liquor with beer or wine is difficult because of the multitude of interactions between the metabolites in the different fermented beverages. This also makes it difficult to estimate the impact of individual aroma compounds on the overall taste and aroma. Considering the threshold values of the different aroma compounds, the esters isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate, which contribute fruity and floral aromas, will probably exert the greatest influence on the aroma of the water kefir liquors of the present study.

In conclusion, a sound water kefir fermentation with good water kefir grain growth was obtained during the present study, which can be used as reference for other water kefir fermentations. The water kefir grain mass increased as long as sucrose was present. The viable counts of the LAB and yeasts in the water kefir liquors and on the water kefir grains remained stable and the majority of the microorganisms remained present on the water kefir grains during the entire fermentation process. Also, the microbial species diversity remained more or less stable during the entire water kefir fermentation process. The main LAB and yeasts were *Lb. paracasei*, *Lb. harbinensis*, *Lb. hilgardii*, *S. cerevisiae*, and *D. bruxellensis*. Additionally, a non-identified *Bifidobacterium* species was detected during the entire water kefir fermentation process. The viable counts of the LAB were higher than the yeasts, but the yeasts produced the major part of the metabolites. The main metabolites produced during the fermentation were ethanol, carbon dioxide, lactic acid, glycerol, and acetic acid. The main aroma compounds in the water kefir liquors of the present study were isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate.

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CHAPTER 4

The water kefir grain inoculum influences the water kefir grain growth, the microbial species diversity, the fermentation rate, and the metabolite concentrations during water kefir fermentation

David Laureys and Luc De Vuyst

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SUMMARY

Three water kefir fermentation processes were started with different water kefir grain inocula to investigate the influence of the inoculum on the characteristics of the resulting water kefir fermentation. The water kefir grain inoculum determined the water kefir grain growth, the viable counts on the water kefir grains, the time until total carbohydrate exhaustion, and the final metabolite concentrations. There were always 2-10 lactic acid bacterial cells for every yeast cell and the majority of these microorganisms was always associated with the water kefir grains. *Lactobacillus paracasei*, *Lactobacillus hilgardii*, *Lactobacillus nagelii*, and *Saccharomyces cerevisiae* were present in all water kefir fermentation processes of the present study, and may be the key microorganisms during water kefir fermentation. Furthermore, the inoculum determined the presence of a non-identified *Bifidobacterium* species, *Dekkera bruxellensis*, *Lactobacillus satsumensis*, *Lactobacillus harbinensis* and *Leuconostoc mesenteroides*, but these microorganisms were not necessary during water kefir fermentation. Low water kefir grain growth was not caused by the absence of exopolysaccharide-producing lactic acid bacteria, but was associated with small grains with high viable counts of microorganisms, fast fermentation, and low pH values during the fermentation.

1 Introduction

Water kefir is a fermented beverage that is drunk worldwide and is believed to possess health-promoting properties (Marsh *et al.*, 2014a; Pothakos *et al.*, 2016). It is made by adding water kefir grains (the inoculum) to a mixture of water, (dried) fruits, and sugar (Gulitz *et al.*, 2011; Marsh *et al.*, 2013b; Laureys & De Vuyst, 2014; Chapter 3). This mixture is fermented for 2 to 4 days at room temperature under anaerobic conditions, after which the water kefir grains are separated from the water kefir liquor by sieving. The liquor is a slightly sweet, acidic, alcoholic, and sparkling beverage that has a yellowish color and a fruity taste and aroma. The grains are brittle and consist of dextran expolysaccharides (EPS) onto which the water kefir microorganisms are attached (Horisberger, 1969; Moinas *et al.*, 1980; Laureys & De Vuyst, 2014; Chapter 3). The water kefir grains obtained after sieving are reused for a next water kefir fermentation process through a backslopping practice.

The main groups of microorganisms found in water kefir are lactic acid bacteria (LAB), yeasts, bifidobacteria, and acetic acid bacteria (AAB) (Magalhães *et al.*, 2010, 2011; Waldherr *et al.*, 2010; Gulitz *et al.*, 2011, 2013; Laureys & De Vuyst, 2014; Chapters 3). Different water kefirs harbor different species diversities, but it is still unclear which are the key microorganisms during a water kefir fermentation process and how the species diversity influences the fermentation process. The LAB species *Lactobacillus hilgardii* is frequently associated with water kefir fermentation and is assumed to be responsible for the water kefir grain growth because of its production of EPS from sucrose (Pidoux, 1989; Waldherr *et al.*, 2010). Recently, a non-identified *Bifidobacterium* species was found in water kefir, but its impact on water kefir fermentation remains unclear (Gulitz *et al.*, 2013; Laureys & De Vuyst, 2014; Chapter 3). The main metabolites produced during water kefir fermentation are ethanol, lactic acid, glycerol, acetic acid, and mannitol; the main aroma compounds are 2-methyl-1-propanol, isoamyl alcohol, ethyl acetate, isoamyl acetate, ethyl hexanoate, and ethyl octanoate (Laureys & De Vuyst, 2014; Chapter 3).

Currently, the water kefir beverage is predominantly produced at household level, whereby water kefir grains are handed over from person to person. This practice is possible because the water kefir grain mass normally increases upon water kefir fermentation. Commercial water kefir is not widely available, because the fermentation process is difficult to control. For example, the fermentation process can become unstable, which yields variable end-products. Also, the water kefir grain growth often decreases, which prevents successful backslopping or upscaling of the production process. To be able to avoid and/or remedy these common problems during water kefir fermentation and allow the development of a stable water kefir production process for commercial purposes, a thorough understanding of the water kefir fermentation process is required.

The comparative study of this chapter aimed to elucidate the influence of the water kefir grain inoculum on the microbial species diversity, community dynamics, pH evolution, water kefir grain growth, substrate consumption profile, and metabolite production course during water kefir fermentation, and to make potential associations between certain of these fermentation characteristics.

2 Materials and methods

2.1 Water kefir grain inocula and prefermentations

Three water kefir grain inocula (A, B, and C) were obtained from different private persons, who maintain a household water kefir fermentation process (Table 1). Each of these

Table 1. Approximate recipes and characteristics of the household water kefir fermentation processes maintained by the private persons, from which the water kefir grain inocula A, B, and C for the present comparative study were obtained. The concentrations are given per liter of water used in the recipe.

Characteristic	Water kefir grain inoculum		
	A	B	C
Origin	Leuven, Belgium	Schiedam, The Netherlands	Lokeren, Belgium
Water kefir grains (g l^{-1})	90	100	250
Sugar (g l^{-1})	110	90	60
Fruits and other ingredients (l^{-1})	2 dried figs 3 dried apricots 2 ml of apple cider vinegar	1 dried fig 20 g of raisins 1 slice of peeled lemon	2 dried figs
Fermentation conditions	15 °C, 2 d	19 °C, 3 d	20 °C, 3 d
Estimated grain growth (%)	20	25	50

water kefir grain inocula (100 g) was cultivated through a series of consecutive prefermentations through backslopping to obtain > 600 g of water kefir grain wet mass. The prefermentations were performed in glass bottles (1, 2, and 5 l) equipped with a polytetrafluoroethylene (PTFE) water lock. They were started by adding 85 ml of autoclaved (121 °C, 2.1 bar, 21 min) water kefir simulation medium (WKSM) per 15 g of water kefir grains. The WKSM contained 6 g of unrefined cane sugar (Candico Bio, Merksem, Belgium), 65 ml of distilled water, and 20 ml of fig extract. The fig extract was prepared as described in Chapter 3. The bottles were incubated in a water bath at 21 °C. Every 3 days, the backslopping practice was applied, whereby the water kefir grains were separated from the water kefir liquor by sieving and recultivated in fresh WKSM and under the same conditions as described above.

2.2 Fermentations

Each of the three water kefir grain inocula, obtained through a series of prefermentations as mentioned above, was used to start a water kefir fermentation process in triplicate. The fermentations were performed in 100-ml glass bottles equipped with a PTFE water lock (several bottles per fermentation), and were started by adding 15 g of water kefir grain inoculum to 85 ml of autoclaved (121 °C, 2.1 bar, 21 min) WKSM. The 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 of the fermentation process and before their sampling.

2.3 Analyses

For water kefir fermentation processes B and C, three fermentation bottles (representing three independent biological replicates) were removed and their contents analyzed after 0, 6, 12, 18, 24, 48, 72, 96, 144, and 192 h of fermentation. For water kefir fermentation process A, only one fermentation bottle was removed and its contents analyzed in triplicate (representing three technical replicates) after 0, 24, and 72 h of fermentation; and three fermentation bottles (representing three independent biological replicates) were removed and their contents analyzed after 48 h of fermentation, because the grain wet mass of water kefir grain inoculum A did not increase during the prefermentations.

The pH, the water kefir grain wet mass, the water kefir grain dry mass, the viable counts of the LAB and yeasts in the water kefir liquors and on the rinsed water kefir grains, and the concentrations of the substrates and metabolites in the water kefir liquors were determined at each sampling point. The viable counts of the *Enterobacteriaceae* and the enterococci plus streptococci in the water kefir liquors and on the water kefir grains were determined after 0 and 72 h of fermentation. The culture-dependent microbial species diversity and community dynamics of the LAB and yeasts in the water kefir liquors and on the rinsed water kefir grains of water kefir fermentation processes B and C were determined after 0, 48, and 192 h; and those in the water kefir liquors and on the rinsed water kefir grains of water kefir fermentation process A after 0 and 48 h of fermentation. The culture-independent microbial species diversity and community dynamics of water kefir fermentation processes B and C were determined after 0, 24, 48, 72, and 192 h, and those of water kefir fermentation process A after 0, 24, 48, and 72 h of fermentation. The relative abundances of minor aroma compounds in the water kefir liquors were determined when the residual total carbohydrate concentrations were < 1 g l^{-1}.

The results are presented as the mean ± standard deviation of the three independent biological replicates performed for each sampling point, except for water kefir fermentation process A, as indicated above.

2.4 pH and water kefir grain wet and dry mass 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 in Chapter 3. Additionally, the water kefir grains were assessed visually at each sampling point.

2.5 Microbial enumerations

The viable counts of the presumptive LAB were determined on de Man-Rogosa-Sharpe (MRS) agar medium, those of the presumptive yeasts on yeast extract-glucose (YG) agar medium, those of the presumptive enterococci plus streptococci on kanamycin-aesculin-azide (KAA) agar medium, and those of the presumptive *Enterobacteriaceae* on violet-red-bile-glucose (VRBG) agar medium, as described in Chapter 3.

2.6 Culture-dependent microbial species diversity and community dynamics analyses

The culture-dependent microbial species diversity and community dynamics analyses of the LAB and yeasts in the water kefir liquors and on the water kefir grains were determined by randomly picking up 10 to 20 % of the total number of colonies from the agar media with 30 to 300 colonies. DNA extracts of the isolates were prepared and used for $(GTG)_5$-PCR fingerprinting in the case of bacteria and M13-PCR fingerprinting in the case of yeasts, as described in Chapter 3. The fingerprint patterns obtained were clustered numerically. Representative bacterial isolates within each cluster were identified by sequencing part of their 16S rRNA gene from genomic DNA, as described in Chapter 3. Representative yeast isolates within each cluster were identified by sequencing of their 26S large subunit (LSU) rRNA gene and internal transcribed spacer (ITS) region from genomic DNA, as described in Chapter 3. The culture-dependent microbial species diversity of each water kefir was presented after pooling the microbial species diversities at each sampling point.

2.7 Exopolysaccharide production

All bacterial isolates were grown on MRS agar medium supplemented with 10 g l^{-1} of sucrose at 30 °C for 7 days to visually assess their EPS production capacity.

2.8 Culture-independent microbial species diversity and community dynamics analyses

The culture-independent microbial species diversity and community dynamics in the water kefir liquors and on the water kefir grains were determined after preparing total DNA extracts from the cell pellets of water kefir liquors and water kefir grain suspensions, respectively, as described in Chapter 3.

The culture-independent microbial community profiles were obtained by amplifying selected genomic fragments in the total DNA with the universal prokaryotic primer pair (V3), the LAB-specific primer pair (LAC), the *Bifidobacterium*-specific primer pair (Bif), and the universal eukaryotic primer pair (Yeast); and separating the PCR amplicons through denaturing gradient gel electrophoresis (DGGE), as described in Chapter 3. Selected bands of the community profiles were cut from the gels and identities were assigned through sequencing, as described in Chapter 3.

2.9 Substrate and metabolite concentration determinations

Samples for substrate and metabolite concentration determinations were prepared as described in Chapter 3. Concentrations of sucrose, glucose, fructose, glycerol, and mannitol were determined through high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), those of D- and L-lactic acid and acetic acid through high-performance liquid chromatography with ultraviolet detection (HPLC-UV), those of ethanol through gas chromatography with flame ionization detection (GC-FID), and those of the aroma compounds through static headspace gas chromatography with mass spectrometry detection (SH-GC-MS), as described in Chapter 3.

The relative abundances of the aroma compounds in the water kefir liquors were determined after a solid phase microextraction of the water kefir liquor headspace with a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco, Bellefonte, PA, USA) and gas chromatography with mass spectrometry detection (HS/SPME-GC-MS). Samples were prepared as for HS-GC-MS described in Chapter 3. The SPME fiber was equilibrated in the water kefir liquor headspace at 40 °C for 30 min at 400 rpm in a MPS2 autosampler (Gerstel, Mülheim-an-der-Ruhr, Germany) before desorption and injection of the compounds from the SPME fiber into the column with a split ratio of 50:1, and elution at 1 ml min^{-1} as described before (Leroy *et al.*, 2009). Briefly, the compounds were desorbed from the fiber at 220 °C for 4 min, helium was used as a carrier gas, and the temperature of the transfer tube was kept at 280 °C. The following temperature gradient was used: 0.0-5.0 min, 40 °C; 5.0-23.5 min, linear gradient at 10 °C min^{-1} until 225 °C; 23.5-28.5 min, 225 °C. The compounds were identified by comparison of the mass spectra with library data (NIST 08 database, http://www.nist.gov) and of the retention times with those of reference compounds (if available). Relative abundances were calculated by normalization of the peak areas with those of the internal standard (4-methyl-2-pentanol; Sigma-Aldrich) and multiplication with a factor of 1000.

2.10 Composition analysis of the water kefir grain exopolysaccharides

The composition of the water kefir grain EPS was determined after rinsing the water kefir grains five times in ultrapure water to remove all soluble compounds. Hereto, 5.0 g of water kefir grain wet mass was supplemented with 45 ml of ultrapure water and mixed by inversion for 5 min, after which the mixture was centrifuged (7,200 x *g* for 15 min) and the supernatant was discarded. For acid hydrolysis, 0.50 g of water kefir grain wet mass was supplemented with 5.0 ml of 2.0 M HCl or 2.0 M H_2SO_4 (Emaga *et al.*, 2012). These mixtures were incubated at 100 °C for 1, 2, 4, and 6 h.

The carbohydrates in the resulting solutions were determined through HPAEC-PAD, as described above, but without adding the internal standard. The organic acids in the resulting solutions were measured through HPLC-UV, as described above; and through HPAEC with the same Dionex chromatograph (Thermo Fisher Scientific) as mentioned above, but equipped with a Dionex IonPac^{TM} AS19 column (Thermo Fisher Scientific) and coupled to a Dionex conductivity under ion suppression (CIS) detector (Thermo Fisher Scientific), as described before (Moens *et al.*, 2014).

2.11 Carbon recovery

The carbon recovery was calculated as the total amount of carbon recovered at a certain sampling time divided by the total amount of carbon recovered at 0 h, and was expressed as % (mol mol^{-1}), as described in Chapter 3.

2.12 Statistics

An ANOVA was performed in R 3.2.0 to test for differences between the water kefir fermentation processes, followed by a series of post-hoc pairwise comparisons with Fisher's least significant difference (LSD) test (de Winter, 2013). Two-tailed Spearman correlation coefficients between test variables were calculated in R 3.2.0. All statistical tests were performed with a significance level of 0.05.

3 Results

3.1 Water kefir grain wet mass, water kefir grain dry mass, and pH

The water kefir grain growth of water kefir A was very low at the end of all prefermentations (Figure 1). Consequently, only a small amount of water kefir grain wet mass was available to start the subsequent fermentations for water kefir fermentation process A. The water kefir grain growth of water kefir fermentation process B was low at the end of the first prefermentation, increased until prefermentation 5, and decreased afterwards. The water kefir grain growth of water kefir C was high at the end of the first prefermentation, and gradually decreased afterwards.

The water kefir grain growth during the water kefir fermentation processes was in line with the water kefir grain growth during their prefermentations (Figure 1). The water kefir grain growth during water kefir fermentation process A remained very low compared to that of water kefir fermentation processes B and C (Figure 2). The water kefir grain growth during water kefir fermentation processes B and C remained comparable during the first 48 h of fermentation, after which it stopped in water kefir B and continued in water kefir C until 144 h of fermentation (Figure 2). The water kefir grain growth always stopped when sucrose was

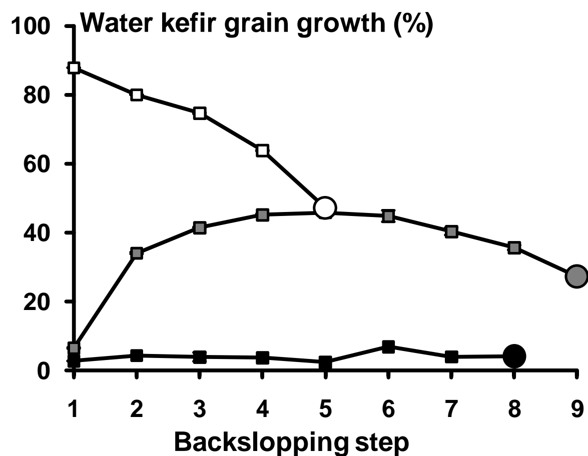

Figure 1. The water kefir grain growth after 72 h of fermentation (%) during the prefermentations of water kefir grain inocula A (■), B (■), and C (□); and during the fermentation processes inoculated with water kefir grain inocula A (●), B (●), and C (○).

depleted. When the total carbohydrate concentrations were < 1 g l^{-1}, the water kefir grain growth was significantly different between the three water kefir fermentation processes (Table 2). The water kefir grain dry mass initially increased during all three water kefir fermentation processes, after which it decreased along with the decrease of the total carbohydrate concentrations to reach a stable value when the latter were < 1 g l^{-1}. Although the water kefir grain dry mass of the water kefir grains was significantly lower in water kefir A than in water kefirs B and C, the differences were small (Table 2). The water kefir grains of water kefir A were noticeably smaller and less transparent than those of water kefirs B and C.

The pH of the WKSM (before inoculation) was 4.82 ± 0.02. The pH of the water kefir liquor decreased fastest during water kefir fermentation process A and slowest during water kefir fermentation process B (Figure 2). After this fast initial decrease, the pH continued to decrease slowly, along with the continued increase of the lactic acid and acetic acid concentrations until the end of the fermentation processes.

3.2 Microbial enumerations

Immediately after the water kefir grain inoculum was added to the WKSM and the bottles were turned gently, the viable counts of the LAB and the yeasts in the water kefir liquors and on the water kefir grains plateaued at a certain level and remained stable during the entire water kefir fermentation processes. When the total residual carbohydrate concentrations were < 1 g l^{-1}, they were representative for the entire water kefir fermentation processes (Table 3). The viable counts of the LAB and yeasts on the water kefir grains were significantly different between the three water kefir fermentation processes, being highest on the water kefir grains of water kefir A. This was also reflected in the viable counts in the water kefir liquors, albeit less pronounced. When the total residual carbohydrate concentrations were < 1 g l^{-1}, the water kefir grain growth correlated negatively with the viable counts of the LAB (– 0.945; $p < 0.001$) and the yeasts (– 0.963; $p < 0.001$) on the water kefir grains, but not with those in the water kefir liquors. No colonies were found on the KAA and VRBG agar media, indicating the absence of enterococci plus streptococci and *Enterobacteriaceae*, respectively.

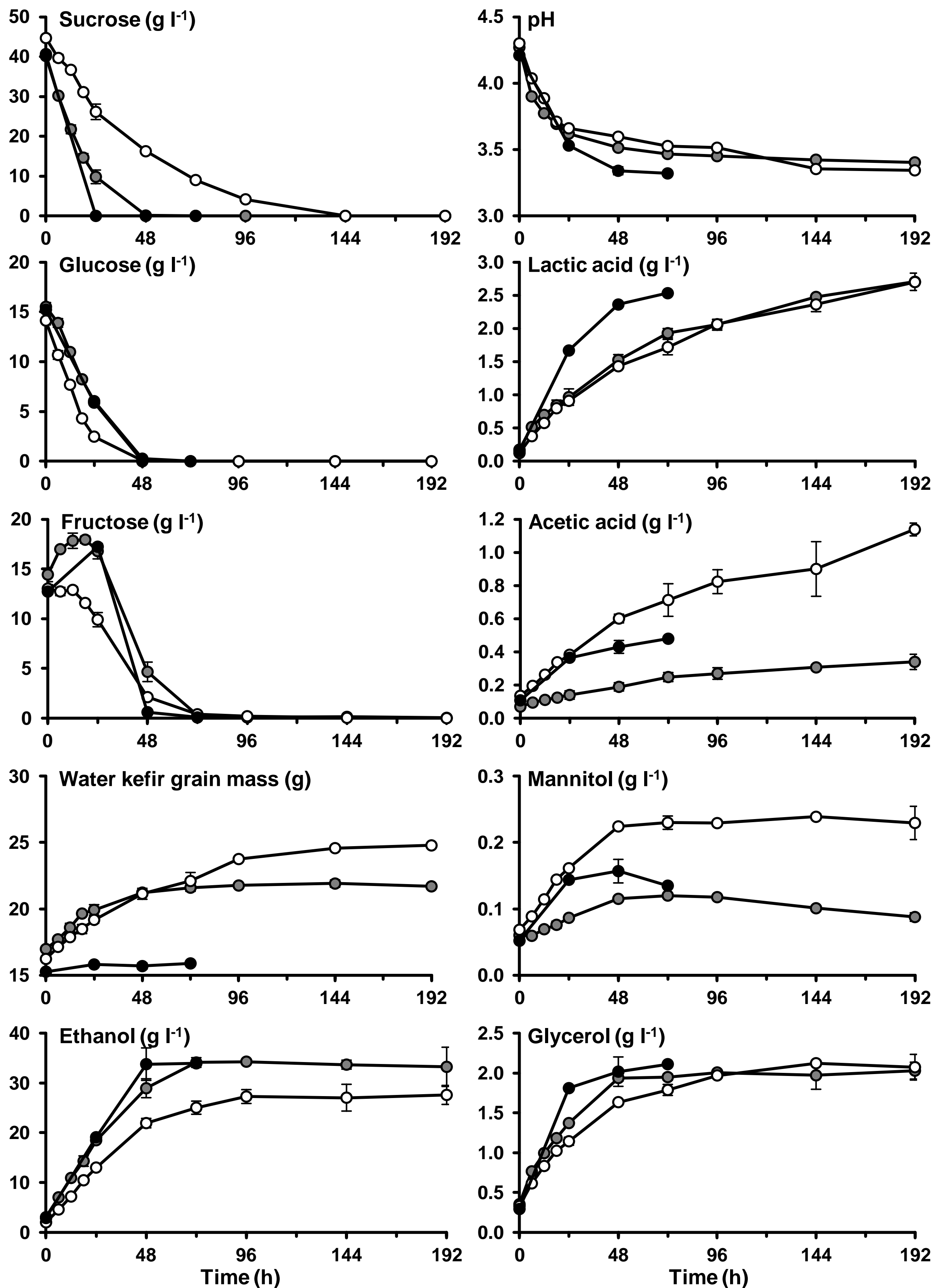

Figure 2. The water kefir grain wet mass, the pH, and the concentrations of substrates and metabolites as a function of time during the fermentation processes inoculated with water kefir grain inocula A (●), B (●), and C (○).

Table 2. Characteristics of the fermentation processes inoculated with water kefir grain inocula A, B, and C. Statistically significant differences ($p < 0.05$) are indicated with superscripts a, b, and c.

Characteristic	Water kefir fermentation process		
	A	B	C
Time when [sucrose] < 1 g l^{-1} (h)	24	48	144
Time when [total carbohydrates] < 1 g l^{-1} (h)	48	72	144
Water kefir grain growth (%)	4.58 ± 1.57 ^{c}	43.98 ± 1.85 ^{b}	63.82 ± 1.19 ^{a}
Water kefir grain dry mass (%)	12.87 ± 0.16 ^{b}	14.41 ± 0.11 ^{a}	14.52 ± 0.02 ^{a}
pH	3.34 ± 0.03 ^{b}	3.47 ± 0.01 ^{a}	3.35 ± 0.01 ^{b}
Ethanol (g l^{-1})	33.77 ± 3.26 ^{a}	34.10 ± 0.97 ^{a}	27.04 ± 2.69 ^{b}
Lactic acid (g l^{-1})	2.36 ± 0.03 ^{a}	1.93 ± 0.07 ^{b}	2.36 ± 0.19 ^{a}
Acetic acid (g l^{-1})	0.43 ± 0.04 ^{b}	0.25 ± 0.03 ^{b}	0.90 ± 0.16 ^{a}
Glycerol (g l^{-1})	2.02 ± 0.18	1.95 ± 0.04	2.12 ± 0.02
Mannitol (g l^{-1})	0.16 ± 0.02 ^{b}	0.12 ± 0.01 ^{c}	0.24 ± 0.01 ^{a}
Ratio glycerol/ethanol (mmol mol^{-1})	30 ± 1 ^{b}	29 ± 1 ^{b}	39 ± 4 ^{a}
Ratio lactic acid/ethanol (mmol mol^{-1})	36 ± 4 ^{b}	29 ± 1 ^{c}	45 ± 4 ^{a}
Ratio acetic acid/ethanol (mmol mol^{-1})	9.9 ± 1.8 ^{b}	5.6 ± 0.5 ^{b}	25.6 ± 4.6 ^{a}
Ratio acetic acid/lactic acid (mol mol^{-1})	0.27 ± 0.02 ^{b}	0.19 ± 0.01 ^{c}	0.57 ± 0.06 ^{a}
D-lactic acid (% of total lactic acid)	36.75 ± 0.82 ^{b}	39.65 ± 1.59 ^{a}	39.98 ± 0.61 ^{a}
Carbon recovery (%)	103.9 ± 7.2	106.7 ± 2.3	98.1 ± 5.3

The ratios of the viable counts of the LAB to those of the yeasts in the water kefir liquors and on the water kefir grains remained stable at approximately 2-10, during the entire courses of the three water kefir fermentation processes. The ratios of the viable counts of the LAB and the yeasts on the water kefir grains (cfu g^{-1}) to those in the water kefir liquors (cfu ml^{-1}) remained also more or less stable around 10-100 during the entire water kefir fermentation processes. When the water kefir grain wet masses and the water kefir liquor volumes were taken into account, the ratios of the total numbers (expressed as total cfu) of the LAB and yeasts on the water kefir grains to those in the water kefir liquors remained around 5-20 during the three entire fermentation processes. The ratios of these viable counts when the total carbohydrate concentrations were < 1 g l^{-1} were representative for the entire water kefir fermentation processes A, B, and C (Table 3).

3.3 Culture-dependent microbial species diversity and community dynamics

The culture-dependent species diversity and community dynamics in the water kefir liquors were similar to those on the corresponding water kefir grains (Figure 3). Furthermore, they remained more or less stable during the entire courses of the three water kefir fermentation processes (data not shown).

Lactobacillus paracasei was found in the liquors and on the grains of water kefirs A, B, and C, with similar relative abundances in the liquors and on the grains; *Lactobacillus hilgardii* was found in water kefirs A and C, with higher relative abundances on the grains than in the liquors; and *Lactobacillus nagelii* was found in water kefir A with higher relative abundances in the liquors than on the grains. Additionally, a low relative abundance of *Lactobacillus satsumensis* was found in the liquors and on the grains of water kefir A, and a low relative abundance of *Lactobacillus harbinensis* was found in the liquors of water kefir C.

Saccharomyces cerevisiae was the most abundant yeast species in the liquors and on the grains of water kefirs A, B, and C, and this species was more abundant on the water kefir

Table 3. Viable counts of the yeasts and the lactic acid bacteria (LAB) in the liquors and on the grains of the fermentation processes inoculated with water kefir grain inocula A, B, and C, when the total carbohydrate concentrations were < 1 g l^{-1}. The ratios between the different viable counts were also calculated. Statistically significant differences ($p < 0.05$) are indicated with superscripts a, b, and c.

Viable counts or ratio	Water kefir fermentation process		
	A	B	C
Yeasts (liquor) (log cfu ml^{-1})	6.44 ± 0.08^{b}	6.72 ± 0.16^{a}	6.11 ± 0.14^{b}
LAB (liquor) (log cfu ml^{-1})	6.92 ± 0.05^{a}	6.86 ± 0.14^{ab}	6.68 ± 0.15^{b}
Yeasts (grains) (log cfu g^{-1})	8.26 ± 0.02^{a}	8.03 ± 0.10^{b}	7.68 ± 0.09^{c}
LAB (grains) (log cfu g^{-1})	8.84 ± 0.07^{a}	8.57 ± 0.07^{b}	8.22 ± 0.07^{c}
LAB /yeasts (liquor)	3.01 ± 0.42^{ab}	1.56 ± 0.86^{b}	4.02 ± 1.73^{a}
LAB /yeasts (grains)	3.78 ± 0.49	3.47 ± 0.75	3.58 ± 1.26
Grains/liquor (yeasts)	66.08 ± 22.84^{a}	22.84 ± 11.36^{b}	39.57 ± 15.91^{b}
Grains/liquor (LAB)	82.58 ± 9.78^{a}	53.10 ± 15.49^{b}	35.93 ± 10.90^{b}
Grains/liquor (total yeasts)	12.32 ± 2.09	6.28 ± 3.07	12.87 ± 5.10
Grains/liquor (total LAB)	15.39 ± 2.08	14.66 ± 4.39	11.69 ± 3.47

grains than in the water kefir liquors. Additionally, *Zygotorulaspora florentina* was found in water kefir A and *Dekkera bruxellensis* was found in water kefirs B and C, whereby their relative abundances were higher in the water kefir liquors than on the water kefir grains.

EPS production was found for 50 and 29 % of the *Lb. hilgardii* strains from water kefirs A and C, respectively; 48 % of the *Lb. nagelii* strains from water kefir A; and for all *Lb. satsumensis* strains from water kefir A. None of the *Lb. paracasei* strains from water kefirs A, B, and C produced EPS. The proportions of EPS-producing *Lb. hilgardii* and *Lb. nagelii* strains were similar in the water kefir liquors and on the water kefir grains. Further, the EPS produced by *Lb. hilgardii* spread out over the entire agar medium, whereas those of *Lb. nagelii* and *Lb. satsumensis* remained localized around the colonies, indicating a ropy- and mucoid-type production, respectively.

3.4 Culture-independent microbial species diversities and community dynamics

The rRNA-PCR-DGGE community profiles obtained with the four different primer pairs (V3, LAC, Bif, and Yeast) of the water kefir liquors and grains for the three biological replicates of water kefir fermentation processes A, B, and C were similar at each sampling point. Furthermore, these community profiles remained more or less stable as a function of time during the entire courses of the three water kefir fermentation processes (data not shown).

The main bands in the community profiles obtained with the V3 and LAC primer pairs for the water kefir liquors and grains of water kefir fermentation processes A, B, and C were attributed to *Lb. nagelii*, *Lb. hilgardii*, and *Lb. paracasei* (Figure 4). The relative intensities of the bands attributed to *Lb. nagelii* were higher for the water kefir liquors than for the water kefir grains, and those of the bands attributed to *Lb. hilgardii* were higher for the grains than for the liquors. The relative intensities of the bands attributed to *Lb. paracasei* were lower for water kefir A than for water kefirs B and C, both for the water kefir liquors and grains. Additionally, bands attributed to *Lb. mali*/*hordei* and *Lb. harbinensis* were found in the community profiles of water kefir C obtained with the V3 and LAC primer pairs, with higher relative intensities for the water kefir liquors than for the grains. Furthermore, a band attributed to a non-identified *Oenococcus* species was found in the community profiles of

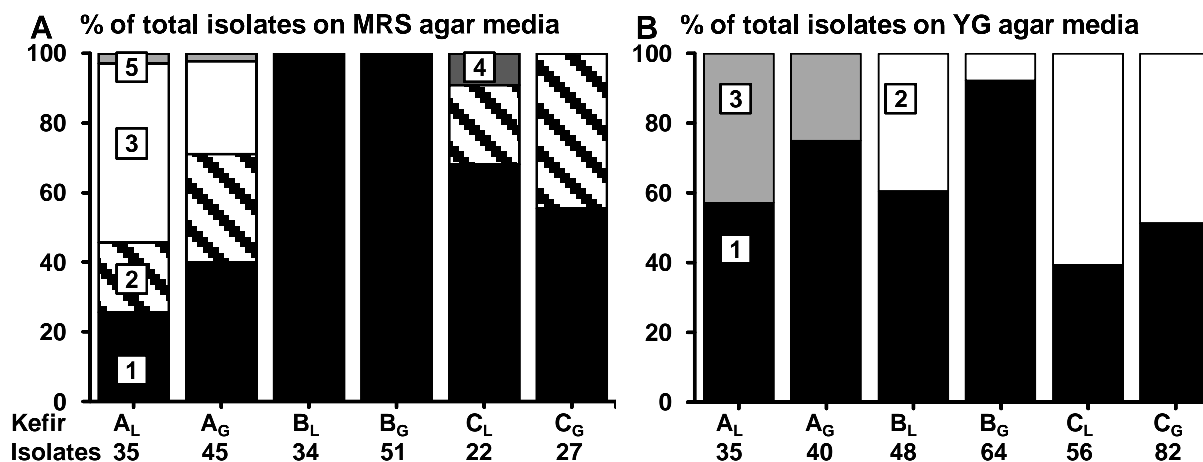

Figure 3. Culture-dependent microbial species diversities and community dynamics for the water kefir liquors (subscript L) and the water kefir grains (subscript G) of the fermentation processes inoculated with water kefir grain inocula A, B, and C, obtained after pooling the samples from the different sampling points. The closest known type strains of the sequenced fragments are given. (A) Isolates on MRS agar media: 1, *Lactobacillus paracasei* (100 % identity, GenBank accession no. AP012541); 2, *Lactobacillus hilgardii* (99 % identity, accession no. LC064898); 3, *Lactobacillus nagelii* (99 % identity, accession no. NR112754); 4, *Lactobacillus harbinensis* (100 % identity, accession no. NR028658); and 5, *Lactobacillus satsumensis* (99 % identity; accession no. NR028658). (B) Isolates on YG agar media: 1, *Saccharomyces cerevisiae* [large subunit rRNA gene (LSU) (99 % identity, accession no. KC881066) and the internal transcribed spacer (ITS) region (99 % identity, accession no. KC881067)]; 2, *Dekkera bruxellensis* [LSU (100 % identity, accession no. AY969049) and ITS (100 % identity, accession no. NR111030)]; and 3, *Zygotorulaspora florentina* [LSU (100 % identity, accession no. U72165) and ITS (100 % identity, accession no. AY046168)].

water kefir C obtained with the V3 primer pair, with higher relative intensities for the water kefir liquors than for the grains. A band attributed to *Bifidobacterium psychraerophilum/crudilactis* was found in the community profiles obtained with the V3 primer pair for the liquors and the grains during the entire water kefir fermentation processes A and C, but not in the liquors or grains of water kefir fermentation process B. The presence of *B. psychraerophilum* (98 % identity; accession no. NR029065) in water kefirs A and C and its absence in water kefir B was confirmed with the community profiles obtained with the Bif primer pair.

The most intense bands in the community profiles obtained with the Yeast primer pair for the liquors and the grains of water kefirs A, B, and C were attributed to *S. cerevisiae* (100 % identity; accession no. KC881066). The relative intensities of these bands were always higher for the water kefir grains than for the liquors. Furthermore, bands with weak relative intensities in the community profiles for the water kefir liquors and grains of water kefirs B and C were attributed to *D. bruxellensis* (100 % identity; accession no. AY969049). The relative intensities of these bands were higher for water kefir C than for water kefir B, and were higher for the water kefir liquors than for the grains, confirming the culture-dependent results. Bands with low relative intensities were present above the band attributed to *S. cerevisiae* in the community profiles for the water kefir liquors and grains of water kefir A, which could not be identified by sequencing, but may be attributed to *Z. florentina*, based on the culture-dependent species diversity data. Further, the relative intensities of these bands

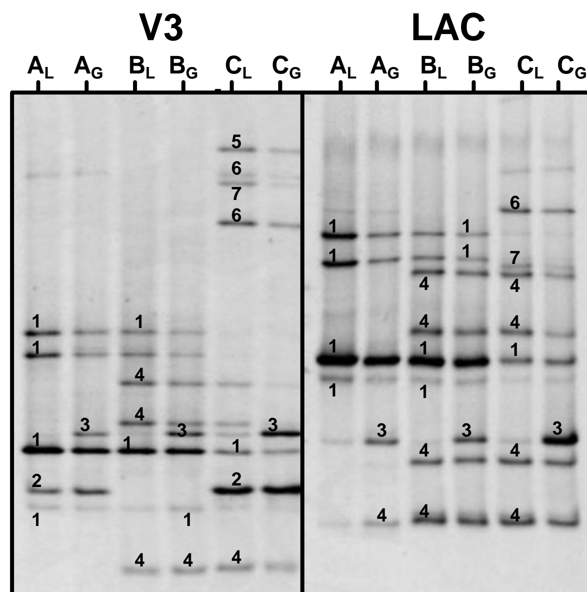

Figure 4. Culture-independent microbial species diversities and community dynamics for the water kefir liquors (subscript L) and the water kefir grains (subscript G) of the water kefir fermentation processes inoculated with water kefir grain inocula A, B, and C after 48 h of fermentation. The numbers indicate the bands that were sequenced. The closest known type strains of the sequenced fragments are given. With the V3 primer pair: 1, *Lactobacillus nagelii/ghanensis* (99 % identity for both species; GenBank accession no. NR112754/NR043896); 2, *Bifidobacterium psychraerophilum/crudilactis* (98 % identity; accession no. NR029065/NR115342); 3, *Lactobacillus hilgardii/diolivorans* (100 % identity; accession no. LC064898/NR037004); 4, *Lactobacillus paracasei/casei/zeae/rhamnosus* (100 % identity; accession no. AP012541/AP012544/NR037122/JQ580982); 5, *Oenococcus kitaharae* (97 % identity; accession no. NR041312); 6, *Lactobacillus mali/hordei* (99 % identity; accession no. LC064888/NR044394); and 7, *Lactobacillus harbinensis* (100 % identity; accession no. NR113969). With the LAC primer pair: 1, *Lb. nagelii* (99 % identity; accession no. NR119275); 3, *Lb. hilgardii/diolivorans* (99 % identity; accession no. LC064898/NR037004); 4, *Lb. paracasei/casei/zeae* (100 % identity; accession no. AP012541/AP012544/NR037122); 6, *Lb. mali/hordei* (99 % identity; accession no. LC064888/NR044394); and 7, *Lb. harbinensis* (100 % identity; accession no. NR113969).

were higher for the water kefir liquors than for the grains, which was in line with the culture-dependent results for *Z. florentina*.

3.5 Substrate consumption and metabolite production profiles

Sucrose was the main carbohydrate at the start of the three water kefir fermentation processes, and was completely consumed (residual concentrations < 1 g l^{-1}) after 24, 48, and 144 h of fermentation for water kefirs A, B, and C, respectively (Table 2). The glucose concentrations decreased continuously during all three water kefir fermentation processes, whereas the fructose concentrations initially increased to reach a maximum after approximately 24 h of fermentation (Figure 2). The total residual carbohydrate concentrations in water kefirs A, B, and C were < 1 g l^{-1} after 48, 72, and 144 h, respectively. The time until the total residual carbohydrate concentrations were < 1 g l^{-1} was higher when the water kefir grain growth was higher (Table 2) and was lower when the viable counts of the LAB and yeasts on the water kefir grains were higher (Table 3).

Ethanol was the most abundant metabolite produced during all three water kefir fermentation processes (Table 2). The production of ethanol was more or less linear during the first 48 h of fermentation, after which the production slowed down (Figure 2). This corresponded with the time that glucose was depleted in the three water kefir fermentation processes. The highest concentrations of ethanol were found in water kefirs A and B, followed by water kefir C. The glycerol production paralleled that of ethanol, and its concentrations were similar for the three water kefirs when the total residual carbohydrate concentrations were < 1 g l^{-1}. The ratios of the concentrations of glycerol to ethanol were highest in water kefir C (Table 2).

The highest concentrations of lactic acid were found in water kefir A, followed by water kefirs B and C, and the proportion of D-lactic acid was lowest in water kefir A (Table 2). The production of acetic acid paralleled that of lactic acid, and the highest concentrations were found in water kefir C and the lowest in water kefir B. The concentrations of lactic acid and acetic acid continued to increase after the total residual carbohydrate concentrations were < 1 g l^{-1} (Figure 2). The production of mannitol followed the same pattern as that of the acetic acid production in the three water kefir fermentation processes until the total residual carbohydrate concentrations were < 1 g l^{-1}.

No major aroma compounds were found in the WKSM (before inoculation) via SH-GC-MS. The concentrations of isoamyl alcohol, 2-methyl-1-propanol, ethyl hexanoate, ethyl octanoate, and isoamyl acetate (SH-GC-MS) increased fast during the first 48 h of the three water kefir fermentation processes, whereafter their concentrations remained more or less stable (Figure 5). In contrast, the concentrations of ethyl acetate and ethyl decanoate increased

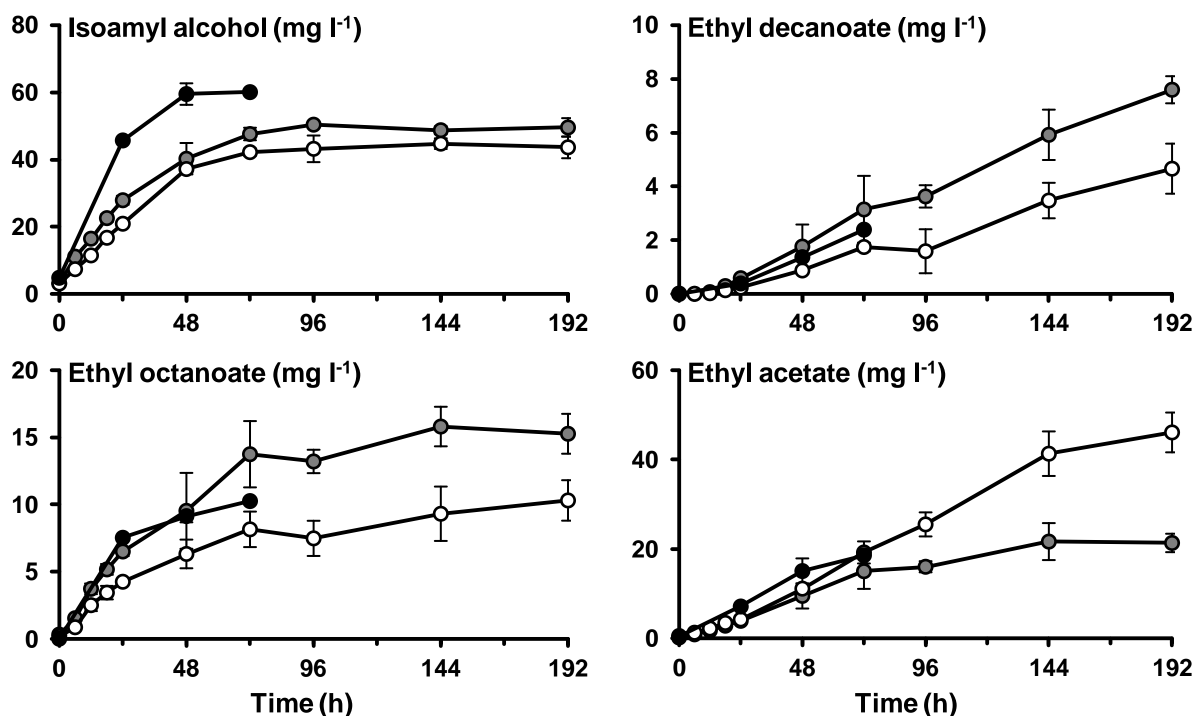

Figure 5. Concentrations of isoamyl alcohol, ethyl octanoate, ethyl decanoate, and ethyl acetate (mg l^{-1}) as a function of time (h), measured in the static headspace (SH) of the liquors of the fermentation processes inoculated with water kefir grain inocula A (●), B (●), and C (○).

Table 4. Concentrations and Kovats indices (KI) of the aroma compounds in the water kefir liquors of the fermentation processes inoculated with water kefir grain inocula A, B, and C, when the total carbohydrate concentrations were < 1 g l^{-1}. The threshold values and aroma descriptors are given for each compound (Corison *et al.*, 1979; Guth, 1997; Ferreira *et al.*, 2000; Lambrechts & Pretorius, 2000; Mamede *et al.*, 2005; Molina *et al.*, 2009). Statistically significant differences ($p < 0.05$) are indicated with superscripts a, b, and c.

Compound	KI	Threshold	Water kefir fermentation process (time)		
		(mg l^{-1})	A (48 h) (mg l^{-1})	B (72 h) (mg l^{-1})	C (144 h) (mg l^{-1})
2-Methyl-1-propanol	1097	40	16.80 ± 1.28^{a}	10.24 ± 1.06^{b}	14.86 ± 0.80^{a}
Isoamyl alcohol	1222	30	59.50 ± 3.20^{a}	47.61 ± 1.87^{b}	44.74 ± 1.59^{b}
Ethyl acetate	831	7.5	15.03 ± 2.83^{b}	15.00 ± 3.95^{b}	41.29 ± 4.98^{a}
Isoamyl acetate	1141	0.03	0.57 ± 0.08^{a}	0.13 ± 0.03^{b}	0.14 ± 0.02^{b}
Ethyl hexanoate	1250	0.014	0.97 ± 0.12^{a}	0.92 ± 0.11^{a}	0.59 ± 0.06^{b}
Ethyl octanoate	1450	0.005	9.13 ± 0.46^{a}	13.74 ± 2.47^{b}	9.31 ± 2.02^{a}
Ethyl decanoate	1659	0.2	1.37 ± 0.47^{b}	3.14 ± 1.25^{a}	3.48 ± 0.66^{a}

only slowly during the first 24 h of fermentation, whereafter their concentrations continued to increase until the end of the fermentation processes. When the total residual carbohydrate concentrations were < 1 g l^{-1}, water kefir A contained the highest isoamyl acetate concentrations and the lowest ethyl decanoate concentrations; water kefir B contained the highest ethyl octanoate concentrations; and water kefir C contained the highest ethyl acetate concentrations and the lowest ethyl hexanoate concentrations (Table 4). The concentrations of the esters found in the water kefir liquors of water kefirs A, B, and C were always well above their threshold values when the total residual carbohydrate concentrations were < 1 g l^{-1}.

Although some esters, higher alcohols, and short- to long-chain fatty acids were already found in the WKSM (before inoculation) via SH-SPME-GC-MS, their relative abundances increased significantly during the three water kefir fermentation processes (Table 5). In contrast, the aldehydes hexanal, furfural, and benzaldehyde were found in the WKSM (before inoculation) but were not found in the water kefir liquors after fermentation. Water kefirs B and C contained higher relative abundances of 4-ethylphenol and 4-ethylguaiacol than water kefir A and the WKSM. Water kefir A contained the highest relative abundances of benzyl alcohol and 2-phenylethyl acetate, and the lowest relative abundances of ethyl 2-methyl-butanoate, ethyl heptanoate, ethyl nonanoate, and decanoic acid (Table 5). Water kefir B contained the lowest relative abundances of ethyl benzenepropanoate and diethyl succinate. Water kefir C contained the highest relative abundances of ethyl lactate, ethyl 2-methyl-butanoate, ethyl benzenepropanoate, decanoic acid, and 1-octanol, whereas it contained the lowest relative abundances of ethyl butanoate and ethyl 9-decenoate. There was no evidence for the presence of 1,3-propanediol neither in WKSM (before inoculation) nor in the three water kefir liquors after fermentation.

3.6 Water kefir grain composition

The water kefir grains were completely hydrolyzed into glucose after 6 h of incubation in either HCl or H_2SO_4. No other monosaccharides neither organic acids were found, indicating that they were composed of glucan-type EPS.

Table 5. Relative abundances in arbitrary units (AU) and the Kovats indices (KI) of the minor aroma compounds found after solid phase microextraction (SPME) of the headspaces of the water kefir simulation medium (WKSM) and the liquors of the fermentation processes inoculated with water kefir grain inocula A, B, and C, when the total carbohydrate concentrations were < 1 g l^{-1}. Statistically significant differences ($p < 0.05$) are indicated with superscripts a, b, c, and d.

Compound	KI	Id	WKSM	Water kefir fermentation process (time)		
			(AU)	A (48 h) (AU)	B (72 h) (AU)	C (144 h) (AU)
2-Phenylethyl acetate	1861	MS/RF	0 ± 0^{a}	635 ± 73^{b}	123 ± 14^{c}	89 ± 7^{c}
Ethyl lactate	1386	MS/RF	1 ± 1^{a}	186 ± 24^{b}	118 ± 10^{bc}	308 ± 89^{c}
Ethyl butanoate	1027	MS	0 ± 0^{a}	64 ± 11^{b}	71 ± 2^{b}	48 ± 2^{c}
Ethyl 2-methyl-butanoate	1045	MS	0 ± 0^{a}	7 ± 2^{b}	19 ± 1^{c}	23 ± 1^{d}
Ethyl heptanoate	1377	MS	0 ± 0^{a}	25 ± 12^{b}	44 ± 4^{c}	47 ± 7^{c}
Ethyl nonanoate	1560	MS	0 ± 0^{a}	41 ± 20^{b}	69 ± 8^{c}	85 ± 18^{c}
Ethyl 9-decenoate	1723	MS	1 ± 1^{a}	171 ± 52^{b}	185 ± 28^{b}	73 ± 15^{c}
Ethyl benzenepropanoate	1928	MS	0 ± 0^{a}	69 ± 8^{b}	49 ± 1^{c}	99 ± 17^{d}
Methyl octanoate	1437	MS	10 ± 1^{a}	21 ± 13^{ac}	46 ± 4^{b}	31 ± 6^{c}
Isoamyl octanoate	1674	MS	1 ± 0^{a}	59 ± 16^{b}	74 ± 12^{bc}	88 ± 21^{c}
Diethyl succinate	1702	MS	2 ± 0^{a}	366 ± 2^{b}	145 ± 7^{c}	344 ± 61^{b}
Hexanoate	1870	MS/RF	16 ± 1^{a}	176 ± 27^{b}	241 ± 19^{c}	217 ± 27^{bc}
Octanoate	2069	MS/RF	18 ± 2^{a}	606 ± 85^{b}	824 ± 112^{c}	710 ± 147^{bc}
Nonanoate	2170	MS	0 ± 0^{a}	11 ± 8^{b}	12 ± 3^{b}	11 ± 2^{b}
Decanoate	2271	MS	6 ± 1^{a}	90 ± 19^{b}	207 ± 34^{c}	258 ± 67^{c}
Hexanal	1092	MS/RF	32 ± 5^{a}	2 ± 1^{b}	1 ± 0^{b}	1 ± 0^{b}
Furfural	1511	MS/RF	66 ± 4^{a}	10 ± 9^{b}	6 ± 6^{b}	1 ± 1^{b}
Benzaldehyde	1570	MS/RF	41 ± 3^{a}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}
1-Octanol	1570	MS	0 ± 0^{a}	45 ± 8^{b}	59 ± 2^{b}	91 ± 17^{c}
1,3-Propanediol	1831	MS/RF	NF	NF	NF	NF
Benzyl alcohol	1913	MS	1 ± 0^{a}	13.5 ± 2.1^{b}	4.8 ± 0.7^{a}	6.4 ± 8.4^{a}
2-Phenylethanol	1954	MS/RF	3 ± 1^{a}	1058 ± 88^{b}	929 ± 53^{b}	911 ± 125^{b}
4-Ethylphenol	2187	MS/RF	1 ± 1^{a}	5 ± 1^{b}	22 ± 1^{c}	16 ± 2^{d}
4-Ethylguaiacol	2063	MS	6 ± 1^{a}	17 ± 4^{a}	241 ± 22^{b}	180 ± 20^{c}
2,4-Di-tert-butylphenol	2102	MS	15 ± 2^{a}	87 ± 9^{b}	162 ± 17^{c}	143 ± 21^{c}
Butylated hydroxytoluene	2310	MS	10 ± 1^{a}	27 ± 5^{b}	36 ± 1^{c}	38 ± 5^{c}
Styrene	1311	MS	1 ± 1^{a}	26 ± 15^{b}	38 ± 1^{b}	39 ± 5^{b}

NF: not found.

3.7 Carbon recovery

The carbon recoveries were approximately 100 % during the entire courses of the three water kefir fermentation processes, indicating that all major substrates and metabolites were recovered from the water kefir fermentation processes studied. The values when the total carbohydrate concentrations were < 1 g l^{-1} were representative for the entire courses of the fermentation processes (Table 2).

4 Discussion

The integrative multiphasic and comparative approach of the present study allowed to determine the influence of the water kefir grain inoculum on the microbial species diversity, community dynamics, substrate consumption, and metabolite production during water kefir

fermentation. Additionally, the comparative nature of this study allowed to reveal associations between the microbial species diversity and certain characteristics of the fermentation process, such as water kefir grain growth.

Microbial growth during water kefir fermentation paralleled the water kefir grain growth. Further, the viable counts of the LAB and yeasts, the most abundant microorganisms during water kefir fermentation, remained stable during the entire courses of the three water kefir fermentation processes studied. Also, the majority of the water kefir microorganisms was always present on the water kefir grains, confirming previous results (Laureys & De Vuyst, 2014; Chapter 3). In addition, the time until total carbohydrate exhaustion was lower when the viable counts of the water kefir microorganisms on the water kefir grains were higher.

The stable character of the viable microbial counts was reflected in a stable microbial species diversity during the entire courses of the three water kefir fermentation processes studied. All three water kefirs harbored *Lb. paracasei* (most abundant), *Lb. hilgardii*, *Lb. nagelii*, and *S. cerevisiae*. These species are also regularly reported in the literature on water kefir, indicating that these microorganisms may be the key microorganisms for water kefir fermentation (Pidoux, 1989; Galli *et al.*, 1995; Magalhães *et al.*, 2010, 2011; Gulitz *et al.*, 2011, 2013; Miguel *et al.*, 2011; Hsieh *et al.*, 2012; Marsh *et al.*, 2013b; Laureys & De Vuyst, 2014; Chapter 3). Additionally, other microorganisms were found in water kefirs A and C, but those were not necessary for water kefir fermentation given their absence in water kefir B.

Lactobacillus hilgardii was more abundant on the water kefir grains than in the liquors, and was more abundant when the water kefir grain growth was high. Indeed, isolated strains of this LAB species produced EPS from sucrose, as has been shown before (Pidoux *et al.*, 1988, 1990; Waldherr *et al.*, 2010). However, the mere presence of EPS-producing strains of *Lb. hilgardii* was not sufficient for good water kefir grain growth, as was found for water kefir A. *Lactobacillus paracasei* was also more abundant when the water kefir grain growth was high, although none of its isolated strains from the water kefirs of the present study produced EPS from sucrose, in contrast with strains of this LAB species of a previous study (Gulitz *et al.*, 2011). This indicated that *Lb. paracasei* was probably not responsible for water kefir grain growth during fermentation. The relative abundances of *Lb. nagelii* were inversely related with water kefir grain growth, even though some strains of this LAB species isolated from water kefir A produced EPS from sucrose. Furthermore, this microorganism was more abundant in the liquors than on the grains, indicating that it was probably not responsible for water kefir grain growth during fermentation.

A non-identified *Bifidobacterium* species was found in water kefirs A and C, but not in water kefir B, indicating that this species was not necessary for water kefir grain growth neither for the course of the water kefir fermentation process. This *Bifidobacterium* species found in water kefirs A and C may represent a novel species, as its partial 16S rRNA gene sequences obtained from strains isolated from water kefirs A and C were only 98 % identical to those of its closest known type strains, but 100 % identical to each other and to those of an uncultivated *Bifidobacterium* species found in a water kefir from Belgium (Laureys & De Vuyst, 2014; Chapter 3) and Germany (Gulitz *et al.*, 2013). The presence of bifidobacteria during water kefir fermentation is remarkable, since these microorganisms are usually adapted to vastly different environments such as the gut ecosystem (Biavati & Mattarelli, 2006). Only recently have *Bifidobacterium* species been found in fermented foods (Delcenserie *et al.*, 2007; Watanabe *et al.*, 2009; Laureys & De Vuyst, 2014; Chapter 3). Bifidobacterial strains are sometimes deliberately added to foods and beverages, because their consumption is associated with positive health effects (Picard *et al.*, 2005).

Low water kefir grain growth was associated with small water kefir grains with high viable counts. Water kefir grains are brittle and break easily during sieving or handling, and insufficient water kefir grain growth may cause the water kefir grains to become small gradually. Small water kefir grains have a large specific surface and can harbor high viable counts, as the water kefir microorganisms are mainly attached onto the surface of the water kefir grains (Moinas *et al.*, 1980; Laureys & De Vuyst, 2014; Chapter 3). Furthermore, the majority of the microorganisms during water kefir fermentation was always associated with the water kefir grains, which further explained why low water kefir grain growth was associated with fast fermentation.

Water kefir grain growth during fermentation resulted from the partial conversion of sucrose into glucan EPS by extracellular glucansucrases (Monsan *et al.*, 2001). The activity of glucansucrases decreases at low pH values (Waldherr *et al.*, 2010), so the low pH values during water kefir fermentation process A may have caused its low water kefir grain growth. However, the pH did not drop so fast to exclude any glucansucrase activity, making it more likely that the production of glucansucrase by *Lb. hilgardii* was suppressed by the low pH values. Acidic stress may thus cause low water kefir grain growth, which should be investigated in more detail. When the water kefir grain growth decreased, less glucose was incorporated into water kefir grain EPS and hence more glucose remained available for acid production, further increasing the acidic stress. Over multiple backsloppings, a continuous increase of the acidic stress may result in a continuous decrease of the water kefir grain growth, as was seen during the prefermentations of the present study. This illustrated that it will be necessary to adjust the process parameters of water kefir fermentation process based on the characteristics to maintain a stable process.

Glucose was the preferred substrate during the water kefir fermentation processes studied, as it was always consumed faster than fructose. Ethanol, lactic acid, glycerol, acetic acid, and mannitol were the main end-metabolites produced. Despite a stable ratio of LAB cells to yeast cells of 2-10, the majority of the metabolites was always produced by the yeasts. The production of mannitol indicated the use of fructose as alternative external electron acceptor by heterofermentative LAB (Zaunmüller *et al.*, 2006), but the concentrations of mannitol remained low, as has been found previously (Laureys & De Vuyst, 2014; Chapter 3). Part of the acetate production may be attributed to the bifidobacteria, as higher acetate concentrations in water kefirs A and C coincided with the presence of bifidobacteria (Biavati & Mattarelli, 2006). Nevertheless, given the low acetate concentrations in these water kefirs, the metabolism of the bifidobacteria was probably only of minor impact during water kefir fermentation.

Continued bacterial metabolism in all the water kefir fermentation processes studied after carbohydrate depletion may be ascribed to the fermentation of other (not measured) carbohydrates, such as starch derived from the figs or glucans composing the water kefir grains. Bifidobacteria were probably not the main cause of this extended metabolism, as they were absent in water kefir B. Although there was no evidence for the degradation of the water kefir grain EPS, dextranase activity has already been shown in certain bifidobacterial strains (Bailey *et al.*, 1961; Kaster & Brown, 1983) and LAB species (Picozzi *et al.*, 2015).

Isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate possess fruity and floral aromas, and may exert an influence on the aroma of the water kefir liquors, as their concentrations were higher than their threshold values (Lambrechts & Pretorius, 2000). In contrast, the concentrations of ethyl acetate, isoamyl alcohol, and 2-methyl-1-propanol were only around their threshold values. The latter compounds may contribute a harsh and unpleasant solvent-like aroma at high concentrations, but may add desirable complexity to fermented beverages in lower concentrations (Lambrechts & Pretorius, 2000).

Additionally, free fatty acids (sour, cheesy, sweaty, rancid, soapy, and/or goaty aroma), short- to medium-chain esters (fruity and floral), long-chain esters (soapy), and 2-phenylethanol (rosy) were produced, whereas hexanal, furfural, and benzaldehyde disappeared during all three water kefir fermentation processes (Vandermerwe & Vanwyk, 1981; Lambrechts & Pretorius, 2000). The compounds 4-ethylphenol (wet horse) and 4-ethylguaiacol (smoky, vanilla, and clove-like) are associated with the metabolism of *D. bruxellensis*, and their relative abundances were indeed higher when this yeast species was present during water kefir fermentation (Lambrechts & Pretorius, 2000). The absence of 1,3-propanediol indicated that glycerol was not further converted by LAB species such as *Lb. hilgardii* (Pasteris & de Saad, 2009; Bauer *et al.*, 2010b).

In conclusion, this comparative study allowed to determine the key microorganisms from the wide range of microbial species found in water kefir, namely *Lb. paracasei*, *Lb. hilgardii*, *Lb. nagelii*, and *S. cerevisiae*. Depending on the water kefir grain inoculum, other microorganisms may occur, but these were not always present during water kefir fermentation. The presence of EPS-producing *Lb. hilgardii* strains was not sufficient for good water kefir grain growth. Low water kefir grain growth seemed to be caused by low pH values during fermentation. Further, the water kefir grain growth seemed to impact the size of the water kefir grains, which may in turn impact the fermentation rate. This study will be of value for the selection of an appropriate water kefir grain inoculum and for developing and maintaining a stable water kefir production process.

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CHAPTER 5

Investigation of the instability and low water kefir grain growth during an industrial water kefir production process

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SUMMARY

An industrial water kefir production process suffering from instability and low water kefir grain growth was investigated to gain more insight into the causes of these two common problems during water kefir fermentation. The water kefir grain inoculum used to start the water kefir production process was stored at -20 °C, thawed, and reactivated during three consecutive prefermentations before the water kefir production process was started. The structure of the water kefir grains was damaged, probably by the freezing and thawing process, and this damage was not restored during the prefermentations nor the production process. *Lactobacillus nagelii*, *Lactobacillus paracasei*, *Lactobacillus hilgardii*, *Leuconostoc mesenteroides*, a non-identified *Bifidobacterium* species, *Gluconobacter roseus/oxydans*, *Gluconobacter cerinus*, *Saccharomyces cerevisiae*, and *Zygotorulaspora florentina* were the main microorganisms found during the water kefir production process. However, the microorganism assumed to be responsible for water kefir grain growth, *Lb. hilgardii*, was not found culture-dependently, which could explain the low water kefir grain growth during the prefermentations and the water kefir production process. The viable counts of lactic acid bacteria and yeasts in the liquors and on the grains were as expected, and those of the acetic acid bacteria were high. Nevertheless, the fermentation processes progressed slowly, probably due to high osmotic stress as a result of the high concentrations of sucrose during the prefermentations and the production process.

1 Introduction

Water kefir is a naturally fermented beverage with health-promoting potential that is produced and drunk at household-scale worldwide (Marsh *et al.*, 2013b; Pothakos *et al.*, 2016). To make water kefir, a mixture of water, (dried) fruits, and sugar is inoculated with water kefir grains and fermented anaerobically at room temperature for 2 to 4 days (Gulitz *et al.*, 2011; Laureys & De Vuyst, 2014; Chapters 3 and 4). At the end of a fermentation process, the water kefir grains are separated from the water kefir liquor by sieving and reused for a next water kefir fermentation process through a backslopping practice. Water kefir grains are brittle, consist of exopolysaccharides (EPS), and harbor the water kefir microorganisms (Moinas *et al.*, 1980; Laureys & De Vuyst, 2014; Chapters 3 and 4). The key microorganisms during water kefir fermentation are the lactic acid bacterial (LAB) species *Lactobacillus paracasei*, *Lactobacillus hilgardii*, and *Lactobacillus nagelii*; and the yeast species *Saccharomyces cerevisiae* (Chapter 4). The end-metabolites of all of them contribute to the flavor of the final water kefir liquor. The viable counts of the acetic acid bacteria (AAB) range from negligible (Laureys & De Vuyst, 2014; Chapter 3) to 8.5 log cfu g^{-1} of water kefir grains, but they do not seem to play a key role (Franzetti *et al.*, 1998; Gulitz *et al.*, 2011; Laureys & De Vuyst, 2014; Chapter 3). Similarly, bifidobacteria are sometimes present, but do not seem to be essential for the water kefir fermentation process (Laureys & De Vuyst, 2014; Chapters 3 and 4).

Usually, sucrose is partly converted into water kefir grain EPS during fermentation, which results in an increase of the water kefir grain wet mass (Laureys & De Vuyst, 2014; Chapters 3 and 4). *Lactobacillus hilgardii* is assumed to be responsible for the water kefir grain growth (Pidoux *et al.*, 1990; Waldherr *et al.*, 2010), but its mere presence is not sufficient for this (Chapter 4). The main end-metabolites produced during water kefir fermentation are ethanol (yeast), lactic acid (LAB), acetic acid (mainly LAB), glycerol (yeast), and mannitol (LAB) (Laureys & De Vuyst, 2014; Chapters 3 and 4). Additionally, a variety of aroma compounds is produced, whereby isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate are most relevant (Laureys & De Vuyst, 2014; Chapters 3 and 4).

At this moment, only a few small companies produce water kefir products, which are mostly sold in health stores as health-promoting supplements for human and animal use (Marsh *et al.*, 2014a). A major reason for the limited industrial production of water kefir is that the water kefir fermentation process is unstable and yields water kefir beverages of variable quality that do not meet the expectations of contemporary consumers. Another major problem during water kefir fermentation is the low water kefir grain growth, which can prevent successful backslopping of the water kefir grains and upscaling of a water kefir production process.

This chapter aimed to investigate an industrial water kefir production process suffering from instability and low water kefir grain growth, to obtain a better understanding of the possible causes behind these two common problems during fermentation.

2 Materials and methods

2.1 Water kefir grain inoculum and prefermentations

The water kefir prefermentation and fermentation processes were carried out in a small Belgian company that produces water kefir. In the company, the water kefir grain inoculum

was stored at -20°C. To start a water kefir production process, the water kefir grains were thawed and reactivated through a series of three consecutive water kefir prefermentations. These were performed in a plastic vessel covered with a muslin cloth, which allowed aerobic fermentation conditions. They were started by adding 5.0 kg of thawed water kefir grains to a mixture of 6.0 l of demineralized water, 1.5 kg of sucrose, and 0.3 kg of dried figs. The fermentation temperature was at 21 °C. After 4 days, a backslopping practice was applied, whereby the water kefir grains were separated from the water kefir liquor by sieving and recultivated in fresh medium under the same conditions as described above.

2.2 Fermentations

The reactivated water kefir grain wet mass, obtained through the series of prefermentations mentioned above, was used by the company to start the actual water kefir production process, which consisted of a first water kefir fermentation process (further referred to as the K1 process), a rest period at low temperature (further referred to as the KR period), and a second water kefir fermentation process (further referred to as the K2 process). The KR period between two subsequent fermentation processes was applied to adjust the production output to the demand for water kefir liquor. These processes were carried out in a plastic fermentation vessel covered with a muslin cloth, as mentioned above. The K1 process was started by adding 5.0 kg of activated water kefir grains from the third prefermentation to a mixture of 15.0 l of demineralized water, 5.0 kg of sucrose, and 1.0 kg of dried figs. This mixture was incubated at 21 °C for 3 days, after which the water kefir grains were separated from the liquor by sieving and used as inoculum for the KR period. Hereto, 5.0 kg of water kefir grains from the K1 process were added to a mixture of 6.0 l of demineralized water and 1.5 kg of sugar. This mixture was incubated at 8 °C for 5 days, after which the water kefir grains were separated from the liquor by sieving and used as inoculum for the K2 process. The K2 process was performed as described above for the K1 process.

2.3 Analyses

Samples were taken in triplicate (representing three technical replicates) during the K1 and K2 processes after 0, 1, 2, and 3 days of fermentation, and during the KR period after 1 and 5 days. No water kefir grains were available at the start of the K1 process (day 0). The water kefir grain wet mass was measured in the company at the end of each prefermentation and fermentation process. The pH and the concentrations of substrates and metabolites were determined at each sampling point. The viable counts of the LAB, AAB, and yeasts in the water kefir liquors and on the non-rinsed water kefir grains were determined at each sampling point (except for the grains after 0 h of fermentation, as no water kefir grains were provided). The culture-dependent microbial species diversity and community dynamics in the water kefir liquors and on the non-rinsed water kefir grains were determined for the K1 and K2 processes after 3 days of fermentation, and for the KR period after 5 days of fermentation. The culture-independent microbial species diversities and community dynamics on the non-rinsed water kefir grains were determined for the K1 process after 0, 1, 2, and 3 days of fermentation; for the KR period after 1 and 5 days of fermentation; and for the K2 process after 1, 2, and 3 days of fermentation. The results are presented as the mean ± standard deviation of the three technical replicates.

2.4 pH and water kefir grain wet mass determinations

The pH of the water kefir liquor was determined with a SenTix 41 glass electrode (WTW, Weilheim, Germany). The water kefir grains were separated from the water kefir liquors by sieving to determine their wet mass. The water kefir grain growth (%; m m^{-1}) was calculated as described in Chapter 3. The water kefir grains were assessed visually throughout the production process by comparison with water kefir grains from a household water kefir fermentation process maintained by a private person described previously (Laureys & De Vuyst, 2014; Chapter 3).

2.5 Microbial enumerations

The viable counts of presumptive LAB were determined on de Man-Rogosa-Sharpe (MRS) agar medium, those of presumptive AAB on modified deoxycholate-mannitol-sorbitol (mDMS) agar medium, and those of presumptive yeasts on yeast extract-glucose (YG) agar medium, as described in Chapter 3.

2.6 Culture-dependent microbial species diversity and community dynamics analyses

The culture-dependent microbial species diversity and community dynamics in the water kefir liquors and on the water kefir grains were determined by randomly picking up 10 to 20 % of the total number of colonies from the agar media with 30 to 300 colonies. DNA extracts from cultures of purified isolates were prepared and used for $(GTG)_5$-PCR fingerprinting for bacteria and M13-PCR fingerprinting for yeasts, as described in Chapter 3. The fingerprint patterns obtained were clustered numerically. Representative bacterial isolates within each cluster were identified by sequencing part of their 16S rRNA gene from genomic DNA, as described in Chapter 3. Representative yeast isolates within each cluster were identified by sequencing their 26S large subunit (LSU) rRNA gene and internal transcribed spacer (ITS) region from genomic DNA, as described in Chapter 3.

2.7 Culture-independent microbial species diversity and community dynamics analyses

The culture-independent microbial species diversity and community dynamics of the water kefir grains were determined after preparing total DNA extracts from the cell pellets of the water kefir grain suspensions, as described in Chapter 3. The culture-independent microbial community profiles were obtained by amplifying selected genomic fragments in the total DNA extracts with the universal prokaryotic primer pair (V3), the LAB-specific primer pair (LAC), the *Bifidobacterium*-specific primer pair (Bif), and the universal eukaryotic primer pair (Yeast); and separating the amplicons through denaturing gradient gel electrophoresis (DGGE), as described in Chapter 3. Selected bands of the community profiles were cut from the gels and identified through sequencing, as described in Chapter 3.

2.8 Substrate and metabolite concentration determinations

The concentrations of sucrose, glucose, and fructose were determined through high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Hereto, 100 µl of cell-free supernatant was added to 400 µl of ultrapure water, and 100 µl of this dilution was added to 900 µl of ultrapure water, after which 50 µl of this dilution was added to 950 µl of deproteinization solution, as described in Chapter 3. The

concentrations of lactic acid and acetic acid were determined through high-performance liquid chromatography with ultraviolet detection (HPLC-UV), those of glycerol and mannitol through HPAEC-PAD, those of ethanol through gas chromatography with flame ionization detection (GC-FID), and those of the aroma compounds through static headspace gas chromatography with mass spectrometry detection (SH-GC-MS), as described in Chapter 3.

3 Results

3.1 Water kefir grain wet mass and pH

The water kefir grains used to start the industrial water kefir production process described in the present study were small and their structure was damaged when compared to water kefir grains from a household water kefir fermentation process (Figure 1). The water kefir grain wet mass remained stable at 5.0 kg during all prefermentations, the K1 process, the KR period, and the K2 process. Thus, the water kefir grain growth was zero during all these fermentation periods.

During the first 2 days of the K1 and K2 processes, the pH decreased fast from 5.88 ± 0.05 to 3.76 ± 0.03, followed by a slower decrease until pH 3.54 ± 0.03 after 3 days of fermentation (Figure 2). During the KR period, the pH decreased slower and reached values of 4.6 ± 0.01 and 3.30 ± 0.01 after 1 and 5 days of fermentation, respectively.

3.2 Microbial enumerations

The viable counts of the LAB, AAB, and yeasts on the water kefir grains remained stable throughout the K1 process, the KR period, and the K2 process, at levels of 7.7 ± 0.5, 5.2 ± 0.5, and 7.1 ± 0.4 log cfu g^{-1}, respectively. In the water kefir liquors, the viable counts of the LAB, AAB, and yeasts remained more or less stable as well, at levels of 7.1 ± 0.3, 5.2 ± 0.4,

Figure 1. Water kefir grains obtained from the industrial water kefir production process investigated in this study (left) and water kefir grains from a household water kefir fermentation process (right).

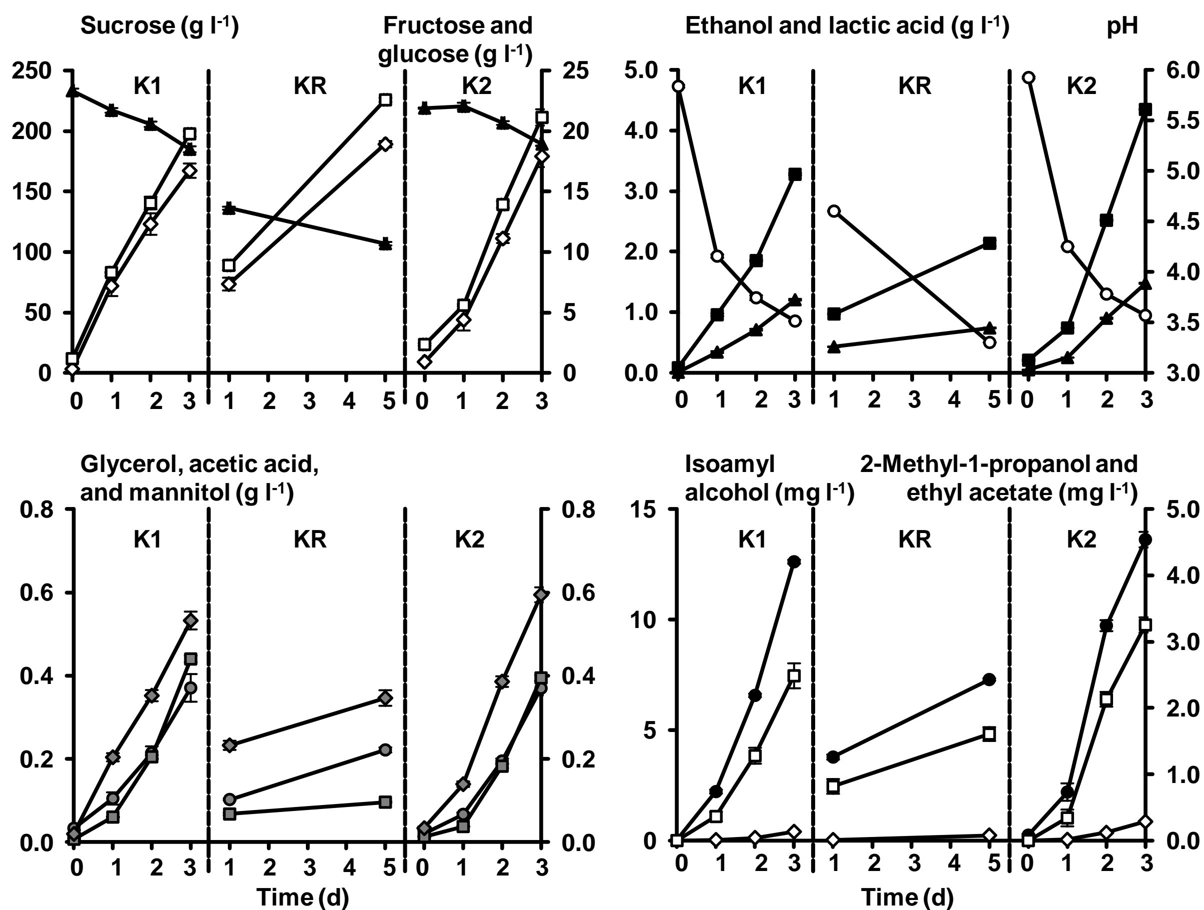

Figure 2. The pH (○) and the concentrations of sucrose (▲), glucose (□), fructose (◊), ethanol (■), lactic acid (▲), glycerol (♦), acetic acid (●), mannitol (■), isoamyl alcohol (●), 2-methyl-1-propanol (□), and ethyl acetate (◊) as a function of time during an industrial water kefir production process consisting of a first water kefir fermentation process (K1), a rest period at lower temperature (KR), and a second water kefir fermentation process (K2).

and 6.1 ± 0.3 log cfu ml^{-1}, respectively. The averages of the ratios of the viable counts of the LAB to those of the yeasts were approximately 4 on the water kefir grains and 11 in the water kefir liquors. The ratios of the viable counts on the water kefir grains to those in the water kefir liquors were approximately 3, 1, and 10 for the LAB, AAB, and yeasts, respectively.

3.3 Culture-dependent microbial species diversity and community dynamics

The culture-dependent microbial species diversity and community dynamics in the water kefir liquors were more or less similar to those on the water kefir grains (Figure 3). Furthermore, they remained more or less stable during the K1 process, the KR period, and the K2 process (data not shown).

The main LAB species recovered from the MRS agar media were *Lb. paracasei*, *Lb. nagelii*, and *Leuconostoc mesenteroides* (Figure 3A). Additionally, *Lactobacillus satsumensis* was found in the water kefir liquors of the K1 process and the KR period. The main AAB

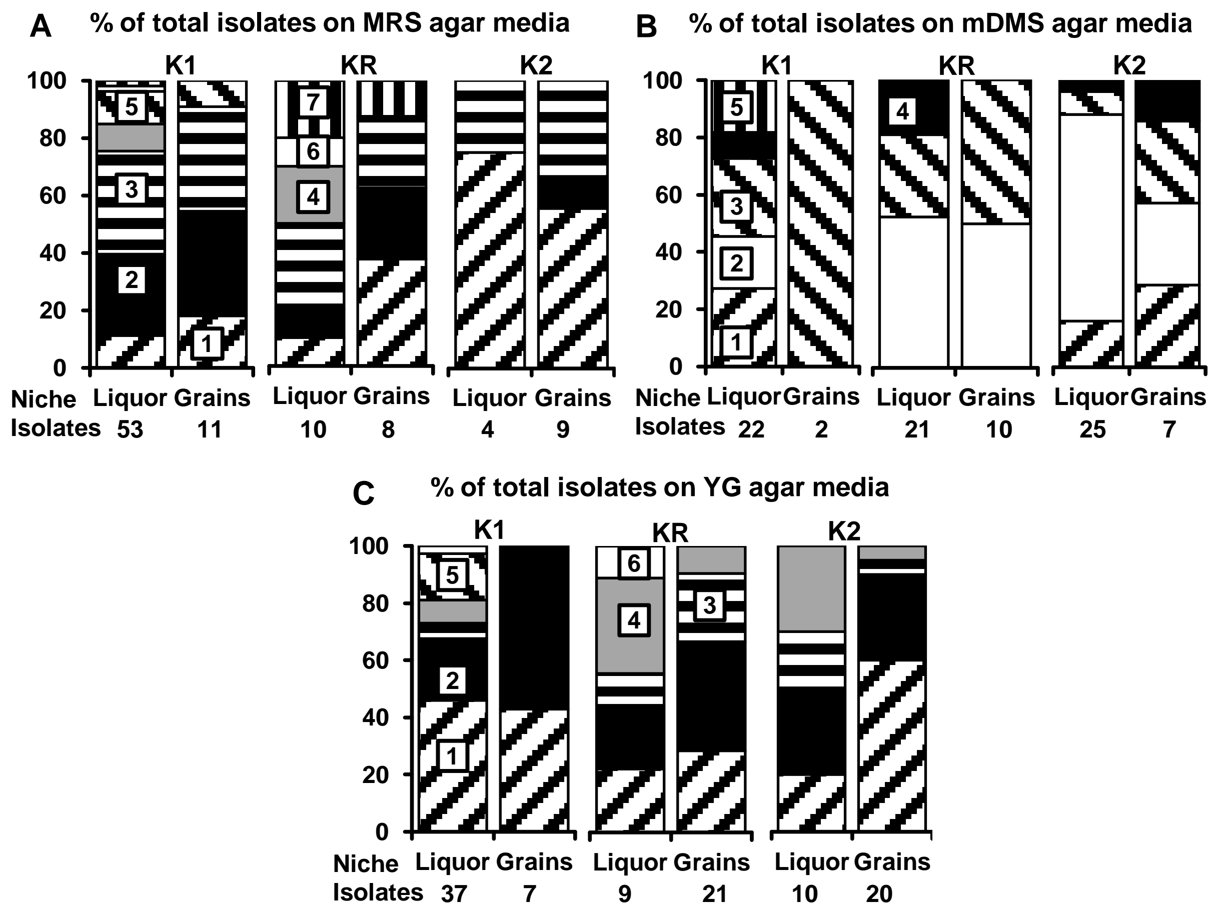

Figure 3. Culture-dependent species diversities and community dynamics for the water kefir liquors and the water kefir grains of an industrial water kefir production process consisting of a first fermentation process (K1), a rest period at lower temperature (KR), and a second fermentation process (K2). The closest known type strains of the sequenced fragments are given. (A) Isolates on MRS agar media: 1, *Lactobacillus paracasei* (99 % identity; GenBank accession no. AP012541); 2, *Lactobacillus nagelii* (99 % identity; accession no. NR112754); 3, *Leuconostoc mesenteroides* (99 % identity; accession no. LC071839); 4, *Lactobacillus satsumensis* (99 % identity; accession no. NR028658); 5, *Gluconobacter cerinus* (99 % identity; accession no. NR118192); 6, *Gluconobacter roseus*/*oxydans* (99 % identity for both species; accession no. NR041049/NR026118); and 7, *Acetobacter orientalis* (98 % identity; accession no. NR113852). (B) Isolates on mDMS agar media: 1, *Lactobacillus paracasei* (99 % identity; accession no. AP012541); 2, *Gluconobacter roseus*/*oxydans* (99 % identity; accession no. NR041049/NR026118); 3, *Gluconobacter cerinus* (99 % identity; accession no. NR118192); 4, *Acetobacter okinawensis* (99 % identity; accession no. NR113546); and 5, *Acetobacter orientalis* (98 % identity; accession no. NR113852). (C) Isolates on YG agar media: 1, *Saccharomyces cerevisiae* [LSU (99 % identity, accession no. KC881066) and ITS (100 % identity, accession no. KC881067)]; 2, *Zygotorulaspora florentina* [LSU (100 % identity, accession no. U72165) and ITS (100 % identity, accession no. AY046168)]; 3, *Dekkera anomala* [LSU (99 % identity, accession no. AY969052) and ITS (99 % identity, accession no. AF043510)]; 4, *Candida boidinii* [LSU (99 % identity, accession no. JQ689009) and ITS (100 % identity, accession no. KM384039)]; 5, *Pichia membranifaciens* [LSU (99 % identity, accession no. NG042444) and ITS (100 % identity, accession no. NR111195)]; and 6, *Wickerhamomyces anomalus* [LSU (100 % identity, accession no. U74592) and ITS (99 % identity, accession no. NR111210)]. LSU, large subunit rRNA gene; ITS, internal transcribed spacer.

species recovered from the mDMS media were *Gluconobacter roseus/oxydans* and *Gluconobacter cerinus*, besides low relative abundances of *Acetobacter okinawensis* and *Acetobacter orientalis* (Figure 3B). The main yeast species recovered from the YG agar media were *S. cerevisiae*, *Zygotorulaspora florentina*, *Dekkera anomala*, and *Candida boidinii*, next to low relative abundances of *Pichia membranifaciens* and *Wickerhamomyces anomalus* (Figure 3C).

3.4 Culture-independent microbial species diversity and community dynamics

The main bands in the rRNA-PCR-DGGE community profiles obtained with the V3 primer pair for the water kefir grains were attributed to *Lb. paracasei*, *Lb. hilgardii/diolivorans*, *Lb. nagelii*, *Leuc. mesenteroides*, a non-identified *Bifidobacterium* species, and the taxon *Acetobacteraceae* (Figure 4). However, the relative intensities of the bands attributed to *Lb. hilgardii/diolivorans* and *Leuc. mesenteroides* decreased, and those of the bands attributed to *Lb. nagelii* and the non-identified *Bifidobacterium* increased over the K1 process, the KR period, and the K2 process. The relative intensities of the bands attributed to *Lb. paracasei* remained stable over these three periods. The results for the LAB were confirmed by the community profiles obtained with the LAC primer pair. The presence of

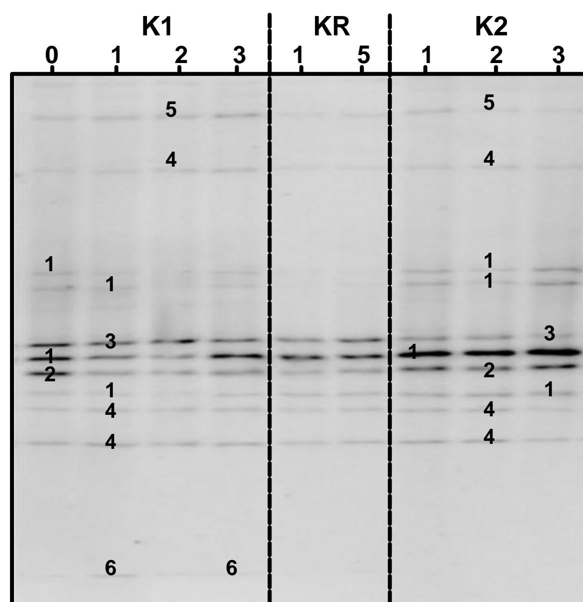

Figure 4. Culture-independent species diversities and community dynamics for the water kefir grains of an industrial water kefir production process consisting of a first fermentation process (K1), a rest period at lower temperature (KR), and a second fermentation process (K2). Samples were taken after 0, 1, 2, and 3 days for the K1 process; after 1 and 5 days for the KR period; and after 1, 2, and 3 days of fermentation for the K2 process. The numbers indicate the bands that were sequenced and the closest known type strains of the sequenced fragments are given. With the V3 primer pair: 1, *Lactobacillus nagelii/ghanensis* (99 % identity for both species; GenBank accession no. NR112754/NR043896); 2, *Bifidobacterium psychraerophilum/crudilactis* (98 % identity; accession no. NR029065/NR115342); 3, *Lactobacillus hilgardii/diolivorans* (100 % identity; accession no. LC064898/NR037004); 4, *Lactobacillus paracasei/casei/zeae/rhamnosus* (99 % identity; accession no. AP012541/AP012544/NR037122/JQ580982); 5, *Leuconostoc mesenteroides/pseudomesenteroides* (99 % identity; accession no. LC071839/LC096220); and 6, *Acetobacteraceae* (100 % identity).

Bifidobacterium psychraerophilum (98 % identity; accession no. NR029065) was confirmed with the community profiles obtained with the Bif primer pair.

The only band in the community profiles obtained with the Yeast primer pair was attributed to *S. cerevisiae*, and its relative intensities remained stable over the three periods.

3.5 Substrate consumption and metabolite production profiles

The total residual carbohydrate concentrations at the start of the K1 and K2 processes were 234 ± 2 and 222 ± 1 g l^{-1}, respectively; and those at the end of these processes were 221 ± 3 and 228 ± 2 g l^{-1}, respectively (Figure 2). Those in the KR period after 1 and 5 days of fermentation were 152 ± 1 and 148 ± 1 g l^{-1}, respectively. Sucrose remained the main carbohydrate during the K1 process, the KR period, and the K2 process. The sucrose consumption and metabolite production was slower at the start of the K2 process than at the start of the K1 process.

The main metabolites produced during the K1 process, the KR period, and the K2 process were ethanol, lactic acid, glycerol, mannitol, and acetic acid (Figure 2). The production of mannitol started slowly and increased upon progression of the water kefir fermentation process, as this resulted in higher fructose concentrations. The ethanol concentrations at the end of the K1 process, the KR period, and the K2 process were 3.28 ± 0.02, 2.14 ± 0.05, and 4.35 ± 0.01 g l^{-1}, respectively; and the lactic acid concentrations were 1.20 ± 0.01, 0.74 ± 0.01, and 1.48 ± 0.01 g l^{-1}, respectively. The only aroma compounds found in the water kefir liquors were ethyl acetate, isoamyl alcohol, and 2-methyl-1-propanol. The production profiles of these aroma compounds paralleled that of ethanol (Figure 2).

4 Discussion

In this chapter, an industrial water kefir production process suffering from instability and low water kefir grain growth was characterized. The water kefir grain inoculum used by the company was stored frozen at -20 °C and, after thawing, three prefermentations were performed to reactivate the water kefir grains. This was followed by the actual water kefir production process consisting of a first water kefir fermentation process (K1), a rest period at a lower temperature than the fermentation temperature (KR), and a second water kefir fermentation process (K2).

The water kefir grain growth remained zero throughout all prefermentations and fermentation processes. This might have been caused by freezing and thawing of the water kefir grains. Indeed, visual inspection of the water kefir grains used for the production process revealed that their structure was damaged compared to water kefir grains from a household water kefir fermentation process. Furthermore, there were no signs that the damage to the water kefir grains was restored over the course of the prefermentations or the water kefir production process. This was in line with literature data indicating that freezing and thawing damages water kefir grains irreversibly, after which they do not recover their original structure nor do they display water kefir grain growth, even after six backslopping steps (Gulitz, 2013). Water kefir grains contain approximately 86 % (m m^{-1}) water (Laureys & De Vuyst, 2014; Chapters 3 and 4), and the growth of ice crystals during a freezing process may damage the polysaccharide structure of the water kefir grains and/or the cell envelope of the water kefir microorganisms. Quick freezing of fresh water kefir grains in liquid nitrogen followed by a freeze-drying process is a more suitable technique for their storage (Gulitz, 2013).

During the water kefir production process studied, the pH decreased as expected from pH 5.0-6.0 to pH 3.4-3.6 after 3 days of fermentation. However, the concentrations of lactic acid and acetic acid after 3 days of fermentation were only approximately 1.2 and 0.4 g l^{-1}, respectively, whereas they are commonly around 3.0 and 1.0 g l^{-1}, respectively (Laureys & De Vuyst, 2014; Chapters 3 and 4). The low pH values at these low lactic acid and acetic acid concentrations were probably caused by the use of demineralized water during the industrial water kefir production process. Demineralization of water removes most of the minerals and buffer capacity, causing a larger pH decrease than normal for a certain level of acid production. During the rest period, the pH decreased toward 3.3, and such low pH values are associated with low water kefir grain growth (Chapter 4).

Low water kefir grain growth has been associated with high viable counts on the water kefir grains (Chapter 4), but the viable counts of the LAB and yeasts on the water kefir grains of the water kefir production process studied were slightly lower than those reported in the literature (Laureys & De Vuyst, 2014; Chapters 3 and 4). Nevertheless, the ratios of the viable counts of the LAB to those of the yeasts (in the water kefir liquors and on the water kefir grains) and the ratios of the viable counts on the water kefir grains to those in the water kefir liquors (for the LAB and the yeasts) were in line with previous results (Laureys & De Vuyst, 2014; Chapters 3 and 4). The aerobic fermentation conditions during the industrial water kefir production process studied explained the high viable counts of AAB in the water kefir liquors and on the water kefir grains. However, the effect of oxygen on the characteristics of water kefir fermentation has not been studied yet. Thus, a possible effect of oxygen on the LAB and/or yeasts during water kefir fermentation cannot be excluded.

Despite the inoculation with high amounts of water kefir grains that contained more or less normal viable counts of water kefir microorganisms, the water kefir fermentation processes of the present study progressed slowly compared to those described in the literature (Gulitz *et al.*, 2011; Laureys & De Vuyst, 2014; Chapters 3 and 4). Indeed, only a small part of the sucrose was converted into glucose, fructose, EPS, and metabolites. This can be explained by the high carbohydrate concentrations during the industrial production process studied, which may have caused substrate inhibition or excessive osmotic pressure, both preventing a normal functioning of the water kefir microbiota (D'Amore *et al.*, 1988), among which EPS production (Hehre, 1946). The lag phase at the start of the K2 process was probably caused by prolonged fermentation at 8 °C during the preceding rest period.

An apparent increase of the total residual carbohydrate concentration during the K2 process could be explained by the diffusion of carbohydrates from the dried figs into the water, as dried figs contain approximately 48 % (m m^{-1}) mono- and disaccharides (release 26, http://ndb.nal.usda.gov/). Glucose seemed to be the preferred substrate for metabolite production during fermentation, as its concentrations increased slower compared to those of fructose, indicating faster consumption of glucose than fructose. The only aroma compounds found in the water kefir liquors were isoamyl alcohol, 2-methyl-1-propanol, and ethyl acetate. The esters ethyl hexanoate, ethyl octanoate, ethyl decanoate, and isoamyl acetate, which might be responsible for the fruity aroma of water kefir beverages (Lambrechts & Pretorius, 2000), were not found in the water kefir liquors produced by the industrial water kefir production process studied.

Three of the four key microorganisms of water kefir fermentation, namely *Lb. paracasei*, *Lb. nagelii*, and *S. cerevisiae*, were present throughout the entire industrial water kefir production process studied, as revealed by both culture-dependent and culture-independent species diversity analyses (Chapter 4). However, the fourth key microorganism, *Lb hilgardii*, which is assumed to be responsible for water kefir grain growth, was only found through culture-independent analyses, whereby its relative abundances decreased over the course of

the production process. Hence, in contrast with the cells of the other key microorganisms, those of *Lb. hilgardii* might have been damaged by the freezing and thawing process, compromising the viability of this LAB species during water kefir fermentation, as has been shown before (Gulitz, 2013). Additionally, *Leuc. mesenteroides*, a non-identified *Bifidobacterium* species, *G. roseus*/*oxydans*, *G. cerinus*, and *Z. florentinus* were present during the industrial water kefir production process studied. All these microorganisms have been found in water kefir fermentations before (Pidoux, 1989; Gulitz *et al.*, 2011, 2013; Gulitz, 2013; Laureys & De Vuyst, 2014; Chapters 3 and 4). The relative abundances of a non-identified *Bifidobacterium* species increased over the entire water kefir production process, indicating that this species is not sensitive to oxygen or acidic stress.

In conclusion, the industrial water kefir production process studied performed poorly. The structure of the industrial water kefir grains that were frozen and thawed was damaged in comparison with that of the grains of a household water kefir fermentation process. The substrate concentrations were very high in comparison with those of common water kefir fermentation processes. Only a small part of the substrate was converted into metabolites and water kefir grain wet mass. Demineralized water should be supplemented with a buffer to avoid excessive acidic stress during water kefir fermentation. Prolonged fermentation at low temperature during a rest period should also be avoided, as this resulted in a lag phase during the subsequent water kefir fermentation process.

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CHAPTER 6

Bifidobacterium aquikefiri sp. nov., isolated from water kefir

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SUMMARY

A novel *Bifidobacterium*, strain LMG 28769^T, was isolated from a household water kefir fermentation process. The cells were Gram-stain-positive, non-motile, non-spore-forming, catalase-negative, oxidase-negative, and facultatively anaerobic short rods. Analysis of its 16S rRNA gene sequence revealed *Bifidobacterium crudilactis* and *Bifidobacterium psychraerophilum* (97.4 % and 97.1 % similarity towards the respective type strain sequences) as nearest phylogenetic neighbors. Its assignment to the genus *Bifidobacterium* was confirmed by the presence of fructose 6-phosphate phosphoketolase (F6PPK) activity. Analysis of the *hsp60* gene sequence revealed a very low similarity with nucleotide sequences in the NCBI nucleotide database. The genotypic and phenotypic analyses allowed to differentiate strain LMG 28769^T from all established *Bifidobacterium* species. Strain LMG 28769^T (= CCUG 67145^T = R 54638^T) therefore represents a new species, for which the name *Bifidobacterium aquikefiri* sp. nov. is proposed.

1 Introduction

Bifidobacteria are Gram-stain-positive, non-motile, non-spore-forming bacteria that are usually associated with the gut microbiota of humans and animals (Simpson *et al.*, 2004; Biavati & Mattarelli, 2006). They are generally obligately anaerobic but some species can also grow aerobically (Simpson *et al.*, 2004; Delcenserie *et al.*, 2007; Watanabe *et al.*, 2009). Bifidobacteria are considered to be non-pathogenic (Borriello *et al.*, 2003) and some species occur in fermented foods and beverages (Delcenserie *et al.*, 2007; Watanabe *et al.*, 2009; Hsieh *et al.*, 2012; Gulitz *et al.*, 2013; Laureys & De Vuyst, 2014; Chapters 3, 4, and 5). Sometimes, bifidobacterial strains are added to foods and beverages because their consumption is associated with positive health effects (Tojo *et al.*, 2014). Recently, an unknown *Bifidobacterium* species was detected in several water kefirs from different origins via their community profiles obtained through 16S rRNA-PCR-denaturing gradient gel electrophoresis (DGGE) with the universal prokaryotic primer pair 357f-GC/518r and the *Bifidobacterium*-specific primer pair bif164f/bif662r-GC (Laureys & De Vuyst, 2014; Chapters 3, 4, and 5). The 0.5-kb partial 16S rRNA gene sequences, obtained with the latter primer pair, were 100 % identical to the 16S rRNA gene sequence of an unknown *Bifidobacterium* species detected in a water kefir fermentation process in Germany (Gulitz *et al.*, 2013), but were only 98 % identical to the 16S rRNA gene sequence of the closest relative *Bifidobacterium psychraerophilum*. These data suggested that a novel *Bifidobacterium* species was present in water kefir, which may even be specific for water kefir.

This chapter aimed to isolate and characterize the non-identified *Bifidobacterium* species found in several water kefirs and to determine if it represents a novel species.

2 Materials and methods

2.1 Isolation

Water kefir liquor was obtained from the household water kefir fermentation process maintained by a private person described in Chapter 3. Strain R 54638^T (= LMG 28769^T) was isolated from the water kefir liquor by plating on modified tryptone-yeast extract (mTY) agar medium (Gulitz *et al.*, 2013), supplemented with cycloheximide (final concentration of 0.1 g l^{-1}; Sigma-Aldrich, Saint Louis, MO, USA), kanamycin sulfate (final concentration of 0.05 g l^{-1}; Sigma-Aldrich), mupirocin (final concentration of 0.05 g l^{-1}; AppliChem, Darmstadt, Germany), and amphotericin B (final concentration of 0.005 g l^{-1}; Sigma-Aldrich). The agar media were incubated anaerobically (AnaeroGen™; Thermo Fisher Scientific, Waltham, MA, USA) at 30 °C for 6 days.

2.2 Genotypic characterization

For its genotypic characterization, strain LMG 28769^T was grown on M144 agar medium [23.0 g l^{-1} special peptone (Oxoid, Basingstoke, UK), 1.0 g l^{-1} soluble starch (Merck, Darmstadt, Germany), 5.0 g l^{-1} NaCl (Merck), 0.3 g l^{-1} cysteine hydrochloride (Sigma-Aldrich), 5.0 g l^{-1} glucose (Merck), and 15.0 g l^{-1} agar (Oxoid)] and DNA was obtained via alkaline lysis of the cells, as described before (Niemann *et al.*, 1997).

The near-complete 16S rRNA gene of strain LMG 28769^T was amplified and sequenced according to both Coenye *et al.* (1999) and Kim *et al.* (2010). The consensus sequence was compared with sequences in the EzTaxon database (Kim *et al.*, 2012), with sequences in the NCBI database (Johnson *et al*., 2008), and with sequence fragments of bifidobacteria formerly

detected in water kefir (Gulitz *et al.*, 2013; Laureys & De Vuyst, 2014; Chapters 3 and 4), using the NCBI BLASTn tool (Zhang *et al.*, 2000). The *hsp60* gene of strain LMG 28769^T was amplified and sequenced with the primer pairs HspF3/HspR4 and HspBF3/HspBR4, as described by Kim *et al.* (2010). The consensus sequence was compared with sequences in the NCBI nucleotide databases (Johnson *et al.*, 2008), as described above. This sequence was also translated into protein sequences and compared with translated sequences in the NCBI nucleotide database using the tBLASTx tool (Altschul *et al.*, 1997).

To reconstruct a phylogenetic tree, the 16S rRNA and *hsp60* gene sequences of the type strains of all *Bifidobacterium* species were retrieved from the NCBI nucleotide database. The MEGA6 software package (Tamura *et al.*, 2013) was used to align the sequences with the MUSCLE algorithm (Edgar, 2004), and to reconstruct the phylogenetic trees with both the maximum-likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993) and the neighbor-joining method (Saitou & Nei, 1987). The statistical reliability of the tree topology was evaluated via a bootstrapping analysis based on 1000 replicates.

The G + C content (%; mol mol^{-1}) of the DNA from strain LMG 28769^T was determined through high-performance liquid chromatography with ultraviolet detection (HPLC-UV) with a high-performance liquid chromatograph equipped with an XBridge BEH Shield RP18 column coupled to an ultraviolet detector (Waters, Milford, MA, USA). Hereto, genomic DNA was hydrolyzed enzymatically, as described before (Mesbah & Whitman, 1989), and the hydrolysate was injected into the column and isocratically eluted with a mixture of 0.02 M $NH_4H_2PO_4$ at pH 4.0 (98.5 %, v v^{-1}) and acetonitrile (1.5 %, v v^{-1}). Non-methylated lambda phage DNA (Sigma-Aldrich) was used as a calibration reference and genomic DNA from *Escherichia coli* LMG 2093 was included as a control.

2.3 Phenotypic characterization

The colony and cell morphologies of strain LMG 28769^T were assessed after 6 days of anaerobic growth (AnaeroGen^{TM}) at 28 °C on M144 agar medium. Growth of strain LMG 28769^T, *B. crudilactis* LMG 23609^T, and *B. psychraerophilum* LMG 21775^T was assessed at 28 °C in M144 broth [23.0 g l^{-1} special peptone (Oxoid), 1.0 g l^{-1} soluble starch (Merck), 5.0 g l^{-1} NaCl (Merck), 0.3 g l^{-1} cysteine hydrochloride (Sigma-Aldrich), and 5.0 g l^{-1} glucose (Merck)] and on M144 agar medium in an anaerobic incubator (95% N_2 and 5% H_2), an anaerobic jar (AnaeroGen^{TM}), a microaerobic jar (CO_2Gen^{TM}, Thermo Fisher Scientific), and under aerobic atmosphere. Growth of strain LMG 28769^T, *B. crudilactis* LMG 23609^T, and *B. psychraerophilum* LMG 21775^T at different pH values was assessed at 28 °C under anaerobic atmosphere (AnaeroGen^{TM}) in M144 broth supplemented with 0.1 M citric acid and adjusted to pH 3.0, 3.5, 4.0, 4.5, 5.0, and 6.0; in M144 broth supplemented with 0.1 M NaH_2PO_4 and adjusted to pH 7.0; and in M144 broth supplemented with 0.1 M Tris-HCl and adjusted to pH 8.0 and 9.0. The pH adjustment was carried out with 1 M NaOH or 1 M HCl. Growth of strain LMG 28769^T, *B. crudilactis* LMG 23609^T, and *B. psychraerophilum* LMG 21775^T was tested at 4, 7, 15, 28, 37, and 45 °C in M144 broth and on M144 agar medium. Growth under different atmospheric conditions, at different pH values, and at different temperatures was visually assessed after 6 and 13 days of incubation. The production of gas during growth was visually assessed with inverted Durham tubes.

Enzyme activities and acid production from different substrates by strain LMG 28769^T, *B. crudilactis* LMG 23609^T, and *B. psychraerophilum* LMG 21775^T were assessed after 4 days of anaerobic growth (AnaeroGen^{TM}) at 28 °C on M144 agar medium. Cells were suspended in 0.85 % (m v^{-1}) NaCl to prepare a suspension with turbidity similar to a McFarland No. 5 standard, from which 65 µl was added to each enzyme test of the API ZYM kit (bioMérieux,

Marcy l'Etoile, France). The results were read after 5 h of anaerobic incubation (AnaeroGen^{TM}) at 28 °C. Cells were also suspended in 0.85 % (m v^{-1}) NaCl to prepare a suspension with turbidity similar to a McFarland No. 2 standard, from which eight drops were added to the API 50CHL medium, which was used to inoculate each substrate test of the API 50CHL kit (bioMérieux). The results were read after 6 and 13 days of anaerobic incubation (AnaeroGen^{TM}) at 28 °C.

To assess the production of metabolites from glucose, strain LMG 28769^T, *B. crudilactis* LMG 23609^T, and *B. psychraerophilum* LMG 21775^T were incubated anaerobically (AnaeroGen^{TM}) at 28 °C in M144 broth. After 24, 48, and 72 h, the cultures were centrifuged (7200 x *g*, 5 min, 4 °C) and the cell-free culture supernatants were used for metabolite analyses. The presence of formic acid and the concentrations of lactic acid and acetic acid were determined through HPLC, making use of a high-performance liquid chromatograph (Waters) equipped with an ICSep ICE-ORH-801 column (Interchim, Montluçon, France) and coupled to a refractive index detector (Waters), as described before (Makras *et al.*, 2005). The concentrations of D- and L-lactic acid were measured through HPLC-UV with a high-performance liquid chromatograph (Waters) equipped with a Shodex ORpak CRX-853 column (Showa Denko, Tokyo, Japan) coupled to an UV detector (Waters). For the above, 250 µl of cell-free culture supernatant was added to a mixture of 500 µl of acetonitrile and 250 µl of ultrapure water. The samples were vortexed, centrifuged (21,000 x *g*, 20 min, 4 °C), and filtered (0.2-µm pore-size Whatman filters; GE Healthcare Life Sciences, Bucks, UK) before they were injected into the columns. Quantifications were performed with an external standard curve with standards prepared in the same way as the samples.

To assess the presence of fructose 6-phosphate phosphoketolase (F6PPK), strain LMG 28769^T, *B. crudilactis* LMG 23609^T, and *B. psychraerophilum* LMG 21775^T were incubated anaerobically (AnaeroGen^{TM}) at 28 °C in M144 broth. After 72 h, the cultures were centrifuged (7200 x *g*, 5 min, 4 °C) and the cell pellets were used for a F6PPK assay, as described before (Orban & Patterson, 2000). Briefly, the cell pellets were washed twice with 0.05 M phosphate buffer supplemented with 0.5 mg l^{-1} cysteine-HCl (adjusted to pH 6.5) and lysed with cetyl-trimethylammonium bromide. After the addition of sodium fluoride, sodium iodoacetate, and dipotassium fructose 6-phospate, the samples were incubated at 37 °C for 30 min. This was followed by addition of hydroxylamine-HCl, trichloroacetic acid, HCl, and $FeCl_3$, after which the color reaction to reddish-violet was visually assessed. A pellet of non-inoculated M144 broth and a cell pellet of strain LMG 28769^T without fructose 6-phosphate were used as negative controls. *Bifidobacterium crudilactis* LMG 23609^T and *B. psychraerophilum* LMG 21775^T were used as positive controls.

To assess gelatine degradation, strain LMG 28769^T, *B. crudilactis* LMG 23609^T, *B. psychraerophilum* LMG 21775^T, and *Serratia marcescens* LMG 2792^T (positive control) were inoculated into test tubes containing 5 ml of M144 broth supplemented with 12 % (m v^{-1}) gelatine. After 6 days of anaerobic incubation (AnaeroGen^{TM}) at 28 °C, the test tubes were cooled to 7 °C and gelatine degradation (liquefaction) was visually assessed. Non-inoculated test tubes were used as negative controls.

To assess casein degradation, strain LMG 28769^T, *B. crudilactis* LMG 23609^T, *B. psychraerophilum* LMG 21775^T, and *Bacillus subtilis* LMG 7135^T (positive control) were grown on M144 agar medium supplemented with 13 g l^{-1} of skimmed milk powder (Oxoid). After 6 days of anaerobic incubation (AnaeroGen^{TM}) at 28 °C, casein degradation (clear zone around the colonies) was visually assessed. Non-inoculated agar media were used as negative controls.

3 Results and discussion

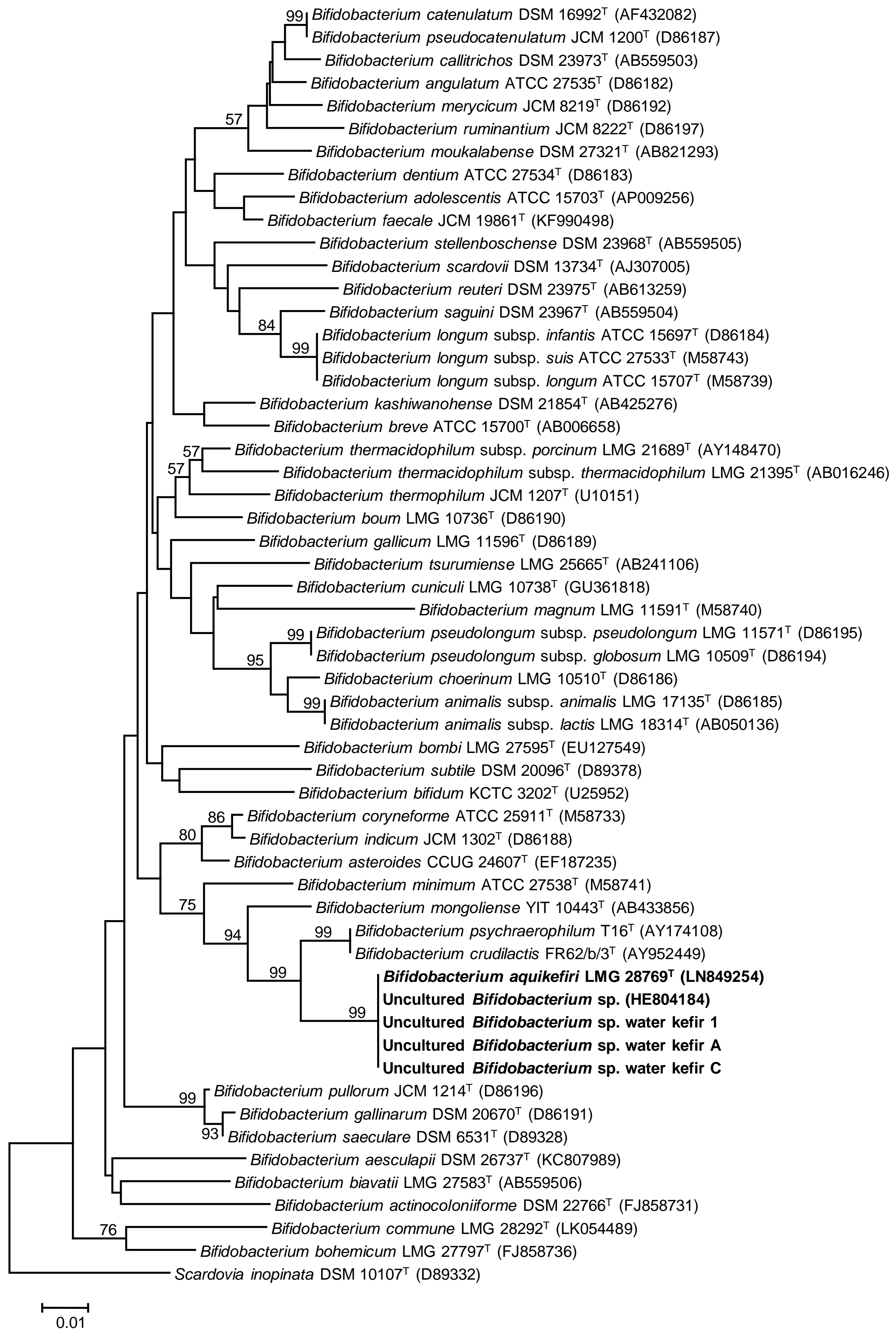
3.1 Genotypic characterization

The 16S rRNA gene sequences obtained from genomic DNA of strain LMG 28769^T with the primer pairs described by Coenye *et al.* (1999) and Kim *et al.* (2010) were identical and the consensus sequence (1445 bp) was deposited in the NCBI nucleotide database (GenBank accession no. LN849254). Comparison of the 16S rRNA gene sequence of strain LMG 28769^T with sequences in the EzTaxon database (Kim *et al.*, 2012) revealed *Bifidobacterium crudilactis* FR62/b/3^T (97.42 % pairwise similarity; accession no. AY952449) and *B. psychraerophilum* T16^T (97.06 % pairwise similarity; accession no. AY174108) as nearest phylogenetic neighbors. Comparison of the 16S rRNA gene sequence of strain LMG 28769^T with sequences in the NCBI nucleotide database confirmed the results obtained via the EzTaxon database, and further revealed that the 16S rRNA gene sequence of strain LMG 28769^T was identical to the 16S rRNA gene sequence of an uncultured *Bifidobacterium* species (100.00 % pairwise similarity; accession no. HE804184) detected in a water kefir sample in Germany (Gulitz *et al.*, 2013). Furthermore, the partial 16S rRNA gene sequences from uncultured *Bifidobacterium* species in a water kefir from Ghent, Belgium (uncultured *Bifidobacterium* sp. water kefir 1; Chapter 3), in a water kefir from Leuven, Belgium (uncultured *Bifidobacterium* sp. water kefir A; Chapter 4), and in a water kefir from Lokeren, Belgium (uncultured *Bifidobacterium* sp. water kefir C; Chapter 4), were all 100 % identical to the 16S rRNA gene sequence obtained from *Bifidobacterium* strain LMG 28769^T (Laureys & De Vuyst, 2014; Chapter 3).

The phylogenetic tree topologies of the 16S rRNA gene sequences reconstructed with the maximum-likelihood method and the neighbor-joining methods were similar and only the phylogenetic tree constructed with the neighbor-joining method is shown (Figure 1).

The *hsp60* gene sequences of strain LMG 28769^T obtained with the primer pairs HspF3/HspR4 and HspBF3/HspBR4 were identical and the consensus sequence (605 bp) was deposited in the NCBI nucleotide database (accession no. LN849255). Comparison of this *hsp60* gene sequence with those of the *Bifidobacterium* type strains retrieved from the NCBI nucleotide database (Johnson *et al.*, 2008) revealed an unexpected low similarity level with all other bifidobacterial species, including its nearest neighbors *B. crudilactis* LMG 23609^T (84.27 % pairwise similarity; accession no. LN849256) and *Bifidobacterium adolescentis* JCM 1275^T (84.16 % pairwise similarity; accession no. AF210319). As a consequence, strain LMG 28769^T appeared only remotely related to other members of the genus *Bifidobacterium* in the phylogenetic tree based on the *hsp60* gene sequences (Figure 2). A comparison of all translated *hsp60* nucleotide sequences revealed that *B. adolescentis* JCM 1275^T (95.02 % pairwise similarity; 98.10 % positives; accession no. AF210319) and *Bifidobacterium kashiwanohense* DSM 21854^T (94.03 % pairwise similarity; 98.01 % positives; accession no. AB491759) had the most similar amino acid sequences to the amino acid sequence of the Hsp60 protein of strain LMG 28769^T (data not shown).

The mean G + C content of the DNA from strain LMG 28769^T was 52.6 ± 0.5 mol%, which is within the range of 50-67 mol% G + C found previously for the genus *Bifidobacterium* (Mattarelli *et al.*, 2014).



99 Bifidobacterium catenulatum DSM 16992T (AF432082)
Bifidobacterium pseudocatenulatum JCM 1200T (D86187)
Bifidobacterium callitrichos DSM 23973T (AB559503)
Bifidobacterium angulatum ATCC 27535T (D86182)
Bifidobacterium merycicum JCM 8219T (D86192)
57
Bifidobacterium ruminantium JCM 8222T (D86197)
Bifidobacterium moukalabense DSM 27321T (AB821293)
Bifidobacterium dentium ATCC 27534T (D86183)
Bifidobacterium adolescentis ATCC 15703T (AP009256)
Bifidobacterium faecale JCM 19861T (KF990498)
Bifidobacterium stellenboschense DSM 23968T (AB559505)
Bifidobacterium scardovii DSM 13734T (AJ307005)
Bifidobacterium reuteri DSM 23975T (AB613259)
Bifidobacterium saguini DSM 23967T (AB559504)
84
Bifidobacterium longum subsp. infantis ATCC 15697T (D86184)
99
Bifidobacterium longum subsp. suis ATCC 27533T (M58743)
Bifidobacterium longum subsp. longum ATCC 15707T (M58739)
Bifidobacterium kashiwanohense DSM 21854T (AB425276)
Bifidobacterium breve ATCC 15700T (AB006658)
57 Bifidobacterium thermacidophilum subsp. porcinum LMG 21689T (AY148470)
57
Bifidobacterium thermacidophilum subsp. thermacidophilum LMG 21395T (AB016246)
Bifidobacterium thermophilum JCM 1207T (U10151)
Bifidobacterium boum LMG 10736T (D86190)
Bifidobacterium gallicum LMG 11596T (D86189)
Bifidobacterium tsurumiense LMG 25665T (AB241106)
Bifidobacterium cuniculi LMG 10738T (GU361818)
Bifidobacterium magnum LMG 11591T (M58740)
99 Bifidobacterium pseudolongum subsp. pseudolongum LMG 11571T (D86195)
95
Bifidobacterium pseudolongum subsp. globosum LMG 10509T (D86194)
Bifidobacterium choerinum LMG 10510T (D86186)
99 Bifidobacterium animalis subsp. animalis LMG 17135T (D86185)
Bifidobacterium animalis subsp. lactis LMG 18314T (AB050136)
Bifidobacterium bombi LMG 27595T (EU127549)
Bifidobacterium subtile DSM 20096T (D89378)
Bifidobacterium bifidum KCTC 3202T (U25952)
86 Bifidobacterium coryneforme ATCC 25911T (M58733)
80
Bifidobacterium indicum JCM 1302T (D86188)
Bifidobacterium asteroides CCUG 24607T (EF187235)
Bifidobacterium minimum ATCC 27538T (M58741)
75
Bifidobacterium mongoliense YIT 10443T (AB433856)
94
99 Bifidobacterium psychraerophilum T16T (AY174108)
Bifidobacterium crudilactis FR62/b/3T (AY952449)
99
Bifidobacterium aquikefiri LMG 28769T (LN849254)
Uncultured Bifidobacterium sp. (HE804184)
99
Uncultured Bifidobacterium sp. water kefir 1
Uncultured Bifidobacterium sp. water kefir A
Uncultured Bifidobacterium sp. water kefir C
99 Bifidobacterium pullorum JCM 1214T (D86196)
Bifidobacterium gallinarum DSM 20670T (D86191)
93 Bifidobacterium saeculare DSM 6531T (D89328)
Bifidobacterium aesculapii DSM 26737T (KC807989)
Bifidobacterium biavatii LMG 27583T (AB559506)
Bifidobacterium actinocoloniiforme DSM 22766T (FJ858731)
76 Bifidobacterium commune LMG 28292T (LK054489)
Bifidobacterium bohemicum LMG 27797T (FJ858736)
Scardovia inopinata DSM 10107T (D89332)
0.01

3.2 Phenotypic characterization

Strain LMG 28769^T, *B. crudilactis* LMG 23609^T, and *B. psychraerophilum* LMG 21775^T grew at 4-37 °C, and under aerobic and anaerobic conditions. The three strains showed activity of F6PPK, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *α*-galactosidase, *β*-galactosidase, *α*-glucosidase, and *β*-glucosidase, but did not show activity of esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, trypsin, *α*-chymotrypsin, *β*-glucuronidase, N-acetyl-*β*-glucosaminidase, *α*-mannosidase, or *α*-fucosidase. None of the three strains degraded gelatine or casein. Strain LMG 28769^T, *B. crudilactis* LMG 23609^T, and *B. psychraerophilum* LMG 21775^T produced acid from D-ribose, D-galactose, D-glucose, D-fructose, methyl *α*-D-glucopyranoside, maltose, melibiose, sucrose, raffinose, and potassium gluconate, but not from glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl *β*-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl *α*-D-mannopyranoside, arbutin, aesculin ferric citrate, trehalose, inulin, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium 2-ketogluconate, or potassium 5-ketogluconate. All three strains produced lactic acid exclusively in the L-isomer form. The differential characteristics between strain LMG 28769^T, *B. crudilactis* LMG 23609^T, and *B. psychraerophilum* LMG 21775^T are shown in Table 1. An overview of the phenotypic characteristics of strain LMG 28769^T is provided in the species description below.

Based on the data presented, strain LMG 28769^T represents a novel species of the genus *Bifidobacterium*, for which the name *Bifidobacterium aquikefiri* sp. nov. was proposed.

4 Description of *Bifidobacterium aquikefiri* sp. nov.

Bifidobacterium aquikefiri (a.qui.ke'fi.ri. L. n. *aqua* water; N.L. gen. n. *kefiri* from kefir; N.L. gen. n. *aquikefiri* from water kefir).

Cells are Gram-stain-positive, non-filamentous, non-motile, non-spore-forming, catalase-negative, and oxidase-negative. They form short rods of 0.5-1.0 µm thick and 1.0-2.0 µm long without bifurcations; some cells are club-shaped. After 6 days of anaerobic growth at 28 °C on M144 agar medium, colonies are around 1 mm in diameter, circular, convex, smooth with smooth edges, translucent, and creamy coloured. Growth occurs under anaerobic, microaerobic, and aerobic conditions, from pH 4.0 to 8.0, and at a temperature of 4-37 °C with an optimum temperature of 28 °C. Growth does not occur at pH 3.5 or pH 9.0, or at 45 °C.

Figure 1. Phylogenetic tree based on the 16S rRNA gene sequences of the genus *Bifidobacterium*, including *Bifidobacterium aquikefiri* LMG 28769^T, an uncultured *Bifidobacterium* species detected in a water kefir sample from Germany (Gulitz et al., 2013), and the partial 16S rRNA gene sequences of a *Bifidobacterium* species found in a water kefir from Ghent, Belgium (uncultured *Bifidobacterium* sp. water kefir 1; chapter 3), a water kefir from Leuven, Belgium (uncultured *Bifidobacterium* sp. water kefir A; chapter 4), and a water kefir from Lokeren, Belgium (uncultured *Bifidobacterium* sp. water kefir C; chapter 4). With the MEGA6 software package (Tamura et al., 2013), the sequences were aligned with the MUSCLE algorithm (Edgar, 2004), and the phylogenetic tree was constructed with the neighbor-joining method (Saitou & Nei, 1987). The bootstrap values were calculated from 1000 replicates, and only values > 50 % are shown. The 16S rRNA gene sequence of *Scardovia inopinata* DSM 10107^T was used as an outgroup. The horizontal length of the bars corresponds to the number of substitutions per nucleotide position.

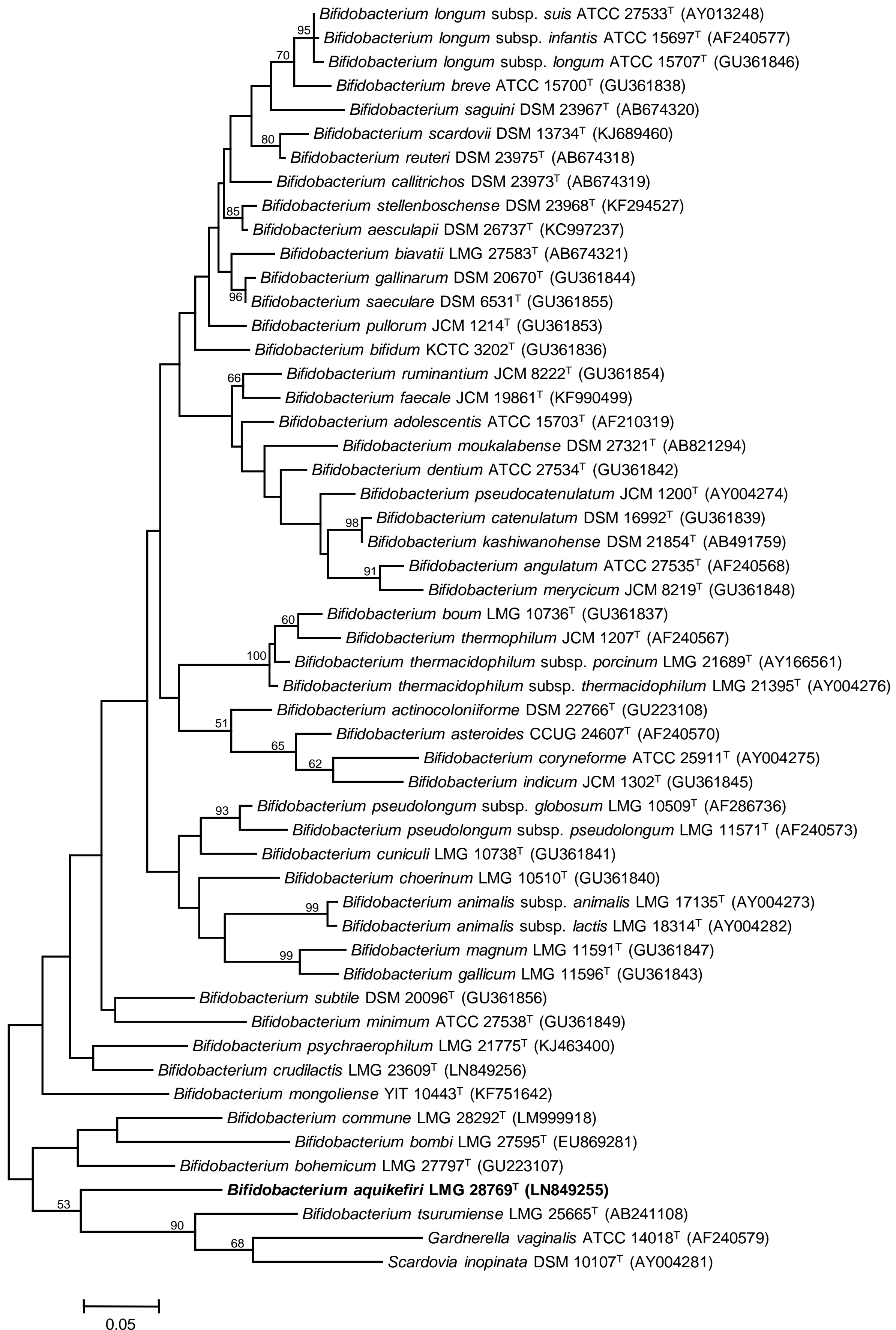

Bifidobacterium longum subsp. *suis* ATCC 27533^{T} (AY013248)
Bifidobacterium longum subsp. *infantis* ATCC 15697^{T} (AF240577)
Bifidobacterium longum subsp. *longum* ATCC 15707^{T} (GU361846)
Bifidobacterium breve ATCC 15700^{T} (GU361838)
Bifidobacterium saguini DSM 23967^{T} (AB674320)
Bifidobacterium scardovii DSM 13734^{T} (KJ689460)
Bifidobacterium reuteri DSM 23975^{T} (AB674318)
Bifidobacterium callitrichos DSM 23973^{T} (AB674319)
Bifidobacterium stellenboschense DSM 23968^{T} (KF294527)
Bifidobacterium aesculapii DSM 26737^{T} (KC997237)
Bifidobacterium biavatii LMG 27583^{T} (AB674321)
Bifidobacterium gallinarum DSM 20670^{T} (GU361844)
Bifidobacterium saeculare DSM 6531^{T} (GU361855)
Bifidobacterium pullorum JCM 1214^{T} (GU361853)
Bifidobacterium bifidum KCTC 3202^{T} (GU361836)
Bifidobacterium ruminantium JCM 8222^{T} (GU361854)
Bifidobacterium faecale JCM 19861^{T} (KF990499)
Bifidobacterium adolescentis ATCC 15703^{T} (AF210319)
Bifidobacterium moukalabense DSM 27321^{T} (AB821294)
Bifidobacterium dentium ATCC 27534^{T} (GU361842)
Bifidobacterium pseudocatenulatum JCM 1200^{T} (AY004274)
Bifidobacterium catenulatum DSM 16992^{T} (GU361839)
Bifidobacterium kashiwanohense DSM 21854^{T} (AB491759)
Bifidobacterium angulatum ATCC 27535^{T} (AF240568)
Bifidobacterium merycicum JCM 8219^{T} (GU361848)
Bifidobacterium boum LMG 10736^{T} (GU361837)
Bifidobacterium thermophilum JCM 1207^{T} (AF240567)
Bifidobacterium thermacidophilum subsp. *porcinum* LMG 21689^{T} (AY166561)
Bifidobacterium thermacidophilum subsp. *thermacidophilum* LMG 21395^{T} (AY004276)
Bifidobacterium actinocoloniiforme DSM 22766^{T} (GU223108)
Bifidobacterium asteroides CCUG 24607^{T} (AF240570)
Bifidobacterium coryneforme ATCC 25911^{T} (AY004275)
Bifidobacterium indicum JCM 1302^{T} (GU361845)
Bifidobacterium pseudolongum subsp. *globosum* LMG 10509^{T} (AF286736)
Bifidobacterium pseudolongum subsp. *pseudolongum* LMG 11571^{T} (AF240573)
Bifidobacterium cuniculi LMG 10738^{T} (GU361841)
Bifidobacterium choerinum LMG 10510^{T} (GU361840)
Bifidobacterium animalis subsp. *animalis* LMG 17135^{T} (AY004273)
Bifidobacterium animalis subsp. *lactis* LMG 18314^{T} (AY004282)
Bifidobacterium magnum LMG 11591^{T} (GU361847)
Bifidobacterium gallicum LMG 11596^{T} (GU361843)
Bifidobacterium subtile DSM 20096^{T} (GU361856)
Bifidobacterium minimum ATCC 27538^{T} (GU361849)
Bifidobacterium psychraerophilum LMG 21775^{T} (KJ463400)
Bifidobacterium crudilactis LMG 23609^{T} (LN849256)
Bifidobacterium mongoliense YIT 10443^{T} (KF751642)
Bifidobacterium commune LMG 28292^{T} (LM999918)
Bifidobacterium bombi LMG 27595^{T} (EU869281)
Bifidobacterium bohemicum LMG 27797^{T} (GU223107)
***Bifidobacterium aquikefiri* LMG 28769^{T} (LN849255)**
Bifidobacterium tsurumiense LMG 25665^{T} (AB241108)
Gardnerella vaginalis ATCC 14018^{T} (AF240579)
Scardovia inopinata DSM 10107^{T} (AY004281)
0.05

Table 1. Differential characteristics between *Bifidobacterium aquikefiri* LMG 28769^T, *Bifidobacterium crudilactis* LMG 23609^T, and *Bifidobacterium psychraerophilum* LMG 21775^T. The biochemical characteristics were evaluated as present (+), weakly present (±), or absent (-).

Characteristic	LMG 28769^T	LMG 23609^T	LMG 21775^T
Growth[c]			
Temperature (°C)	4-37	4-37 (45[a])	4-37
Optimal temperature (°C)	28	37	37
pH	4.0-8.0	4.0 (4.7[a])-8.0	4.5-8.0
Enzyme activity[d]			
Alkaline phosphatase	-	±	+(-[b])
Cystine arylamidase	-	±	-
Acid phosphatase	±	±	+
Naphthol-AS-BI-phosphohydrolase	±	±	+
β-Glucosidase	+	±	+
Production of acid from[c]			
L-Arabinose	+	-	+
D-Xylose	-	-	+
D-Mannose	+	-	-
D-Mannitol	±	-	-
Methyl α-D-glucopyranoside	+	+ (-[a])	+
N-acetyl-glucosamine	±	-	-
Amygdaline	±	-	+
Salicin	-	-	+
Cellobiose	-	+	-(+[b])
Maltose	+	+	+(-[b])
Lactose	-	+	-
Melezitose	-	+(-[a])	+
Gentiobiose	+	-	+
Turanose	+	+	-
Potassium gluconate	+	+(-[a])	+
Metabolites in M144 broth[e]			
Molar ratio of acetic acid/lactic acid	4.83 ± 0.35	1.49 ± 0.01	2.77 ± 0.16
Production of formic acid	+	-	+
DNA G + C content (%; mol mol^{-1})	52.6	56.4[a]	59.2[b]

[a] Delcenserie *et al.* (2007).
[b] Simpson *et al.* (2004).
[c] Assessed after 13 days of anaerobic incubation at 28 °C.
[d] Assessed after 5 h of anaerobic incubation at 28 °C.
[e] Assessed after 72 h of anaerobic incubation at 28 °C.

Figure 2. Phylogenetic tree based on the *hsp60* gene sequences of the genus *Bifidobacterium*, including *Bifidobacterium aquikefiri* LMG 28769^T. With the MEGA6 software package (Tamura *et al.*, 2013), the sequences were aligned with the MUSCLE algorithm (Edgar, 2004), and the phylogenetic tree was constructed with the neighbor-joining method (Saitou & Nei, 1987). The bootstrap values were calculated from 1000 replications, and only values > 50 % are shown. The *hsp60* gene sequence of *Scardovia inopinata* DSM 10107^T and *Gardnerella vaginalis* ATCC 14018^T were used as an outgroup. The horizontal lengths of the bars correspond to the number of substitutions per nucleotide position.

When grown on glucose in M144 broth, no gas is produced and the main metabolites are acetic acid, lactic acid, and formic acid. The molar ratio of acetic acid to lactic acid is 4.8, and lactic acid is produced exclusively in the L-isomer form. Acid is produced from L-arabinose, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, D-mannitol (weak), methyl *α*-D-glucopyranoside, N-acetylglucosamine (weak), amygdalin (weak), maltose, melibiose, sucrose, raffinose, gentiobiose, turanose, and potassium gluconate. Acid is not produced from glycerol, erythritol, D-arabinose, D-xylose, L-xylose, D-adonitol, methyl *β*-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl *α*-D-mannopyranoside, arbutin, aesculin ferric citrate, salicin, cellobiose, lactose, trehalose, inulin, melezitose, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium 2-ketogluconate, or potassium 5-ketogluconate. Activity of F6PPK, leucine arylamidase, acid phosphatase (weak) naphthol-AS-BI-phosphohydrolase (weak), *α*-galactosidase, *β*-galactosidase, *α*-glucosidase, and *β*-glucosidase is present. Activity of alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, *α*-chymotrypsin, *β*-glucuronidase, N-acetyl-*β*-glucosaminidase, *α*-mannosidase, and *α*-fucosidase is not present, and gelatin and casein are not degraded.

The type strain, LMG 28769^T (= CCUG 67145^T = R 54638^T), was isolated from a household water kefir fermentation process carried out in Brussels, Belgium, in 2014. Its DNA G + C content is 52.6 %.

Acknowledgements

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CHAPTER 7

The buffer capacity and calcium concentration of the water influence the microbial species diversity, grain growth, and metabolite production during water kefir fermentation

David Laureys, Maarten Aerts, Peter Vandamme, and Luc De Vuyst

SUMMARY

Eight water kefir fermentation series differing in buffer capacity and calcium concentration of the water were studied during eight backslopping steps. A high buffer capacity resulted in high pH values and a high calcium concentration in low pH values at the end of each backslopping step. When the buffer capacity and/or calcium concentration of the water were below certain minima, the water kefir grain growth decreased gradually over multiple backsloppings. High buffer capacity of the water resulted in higher concentrations of residual total carbohydrate concentrations and lower metabolite concentrations. Further, high buffer capacity of the water resulted in high ratios of lactic acid bacteria to yeasts, which was reflected in high molar ratios of the concentrations of lactic acid to ethanol and acetic acid to ethanol. The most prevalent microorganisms on the water kefir grain inoculum and on the grains of the eight different fermentation series at the end of backslopping step 8 were *Lactobacillus hilgardii*, *Lactobacillus nagelii*, *Lactobacillus paracasei*, *Bifidobacterium aquikefiri*, *Saccharomyces cerevisiae*, and *Dekkera bruxellensis*. The buffer capacity of the water influenced the microbial communities, which in turn impacted the substrate consumption and metabolite production during water kefir fermentation.

1 Introduction

Water kefir is a traditional fermented beverage that is produced worldwide under a variety of names (Pothakos *et al.*, 2016). The water kefir fermentation process is started by adding water kefir grains (the inoculum) to a mixture of water, (dried) fruits, and sugar; and is usually performed at room temperature under anaerobic conditions for two to four days (Gulitz *et al.*, 2011, 2013; Marsh *et al.*, 2013b; Stadie *et al.*, 2013; Laureys & De Vuyst, 2014; Chapters 3 and 4). After fermentation, the water kefir liquor is separated from the water kefir grains by sieving to obtain a slightly sweet, alcoholic, acidic, sparkling beverage with a yellowish color and a fruity taste and aroma.

The water-insoluble, translucent, and brittle water kefir grains are composed of glucan-type exopolysaccharides (EPS) (Horisberger, 1969; Waldherr *et al.*, 2010; Chapter 4), and harbor the water kefir microorganisms (Waldherr *et al.*, 2010; Gulitz *et al.*, 2011; Laureys & De Vuyst, 2014; Chapters 3, 4, and 5). When the water kefir grain inoculum is added to the water kefir liquor, part of the microorganisms detach from the grains into the liquor, but the majority remains always associated with the water kefir grains (Laureys & De Vuyst, 2014; Chapters 3 and 4). The key microorganisms of water kefir fermentation are *Lactobacillus paracasei*, *Lactobacillus hilgardii*, *Lactobacillus nagelii*, and *Saccharomyces cerevisiae* (Chapter 4). Other species of lactic acid bacteria (LAB), yeasts, acetic acid bacteria (AAB), and/or bifidobacteria may occur too (Waldherr *et al.*, 2010; Gulitz *et al.*, 2011, 2013; Laureys & De Vuyst, 2014; Laureys *et al.*, 2016; Chapters 3, 4, 5, and 6). The water kefir microorganisms convert sucrose into water kefir grain EPS, ethanol, carbon dioxide, lactic acid, glycerol, acetic acid, mannitol, and a variety of aroma compounds (Laureys & De Vuyst, 2014; Chapters 3 and 4). The water kefir grain mass usually increases during fermentation, due to the production of glucan EPS from sucrose by glucansucrases (Pidoux *et al.*, 1988, 1990; Pidoux, 1989; Waldherr *et al.*, 2010; Laureys & De Vuyst, 2014; Chapters 3 and 4). The activity of these extracellular enzymes depends on the environmental conditions, which may thus influence the water kefir grain growth during fermentation (Waldherr *et al.*, 2010). Low water kefir grain growth is a common problem during water kefir fermentation, and can prevent successful continuation and upscaling of a water kefir production process (Chapters 4 and 5).

Water kefir grain growth during fermentation is greatly influenced by the water kefir grain inoculum and can change gradually over the course of multiple backslopping steps (Chapter 4). *Lactobacillus hilgardii* is probably responsible for water kefir grain growth (Pidoux *et al.*, 1990; Waldherr *et al.*, 2010), but other LAB strains isolated from water kefir fermentations can also produce EPS from sucrose, as is the case for *Lb. nagelii*, *Leuconostoc mesenteroides*, and *Lactobacillus hordei* (Gulitz *et al.*, 2011; Chapter 4). However, the presence of EPS-producing *Lb. hilgardii* strains is not sufficient for good grain growth during fermentation. The water kefir grain growth may decrease as a result of excessive acidic stress during fermentation (Chapter 4). Indeed, the activity of glucansucrase from *Lb. hilgardii* decreases from 60 % at pH 3.6 to 10 % at pH 3.2 (Waldherr *et al.*, 2010), indicating that the pH during fermentation may have an effect on the water kefir grain growth. Hence, the influence of acidic stress on the water kefir grain growth and other characteristics of the water kefir fermentation process needs to be investigated in detail, for instance through the buffer capacity of the water used for fermentation.

Glucansucrases have a calcium-binding region near their active centre and need calcium ions for optimal activity (Yokoi & Watanabe, 1992; Kralj *et al.*, 2004; Vujičić-Žagar *et al.*, 2010; Leemhuis *et al.*, 2013). This suggests that calcium may influence the water kefir grain growth during fermentation. Calcium is one of the most abundant minerals in water, but its

concentration varies widely depending on the water source (Misund *et al.*, 1999). Hence, the influence of the calcium concentration of the water on the water kefir grain growth and other characteristics of the water kefir fermentation process needs to be investigated in detail.

This chapter aimed to investigate the influence of the buffer capacity and calcium concentration of the water used for fermentation 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 described in Chapter 3. 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 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 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 mentioned above, was used to start eight series of water kefir fermentations differing in the buffer capacity and calcium concentration of the water used for fermentation. Hereto, ultrapure water (18.2 MΩ·cm at 25 °C) was obtained from a gradient A10 Milli-Q water purification system (EMD Millipore, Billerica, MA, USA) and supplemented with 0 (in fermentation series 0B0Ca and 0B1Ca), 313 (1B0Ca, 1B1Ca, and 1B4Ca), or 626 (2B1Ca and 2B4Ca) mg l^{-1} of HCO_3^-, added as $KHCO_3$ (Sigma-Aldrich, Saint Louis, MO, USA); and with 0 (0B0Ca and 1B0Ca), 50 (0B1Ca, 1B1Ca, and 2B1Ca), or 200 (1B4Ca and 2B4Ca) mg l^{-1} of Ca^{2+}, added as $CaCl_2.2H_2O$ (Merck, Darmstadt, Germany). Untreated tap water (Brussels, Belgium) was used for a control fermentation (TAP). A buffer capacity of 313 mg l^{-1} of HCO_3^- solution was chosen to correspond with the buffer capacity of the untreated tap water, so that identical volumes of 0.125 M HCl were required for titration until pH 3.5, which is the common end-pH of a water kefir fermentation.

Each fermentation series was performed in independent biological triplicates. All fermentations were carried out in 250-ml glass bottles equipped with a water lock (PTFE). They were started by adding 50 g of non-rinsed water kefir grains to 10 g of sugar (Candico Bio), 5 g of dried figs (King Brand), and 160 ml of water with the appropriate composition. The 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. Every 3 days, the backslopping practice was applied for each fermentation bottle, whereby the water kefir grains were separated from the water kefir liquors by sieving, after which 50 g of non-rinsed water kefir grains were recultivated in fresh medium with the same composition 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 grains of the eight fermentation series at the end of backslopping step 8 were assessed visually. 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 and yeasts 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-independent microbial species diversity were determined for the water kefir liquors and the non-rinsed water kefir grains of the inoculum and the eight fermentation series at the end of backslopping step 8. The substrate and metabolite concentrations were determined for the liquors of the eight fermentation series at the end of backslopping steps 1 and 8.

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

2.4 pH and water kefir grain wet and dry mass 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 in Chapter 3, 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 in Chapter 3, except for the fact that an additional antibiotic, amphotericin B (final concentration of 0.0025 g l^{-1}; Sigma-Aldrich), 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), as described in Chapter 3.

2.6 Culture-dependent microbial species diversity analyses

The culture-dependent microbial species diversity analyses of the LAB and yeasts on the water kefir grains were determined by randomly picking up 10 to 20 % of the total number of colonies from the respective agar media with 30 to 300 colonies. Each isolate was subcultivated on its respective agar medium until the third generation, which was stored at -80 °C in YPD medium supplemented with 25 % (v v^{-1}) of glycerol, and used for dereplication via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) fingerprinting, as described before (Spitaels *et al.*, 2014).

Briefly, an ent loop of cell mass was suspended in 300 µl of ultrapure water, after which 900 µl of ethanol was added. This suspension was centrifuged (21,000 x *g*, 3 min, 4 °C) and stored at -20 °C. Before analysis, the suspensions were centrifuged (21,000 x *g*, 3 min, 4 °C), the supernatants were removed, and 50 µl of 70 % formic acid (Merck) and 50 µl of acetonitrile (Fluka Chemie, Buchs, Switzerland) were added. After vortexing and centrifugation (21,000 x *g*, 3 min, 4 °C), 1 µl of these solutions were spotted in duplicate onto an OPTI-TOF 384 stainless steel plate (AB SCIEX, Framingham, MA, USA) and overlaid with 1 µl of matrix solution [5 mg ml^{-1} of α-cyano-4-hydroxycinnamic acid in

water:acetonitrile:trifluoroacetic acid (48:50:2)]. Mass spectra were acquired on a 4800 Plus MALDI TOF/TOF Analyzer (AB SCIEX) (Spitaels *et al.*, 2014).

The fingerprint peptide patterns, ranging from 2 to 20 kDa, were clustered numerically into similarity trees using the Pearson correlation coefficient (PCC) and the unweighted pair group method with arithmetic mean (UPGMA) algorithm by means of the BioNumerics software version 5.10 (Applied Maths, Sint-Martens-Latem, Belgium). Representative bacterial isolates within each cluster were identified by sequencing part of their 16S rRNA gene from genomic DNA, and representative yeast isolates within each cluster were identified by sequencing part of their 26S large subunit (LSU) rRNA gene and internal transcribed spacer (ITS) region from genomic DNA, as described in Chapter 3.

2.7 Exopolysaccharide production

All bacterial isolates were grown on MRS agar medium supplemented with 10 g l^{-1} of sucrose at 30 °C for 7 days to visually assess their EPS production capacity.

2.8 Culture-independent microbial species diversity analyses

The culture-independent microbial species diversity of bacteria and yeasts in the water kefir liquors and on the water kefir grains were determined after preparing total DNA extracts from the cell pellets of the water kefir liquors and 0.2 g of crushed water kefir grains, respectively. Cell pellets of the water kefir liquors were obtained after centrifugation (7,200 x *g*, 20 min, 4 °C) of 40 ml of water kefir liquors and discarding the supernatants.

An optimized protocol was used for DNA extraction, as follows. The pellets of the liquors and the grains were resuspended in 1 ml of TES buffer [6.7 % (m v^{-1}) sucrose, 50 mM Tris-base, 1 mM EDTA, pH 8.0], after which the suspensions were centrifuged (21,000 x *g*, 20 min, 4 °C) and the supernatants were discarded. The resulting pellets were resuspended in 1 ml of sorbitol buffer [1.2 M sorbitol, 50 mM Tris-base, pH 7.5] supplemented with 30 mM *β*-mercaptoethanol, 200 U of lyticase (Sigma-Aldrich), and 15 U of Zymolyase (G-Biosciences, Saint Louis, MO, USA). These suspensions were incubated at 30 °C for 1 h, after which they were centrifuged (10,000 x *g*, 10 min) and the supernatants were discarded. Then, the pellets were resuspended in 1 ml of sorbitol buffer, after which the suspensions were centrifuged (10,000 x *g*, 10 min) and the supernatants were discarded. Finally, the pellets were resuspended in 400 µl of STET buffer [8.0 % (m v^{-1}) sucrose, 50 mM Tris-base, 50 mM EDTA, 5.0 % (v v^{-1}) Triton X-100, pH 8.0] supplemented with 12.5 U of mutanoysin (Sigma-Aldrich) and 20 mg ml^{-1} of lysozyme (Merck). These suspensions were incubated at 37 °C for 1 h. A pinch of acid washed glass beads (Sigma-Aldrich), 40 µl of 0.2 g ml^{-1} of sodium dodecyl sulfate (SDS), and 50 µl of 2 mg ml^{-1} proteinase K solution (Merck) were added. These suspensions were vortexed for 60 s and incubated at 56 °C for 2 h. The suspensions were heated until 65 °C and supplemented with 100 µl of 5 M NaCl and 80 µl of 10 % (m v^{-1}) cetyl trimethylammonium bromide (CTAB) in 0.7 M NaCl at 65 °C, vortexed, and incubated at 65 °C for 10 min. Finally, the suspensions were supplemented with 600 µl of chloroform:phenol:isoamylalcohol (49.5:49.5:1.0), vortexed, and centrifuged (13,000 x *g*, 5 min). The DNA obtained was purified with the Nucleospin® tissue 96 kit (Macherey-Nagel, Düren, Germany), according to the instructions of the manufacturer, and the DNA solutions were adjusted at approximately 50 ng $µl^{-1}$.

The culture-independent microbial community profiles were obtained by amplifying selected genomic fragments in the total DNA with the universal prokaryotic primer pair (V3), the LAB-specific primer pair (LAC), the *Bifidobacterium*-specific primer pair (Bif), and the

universal eukaryotic primer pair (Yeast); and separating the PCR amplicons through denaturing gradient gel electrophoresis (DGGE), as described in Chapter 3. Selected bands of the community profiles were cut from the gels and identified through sequencing, as described in Chapter 3.

2.9 Substrate and metabolite concentration determinations

Samples were prepared as described in Chapter 3. The concentrations of sucrose, glucose, and fructose were determined through high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), as described in Chapter 3, except that 100 µl of cell-free supernatant was added to 400 µl of ultrapure water, and 100 µl of this dilution was added to 900 µl of deproteinization solution (Chapter 3). The concentrations of D- and L-lactic acid and acetic acid were determined through high-performance liquid chromatography with ultraviolet detection (HPLC-UV), those of glycerol and mannitol through HPAEC-PAD, those of ethanol through gas chromatography with flame ionization detection (GC-FID), and those of the aroma compounds through static headspace gas chromatography with mass spectrometry detection (SH-GC-MS), as described in Chapter 3.

2.10 Carbon recovery

The carbon recovery at the end of a backslopping step was calculated as described in Chapter 3, whereby the total amount of carbon including that of the figs added to the fermentation was taken into account. The mono- and disaccharide content ($m\ m^{-1}$) of dried figs (48 %) was obtained from the national nutrient database for standard reference (release 26, http://ndb.nal.usda.gov/).

2.11 Statistics

An ANOVA was performed to test for differences between the eight water kefir fermentation series, followed by a series of post-hoc pairwise comparisons with Fisher's least significant difference (LSD) test (de Winter, 2013). Two-tailed Spearman correlation coefficients between test variables were calculated for all fermentation series with defined buffer capacity and calcium concentration of the water, excluding the control fermentation with tap water. The correlation coefficients between the buffer capacity of the water and the characteristics of the water kefir fermentation processes were always controlled for the calcium concentration of the water, and those between the calcium concentration of the water and the characteristics of the water kefir fermentation processes were always controlled for the buffer capacity. The correlation coefficients between different characteristics of the water kefir fermentation processes were not controlled.

All statistical tests were performed in R 3.2.0 with a significance level of 0.05.

3 Results

3.1 pH and water kefir grain wet and dry mass

At the end of backslopping step 1, a high buffer capacity of the water resulted in high pH values and a high calcium concentration in low pH values (Table 1). Indeed, the pH at the end of backslopping step 1 correlated positively with the buffer capacity (controlled for the calcium concentration) and negatively with the calcium concentration (controlled for the buffer capacity) (Table 2). The buffer capacity and calcium concentration of the water had no

significant influence on the water kefir grain growth, which was approximately 58 % for all fermentation series (Table 1).

Over the course of the eight backslopping steps, the pH values of the eight fermentation series decreased slightly, and this was more pronounced for the fermentation series with a large decrease of the water kefir grain growth (Figure 1; Tables 3 and 4). When the buffer capacity and/or calcium concentration of the water were below certain minima, the water kefir grain growth decreased significantly already at the end of backslopping step 2. This decrease continued gradually over the course of the eight backslopping steps (Figure 1). The minimum buffer capacity and calcium concentration to obtain a water kefir grain growth similar to that of the control fermentation series with tap water at the end of backslopping step 8 were 313 mg l^{-1} of HCO_3^- and 200 mg l^{-1} of Ca^{2+} (fermentation series 1B4Ca), or 626 mg l^{-1} of HCO_3^- and 50 mg l^{-1} of Ca^{2+} (2B1Ca). A buffer capacity and/or calcium concentration of the water above these minima did not further increase the water kefir grain growth.

The results at the end of backslopping step 8 were in line with those at the end of backslopping step 1, whereby a high buffer capacity of the water resulted in high pH values and a high calcium concentration in low pH values (Table 5). Indeed, the pH at the end of backslopping step 8 correlated again positively with the buffer capacity of the water (controlled for the calcium concentration) and negatively with the calcium concentration in the water (controlled for the buffer capacity) (Table 2). The water kefir grain growth at the end of backslopping step 8 ranged from 2.7 ± 0.5 % for fermentation series 0B0Ca to 52.0 ± 2.3 % for fermentation series 2B4Ca (Table 5), and correlated positively with the buffer capacity and the calcium concentration of the water (Table 2) and with the pH (0.801; $p < 0.001$).

The water kefir grain dry mass at the end of backslopping step 8 was approximately 14 % (m m^{-1}) for all fermentation series (Table 5). Visual assessment of the water kefir grains at the end of backslopping step 8 indicated that they were smaller when the water kefir grain growth was lower.

3.2 Substrate consumption and metabolite production

The total residual carbohydrate concentrations in all fermentation series were 5.3 to 10.9 g l^{-1} at the end of backslopping step 1 (Table 1), and 3.9 to 14.6 g l^{-1} at the end of backslopping step 8 (Table 5), whereby fructose was always the main residual carbohydrate. Although the concentrations of the total residual carbohydrates did not differ significantly between the fermentation series at the end of backslopping steps 1 and 8, they were always lowest in the fermentation series with the lowest buffer capacity of the water (0B0Ca and 0B1Ca) and always highest in the fermentation series with the highest buffer capacity of the water (TAP, 2B1Ca, and 2B4Ca). The total residual carbohydrate concentrations correlated positively with the pH at the end of backslopping steps 1 (0.597; $p = 0.005$) and 8 (0.491; $p = 0.025$).

The fermentation series with the lowest buffer capacity and calcium concentration of the water (0B0Ca) resulted in the highest concentrations of ethanol at the end of backslopping step 1 (Table 1), and the highest concentrations of ethanol, lactic acid, and glycerol at the end of backslopping step 8 (Table 5). Indeed, the buffer capacity of the water correlated negatively with the concentrations of ethanol and positively with the concentrations of acetic acid and mannitol at the end of backslopping step 1 (Table 2). The buffer capacity of the

Table 1. Characteristics of eight water kefir fermentation series differing in the buffer capacity and calcium concentration of the water used for fermentation [control fermentation with tap water (TAP); 0 (0B0Ca and 0B1Ca), 313 (1B0Ca, 1B1Ca, and 1B4Ca), and 626 (2B1Ca and 2B4Ca) mg l^{-1} of HCO_3^-; and 0 (0B0Ca and 1B0Ca), 50 (0B1Ca, 1B1Ca, and 2B1Ca), and 200 (1B4Ca and 2B4Ca) mg l^{-1} of Ca^{2+}] at the end of backslopping step 1. Significant differences between the series are indicated with different superscripts (a, b, c, d, and e).

Characteristic	TAP	0B0Ca	0B1Ca	1B0Ca	1B1Ca	1B4Ca	2B1Ca	2B4Ca
Water kefir grain growth (%)	58.9 ± 1.8	57.5 ± 0.3	58.0 ± 1.5	58.6 ± 1.0	60.0 ± 1.0	58.1 ± 0.8	59.0 ± 1.4	58.5 ± 1.5
pH	3.50 ± 0.04^{bc}	3.29 ± 0.06^{e}	3.33 ± 0.02^{e}	3.53 ± 0.05^{abc}	3.45 ± 0.05^{cd}	3.41 ± 0.06^{d}	3.59 ± 0.06^{a}	3.56 ± 0.02^{ab}
Sucrose (g l^{-1})	1.4 ± 0.1	1.5 ± 0.1	1.5 ± 0.2	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.0	1.4 ± 0.1	1.5 ± 0.0
Glucose (g l^{-1})	0.3 ± 0.3	0.0 ± 0.1	0.2 ± 0.3	0.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3	0.3 ± 0.2
Fructose (g l^{-1})	9.1 ± 2.7	3.8 ± 1.3	6.5 ± 4.9	7.3 ± 5.5	5.6 ± 1.4	5.8 ± 0.7	8.7 ± 3.6	8.6 ± 2.6
Total residual carbohydrates (g l^{-1})	10.9 ± 3.0	5.3 ± 1.2	8.2 ± 5.0	9.1 ± 5.8	7.1 ± 1.5	7.2 ± 0.6	10.5 ± 3.8	10.4 ± 2.9
Ethanol (g l^{-1})	16.4 ± 1.4	19.4 ± 0.3	17.8 ± 2.1	16.9 ± 2.3	18.4 ± 1.2	18.0 ± 0.5	16.5 ± 1.0	16.9 ± 1.3
Lactic acid (g l^{-1})	2.61 ± 0.25	2.68 ± 0.05	2.49 ± 0.36	2.60 ± 0.27	2.76 ± 0.08	2.79 ± 0.07	2.67 ± 0.25	2.75 ± 0.11
Acetic acid (g l^{-1})	1.08 ± 0.07^{ab}	1.00 ± 0.03^{b}	1.00 ± 0.11^{b}	1.07 ± 0.08^{ab}	1.16 ± 0.04^{a}	1.18 ± 0.09^{a}	1.14 ± 0.04^{a}	1.19 ± 0.04^{a}
Glycerol (g l^{-1})	1.90 ± 0.05	1.98 ± 0.09	1.90 ± 0.14	1.87 ± 00.16	1.95 ± 0.06	1.97 ± 0.10	1.89 ± 0.08	1.98 ± 0.10
Mannitol (g l^{-1})	0.81 ± 0.04^{ab}	0.67 ± 0.09^{cd}	0.65 ± 0.08^{d}	0.71 ± 0.08^{cd}	0.73 ± 0.03^{bcd}	0.76 ± 0.05^{abc}	0.82 ± 0.02^{ab}	0.85 ± 0.06^{a}
Glycerol/ethanol (mmol/mol)	58 ± 4	51 ± 3	54 ± 3	56 ± 3	53 ± 2	55 ± 2	57 ± 3	59 ± 4
Lactic acid/ethanol (mmol/mol)	81 ± 3^{ab}	71 ± 2^{c}	72 ± 3^{c}	79 ± 3^{ab}	77 ± 3^{b}	79 ± 3^{ab}	83 ± 3^{a}	83 ± 3^{a}
Acetic acid/ethanol (mmol/mol)	51 ± 3^{ab}	40 ± 1^{d}	43 ± 2^{cd}	49 ± 4^{ab}	48 ± 5^{bc}	50 ± 5^{ab}	53 ± 2^{ab}	54 ± 2^{a}
Acetic acid/lactic acid (mol/mol)	0.62 ± 0.02	0.56 ± 0.03	0.61 ± 0.03	0.62 ± 0.03	0.63 ± 0.04	0.63 ± 0.04	0.64 ± 0.04	0.65 ± 0.01
D-lactic acid (% of total)	45.0 ± 1.0	45.7 ± 1.7	45.1 ± 0.7	44.6 ± 0.5	45.0 ± 0.4	45.0 ± 1.4	45.6 ± 1.5	45.2 ± 0.7
Carbon recovery (%)	100.2 ± 0.8	100.9 ± 0.3	100.1 ± 1.1	99.6 ± 0.3	101.6 ± 1.2	100.5 ± 0.2	100.2 ± 1.0	100.9 ± 0.2
2-Methyl-1-propanol (mg l^{-1})	8.4 ± 0.7	10.4 ± 0.6	9.7 ± 1.2	9.1 ± 0.5	9.9 ± 0.9	9.7 ± 0.7	9.4 ± 0.4	9.3 ± 0.5
Isoamyl alcohol (mg l^{-1})	41.3 ± 2.9	49.5 ± 1.2	45.3 ± 4.0	44.0 ± 2.1	46.7 ± 3.1	45.9 ± 1.5	45.1 ± 1.4	44.6 ± 2.9
Ethyl acetate (mg l^{-1})	9.9 ± 1.2	11.9 ± 0.8	11.6 ± 2.0	10.1 ± 1.2	12.5 ± 1.9	12.2 ± 0.7	11.5 ± 0.4	13.4 ± 2.0
Isoamyl acetate (mg l^{-1})	0.10 ± 0.01	0.14 ± 0.01	0.12 ± 0.03	0.12 ± 0.03	0.13 ± 0.02	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
Ethyl hexanoate (mg l^{-1})	0.21 ± 0.01	0.27 ± 0.03	0.25 ± 0.04	0.28 ± 0.07	0.29 ± 0.04	0.26 ± 0.07	0.26 ± 0.01	0.25 ± 0.04
Ethyl octanoate (mg l^{-1})	0.25 ± 0.02	0.41 ± 0.15	0.31 ± 0.04	0.30 ± 0.04	0.30 ± 0.04	0.33 ± 0.06	0.27 ± 0.02	0.26 ± 0.03

Table 2. Spearman correlation coefficients (SCC) between the buffer capacity (controlled for the calcium concentrations) or the calcium concentration (controlled for the buffer capacity) of the water and the characteristics of the water kefir fermentation processes, at the end of backslopping steps 1 and 8. Significant correlations have a grey background.

Characteristic	Backslopping step 1				Backslopping step 8			
	Buffer capacity		Calcium concentrations		Buffer capacity		Calcium concentrations	
	SCC	p	SCC	p	SCC	p	SCC	p
Yeasts (log cfu g^{-1})	NA	NA	NA	NA	-0.735	0.000	-0.191	0.421
Lactic acid bacteria (log cfu g^{-1})	NA	NA	NA	NA	-0.235	0.318	-0.281	0.230
Acetic acid bacteria (log cfu g^{-1})	NA	NA	NA	NA	0.638	0.002	0.147	0.537
Lactic acid bacteria/yeasts (cfu g^{-1}/cfu g^{-1})	NA	NA	NA	NA	0.711	0.000	-0.198	0.402
Water kefir grain growth (%)	0.325	0.161	-0.095	0.690	0.946	0.000	0.811	0.000
Water kefir grain dry mass (%)	NA	NA	NA	NA	-0.214	0.364	-0.418	0.066
pH	0.901	0.000	-0.494	0.027	0.955	0.000	-0.652	0.002
Total residual carbohydrates (g l^{-1})	0.342	0.140	0.066	0.782	0.435	0.055	-0.139	0.558
Ethanol (g l^{-1})	-0.457	0.043	0.028	0.908	-0.816	0.000	-0.202	0.392
Lactic acid (g l^{-1})	0.025	0.917	0.225	0.340	-0.341	0.141	0.026	0.914
Acetic acid (g l^{-1})	0.563	0.010	0.511	0.021	0.030	0.899	0.121	0.612
Glycerol (g l^{-1})	-0.139	0.560	0.151	0.526	-0.842	0.000	-0.347	0.134
Mannitol (g l^{-1})	0.760	0.000	0.221	0.348	-0.012	0.960	0.096	0.687
Ratio glycerol/ethanol (mol/mol)	0.598	0.005	-0.031	0.895	0.577	0.008	0.008	0.974
Ratio lactic acid/ethanol (mol/mol)	0.801	0.000	0.033	0.889	0.923	0.000	0.513	0.021
Ratio acetic acid/ethanol (mol/mol)	0.726	0.000	0.311	0.182	0.768	0.000	0.412	0.071
Ratio acetic acid/lactic acid (mol/mol)	0.500	0.025	0.334	0.150	0.345	0.136	0.197	0.405
D-lactic acid (% of total)	-0.018	0.940	0.054	0.821	0.442	0.051	0.014	0.954
2-Methyl-1-propanol (mg l^{-1})	-0.280	0.231	-0.006	0.979	-0.674	0.001	-0.080	0.737
Isoamyl alcohol (mg l^{-1})	-0.330	0.155	0.000	1.000	-0.609	0.004	-0.057	0.811
Ethyl acetate (mg l^{-1})	-0.148	0.533	0.385	0.094	-0.604	0.005	0.181	0.446
Ethyl butanoate (AU)	-0.278	0.235	-0.150	0.529	-0.163	0.493	-0.273	0.244
Ethyl-2-methyl butanoate (AU)	0.604	0.005	-0.498	0.025	0.763	0.000	-0.604	0.005
Isoamyl acetate (mg l^{-1})	-0.427	0.060	-0.055	0.816	-0.388	0.091	0.000	1.000
Ethyl hexanoate (mg l^{-1})	0.136	0.568	-0.264	0.260	-0.205	0.385	-0.132	0.578
Ethyl octanoate (mg l^{-1})	-0.494	0.027	0.050	0.835	-0.788	0.000	-0.043	0.856

AU, arbitratry units; NA, not available.

water correlated negatively with the concentrations of ethanol and glycerol at the end of backslopping step 8 (Table 2). Further, the buffer capacity of the water correlated positively with the ratios of the concentrations of glycerol to ethanol, lactic acid to ethanol, acetic acid to ethanol, and acetic acid to lactic acid at the end of backslopping steps 1 and 8. At the end of backslopping steps 1 and 8, the buffer capacity of the water correlated positively with the concentrations of ethyl-2-methyl butanoate and negatively with the concentrations of ethyl decanoate, whereas the calcium concentration of the water correlated negatively with the concentrations of ethyl-2-methyl butanoate. The calcium concentration of the water correlated positively with the concentration of acetic acid at the end of backslopping step 1 and the ratios of the concentrations of lactic acid to ethanol at the end of backslopping step 8.

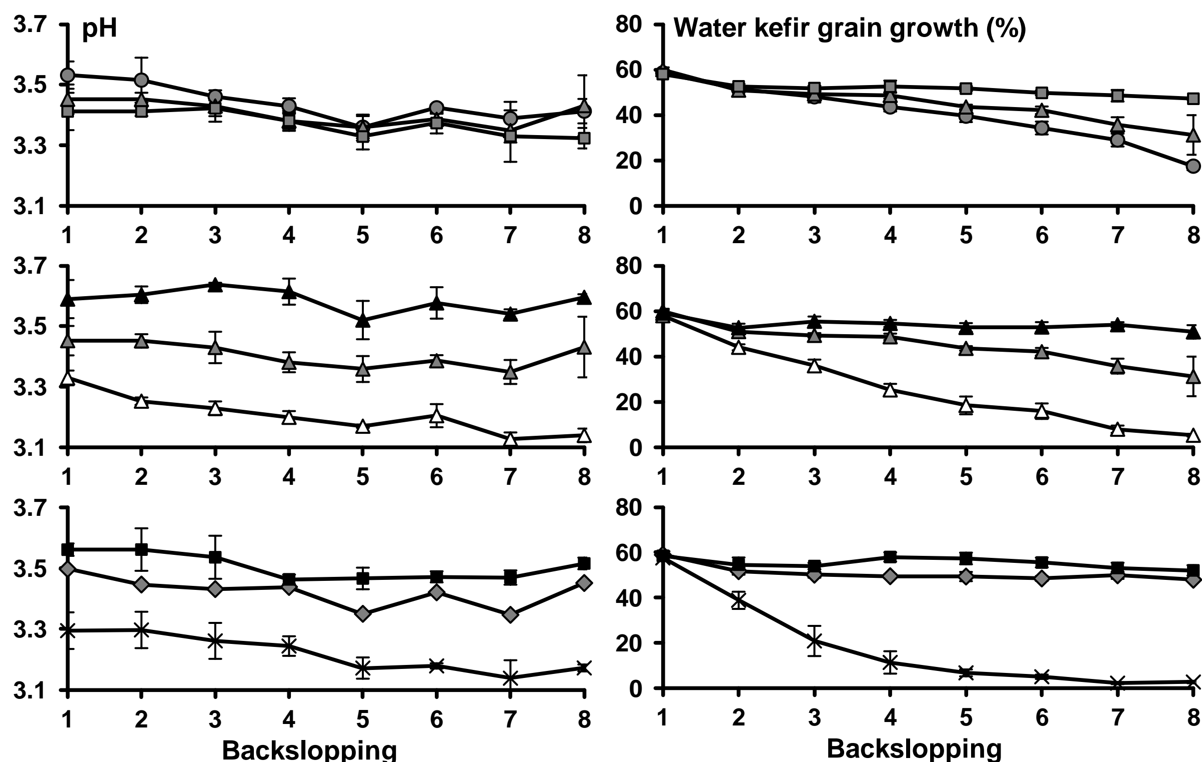

Figure 1. The pH and water kefir grain growth at the end of each backslopping step for eight water kefir fermentation series differing in the buffer capacity and calcium concentration of the water: increasing calcium concentrations [1B0Ca (●), 1B1Ca (▲), and 1B4Ca (■)] (top); increasing buffer capacity [0B1Ca (Δ), 1B1Ca (▲), and 2B1Ca (▲)] (middle); high buffer capacity and calcium concentration [2B4Ca (■)]; low buffer capacity and calcium concentration [0B0Ca (X)]; and tap water [TAP (♦)] (bottom). For differences of significance, see Tables 3 and 4.

At the end of backslopping step 1, the concentrations of ethanol correlated positively with the concentrations of glycerol (0.662; $p < 0.001$) and total lactic acid (0.588; $p = 0.006$), but not with those of acetic acid (-0.114; $p = 0.613$). At the end of backslopping step 8, the concentrations of ethanol correlated positively with the concentrations of glycerol (0.932; $p < 0.001$) and lactic acid (0.645; $p = 0.002$), but not with those of acetic acid (-0.032; $p = 0.890$).

At the end of backslopping step 1, the concentrations of ethanol correlated positively with the concentrations of ethyl butanoate (0.895; $p < 0.001$), 2-methyl-1-propanol (0.753; $p < 0.001$), isoamyl alcohol (0.736; $p < 0.001$), isoamyl acetate (0.945; $p < 0.001$), ethyl hexanoate (0.658; $p = 0.002$), and ethyl octanoate (0.736; $p < 0.001$), but not with the concentrations of ethyl acetate (0.377; $p = 0.093$) and ethyl-2-methylbutanoate (0.143; $p = 0.535$). At the end of backslopping step 8, the concentrations of ethanol correlated positively with the concentrations of ethyl acetate (0.677; $p < 0.001$), ethyl butanoate (0.561; $p = 0.009$), 2-methyl-1-propanol (0.879; $p < 0.001$), isoamyl alcohol (0.848; $p < 0.001$), isoamyl acetate (0.648; $p = 0.002$), ethyl hexanoate (0.547; $p = 0.011$), and ethyl octanoate (0.857; $p < 0.001$), and negatively with the concentrations of ethyl-2-methylbutanoate (-0.536; $p = 0.013$).

Table 3. The pH of eight water kefir fermentation series differing in the buffer capacity and calcium concentration of the water used for fermentation [control fermentation with tap water (TAP); 0 (0B0Ca and 0B1Ca), 313 (1B0Ca, 1B1Ca, and 1B4Ca), and 626 (2B1Ca and 2B4Ca) mg l^{-1} of HCO_3^-; and 0 (0B0Ca and 1B0Ca), 50 (0B1Ca, 1B1Ca, and 2B1Ca), and 200 (1B4Ca and 2B4Ca) mg l^{-1} of Ca^{2+}] at the end of backslopping steps 1-8. Significant differences between the series are indicated with different superscripts (a, b, c, d, and e).

Backslopping step	TAP	0B0Ca	0B1Ca	1B0Ca	1B1Ca	1B4Ca	2B1Ca	2B4Ca
1	3.50 ± 0.04 ^{bc}	3.29 ± 0.06 ^{e}	3.33 ± 0.02 ^{e}	3.53 ± 0.05 ^{abc}	3.45 ± 0.05 ^{cd}	3.41 ± 0.06 ^{d}	3.59 ± 0.06 ^{a}	3.56 ± 0.02 ^{ab}
2	3.45 ± 0.03 ^{cd}	3.30 ± 0.06 ^{e}	3.25 ± 0.01 ^{e}	3.52 ± 0.07 ^{bc}	3.45 ± 0.02 ^{cd}	3.41 ± 0.02 ^{d}	3.60 ± 0.03 ^{a}	3.56 ± 0.07 ^{ab}
3	3.43 ± 0.05 ^{c}	3.26 ± 0.06 ^{d}	3.23 ± 0.0 ^{d}	3.46 ± 0.01 ^{c}	3.43 ± 0.05 ^{c}	3.42 ± 0.03 ^{c}	3.64 ± 0.01 ^{a}	3.54 ± 0.07 ^{b}
4	3.44 ± 0.02 ^{b}	3.24 ± 0.03 ^{e}	3.20 ± 0.02 ^{e}	3.43 ± 0.03 ^{bc}	3.38 ± 0.03 ^{d}	3.38 ± 0.03 ^{cd}	3.61 ± 0.04 ^{a}	3.46 ± 0.02 ^{b}
5	3.35 ± 0.01 ^{b}	3.17 ± 0.03 ^{c}	3.17 ± 0.01 ^{c}	3.36 ± 0.04 ^{b}	3.36 ± 0.04 ^{b}	3.33 ± 0.04 ^{b}	3.52 ± 0.06 ^{a}	3.47 ± 0.04 ^{a}
6	3.42 ± 0.02 ^{cd}	3.18 ± 0.01 ^{e}	3.20 ± 0.04 ^{e}	3.42 ± 0.01 ^{bc}	3.39 ± 0.02 ^{cd}	3.37 ± 0.03 ^{d}	3.58 ± 0.05 ^{a}	3.47 ± 0.02 ^{b}
7	3.35 ± 0.03 ^{b}	3.14 ± 0.06 ^{c}	3.13 ± 0.02 ^{c}	3.39 ± 0.06 ^{b}	3.35 ± 0.04 ^{b}	3.33 ± 0.09 ^{b}	3.54 ± 0.02 ^{a}	3.47 ± 0.02 ^{a}
8	3.45 ± 0.01 ^{bc}	3.17 ± 0.01 ^{e}	3.14 ± 0.02 ^{e}	3.41 ± 0.04 ^{c}	3.43 ± 0.10 ^{c}	3.32 ± 0.03 ^{d}	3.60 ± 0.01 ^{a}	3.52 ± 0.02 ^{b}

Table 4. The water kefir grain growth of eight water kefir fermentation series differing in the buffer capacity and calcium concentration of the water use for fermentation [control fermentation with tap water (TAP); 0 (0B0Ca and 0B1Ca), 313 (1B0Ca, 1B1Ca, and 1B4Ca), and 626 (2B1Ca and 2B4Ca) mg l^{-1} of HCO_3^-; and 0 (0B0Ca and 1B0Ca), 50 (0B1Ca, 1B1Ca, and 2B1Ca), and 200 (1B4Ca and 2B4Ca) mg l^{-1} of Ca^{2+}] at the end of backslopping steps 1-8. Significant differences between the series are indicated with different superscripts (a, b, c, d, e, and f).

Backslopping step	TAP	0B0Ca	0B1Ca	1B0Ca	1B1Ca	1B4Ca	2B1Ca	2B4Ca
1	58.9 ± 1.8	57.5 ± 0.3	58.0 ± 1.5	58.6 ± 1.0	60.0 ± 1.0	58.1 ± 0.8	59.0 ± 1.4	58.5 ± 1.5
2	51.7 ± 1.5 ^{ab}	38.8 ± 3.8 ^{d}	44.1 ± 1.3 ^{c}	51.3 ± 0.1 ^{ab}	51.0 ± 0.6 ^{b}	52.6 ± 1.1 ^{ab}	52.7 ± 1.7 ^{ab}	54.6 ± 3.2 ^{a}
3	50.1 ± 1.3 ^{bc}	20.9 ± 6.7 ^{e}	35.9 ± 2.7 ^{d}	48.1 ± 2.5 ^{c}	49.3 ± 1.0 ^{bc}	51.9 ± 1.0 ^{ac}	55.5 ± 2.1 ^{a}	54.0 ± 1.4 ^{ab}
4	49.5 ± 0.9 ^{c}	11.4 ± 4.9 ^{f}	25.3 ± 2.7 ^{e}	43.6 ± 2.0 ^{d}	48.7 ± 1.4 ^{c}	52.5 ± 2.7 ^{bc}	54.6 ± 1.5 ^{ab}	58.0 ± 2.1 ^{a}
5	49.3 ± 2.0 ^{b}	6.8 ± 1.5 ^{f}	18.5 ± 3.9 ^{e}	39.5 ± 2.6 ^{d}	43.6 ± 1.0 ^{c}	51.7 ± 1.1 ^{b}	52.8 ± 1.9 ^{b}	57.3 ± 2.6 ^{a}
6	48.5 ± 1.1 ^{c}	5.1 ± 1.0 ^{g}	15.9 ± 3.4 ^{f}	34.3 ± 2.8 ^{e}	42.1 ± 1.7 ^{d}	49.8 ± 1.0 ^{bc}	53.0 ± 2.2 ^{ab}	55.5 ± 2.2 ^{a}
7	49.9 ± 1.6 ^{bc}	2.2 ± 0.4 ^{g}	8.0 ± 1.6 ^{f}	29.0 ± 2.8 ^{e}	35.8 ± 3.3 ^{d}	48.5 ± 2.6 ^{c}	54.0 ± 1.1 ^{a}	53.1 ± 2.3 ^{ab}
8	47.9 ± 0.8 ^{a}	2.7 ± 0.5 ^{d}	5.4 ± 0.6 ^{d}	17.5 ± 1.8 ^{c}	31.2 ± 8.7 ^{b}	47.2 ± 0.5 ^{a}	50.9 ± 3.0 ^{a}	52.0 ± 2.3 ^{a}

Table 5. Characteristics of eight water kefir fermentation series differing in the buffer capacity and calcium concentration of the water used for fermentation [control fermentation with tap water (TAP); 0 (0B0Ca and 0B1Ca), 313 (1B0Ca, 1B1Ca, and 1B4Ca), and 626 (2B1Ca and 2B4Ca) mg l^{-1} of HCO_3^-; and 0 (0B0Ca and 1B0Ca), 50 (0B1Ca, 1B1Ca, and 2B1Ca), and 200 (1B4Ca and 2B4Ca) mg l^{-1} of Ca^{2+}] at the end of backslopping step 8. Significant differences between the series are indicated with different superscripts (a, b, c, d, and e).

Characteristic	TAP	0B0Ca	0B1Ca	1B0Ca	1B1Ca	1B4Ca	2B1Ca	2B4Ca
Yeasts (log cfu g^{-1})	7.5 ± 0.1 ^{bc}	7.7 ± 0.1 ^{a}	7.7 ± 0.1 ^{ab}	7.5 ± 0.1 ^{c}	7.4 ± 0.1 ^{c}	7.3 ± 0.1 ^{cd}	7.2 ± 0.1 ^{d}	7.4 ± 0.2 ^{cd}
Lactic acid bacteria (log cfu g^{-1})	8.4 ± 0.1	8.6 ± 0.1	8.5 ± 0.1	8.4 ± 0.2	8.3 ± 0.1	8.3 ± 0.1	8.4 ± 0.1	8.4 ± 0.1
Acetic acid bacteria (log cfu g^{-1})	4.7 ± 0.3 ^{a}	3.5 ± 0.4 ^{d}	3.7 ± 0.5 ^{cd}	3.9 ± 0.6 ^{bd}	4.2 ± 0.3 ^{abc}	4.4 ± 0.2 ^{ab}	4.8 ± 0.1 ^{a}	4.4 ± 0.5 ^{abc}
Lactic acid bacteria/yeasts (cfu/cfu)	8.9 ± 0.8 ^{bc}	7.7 ± 2.3 ^{c}	7.2 ± 0.8 ^{c}	9.8 ± 2.8 ^{bc}	9.0 ± 3.1 ^{bc}	9.1 ± 2.3 ^{bc}	14.8 ± 2.1 ^{a}	12.1 ± 3.3 ^{ab}
Water kefir grain growth (%)	47.9 ± 0.7 ^{a}	2.7 ± 0.5 ^{d}	5.4 ± 0.6 ^{d}	17.5 ± 1.8 ^{c}	31.2 ± 8.7 ^{b}	47.2 ± 0.5 ^{a}	50.9 ± 2.9 ^{a}	52.0 ± 2.3 ^{a}
Water kefir grain dry mass (%)	14.1 ± 0.3 ^{bc}	14.2 ± 0.3 ^{bc}	14.4 ± 0.5 ^{ac}	14.6 ± 0.2 ^{ab}	15.0 ± 0.4 ^{a}	13.9 ± 0.4 ^{c}	14.0 ± 0.3 ^{bc}	13.0 ± 0.6 ^{d}
pH	3.45 ± 0.01 ^{bc}	3.17 ± 0.01 ^{e}	3.14 ± 0.02 ^{e}	3.41 ± 0.04 ^{c}	3.43 ± 0.10 ^{c}	3.32 ± 0.03 ^{d}	3.60 ± 0.01 ^{a}	3.52 ± 0.02 ^{b}
Sucrose (g l^{-1})	1.3 ± 0.1 ^{bc}	1.0 ± 0.3 ^{c}	2.6 ± 1.7 ^{ab}	3.9 ± 1.4 ^{a}	2.1 ± 0.5 ^{bc}	1.4 ± 0.2 ^{bc}	1.3 ± 0.2 ^{bc}	1.5 ± 0.1 ^{bc}
Glucose (g l^{-1})	0.7 ± 0.6	0.2 ± 0.1	0.6 ± 0.7	1.2 ± 0.4	1.8 ± 1.4	0.3 ± 0.4	0.7 ± 0.3	0.1 ± 0.1
Fructose (g l^{-1})	10.1 ± 4.0 ^{a}	2.7 ± 1.0 ^{b}	3.7 ± 2.8 ^{b}	6.7 ± 1.9 ^{ab}	10.8 ± 5.1 ^{a}	7.5 ± 4.7 ^{ab}	10.6 ± 2.1 ^{a}	7.5 ± 0.3 ^{ab}
Total residual carbohydrates (g l^{-1})	12.1 ± 4.7	3.9 ± 1.4	7.0 ± 5.2	11.8 ± 0.9	14.6 ± 7.1	9.2 ± 5.3	12.6 ± 2.7	9.1 ± 0.3
Ethanol (g l^{-1})	17.7 ± 2.2 ^{cd}	31.6 ± 0.4 ^{a}	29.5 ± 2.5 ^{a}	22.8 ± 0.6 ^{b}	18.8 ± 5.0 ^{bc}	18.7 ± 2.5 ^{c}	14.5 ± 0.2 ^{d}	17.1 ± 0.6 ^{cd}
Lactic acid (g l^{-1})	2.63 ± 0.38 ^{d}	3.40 ± 0.12 ^{a}	3.30 ± 0.29 ^{ab}	2.92 ± 0.25 ^{ad}	2.73 ± 0.41 ^{cd}	2.91 ± 0.15 ^{ad}	2.83 ± 0.33 ^{d}	3.2 ± 0.22 ^{cd}
Acetic acid (g l^{-1})	1.05 ± 0.10	1.26 ± 0.03	1.21 ± 0.08	1.00 ± 0.08	1.05 ± 0.20	1.14 ± 0.10	1.25 ± 0.16	1.24 ± 0.07
Glycerol (g l^{-1})	1.87 ± 0.27 ^{cd}	2.76 ± 0.10 ^{a}	2.50 ± 0.14 ^{b}	2.01 ± 0.07 ^{c}	1.84 ± 0.21 ^{cd}	1.82 ± 0.06 ^{cd}	1.67 ± 0.07 ^{d}	1.74 ± 0.02 ^{d}
Mannitol (g l^{-1})	0.59 ± 0.04 ^{bc}	0.74 ± 0.18 ^{b}	0.68 ± 0.06 ^{b}	0.43 ± 0.15 ^{c}	0.56 ± 0.11 ^{bc}	0.67 ± 0.16 ^{bc}	1.00 ± 0.22 ^{a}	0.58 ± 0.12 ^{bc}
Glycerol/ethanol (mmol/mol)	53 ± 3 ^{ab}	44 ± 2 ^{cd}	42 ± 2 ^{d}	44 ± 3 ^{cd}	50 ± 7 ^{bc}	49 ±7 ^{bcd}	58 ± 2 ^{a}	51 ± 2 ^{ac}
Lactic acid/ethanol (mmol/mol)	76 ± 5 ^{bc}	55 ± 1 ^{d}	57 ± 1 ^{d}	65 ± 6 ^{cd}	75 ± 10 ^{bc}	80 ± 7 ^{b}	100 ± 11 ^{a}	95 ± 4 ^{a}
Acetic acid/ethanol (mmol/mol)	45 ± 3 ^{bc}	30 ± 1 ^{e}	32 ± 1 ^{e}	34 ± 4 ^{de}	44 ± 12 ^{cd}	48 ± 10 ^{bc}	66 ± 8 ^{a}	55 ± 2 ^{ab}
Acetic acid/lactic acid (mol/mol)	0.60 ± 0.03	0.56 ± 0.03	0.55 ± 0.02	0.51 ± 0.05	0.58 ± 0.09	0.59 ± 0.08	0.66 ± 0.04	0.58 ± 0.2
D-lactic acid (% of total)	46.2 ± 0.2	45.6 ± 0.1	45.5 ± 1.0	46.6 ± 0.7	46.5 ± 0.8	46.3 ± 0.6	46.4 ± 1.3	46.9 ± 0.8
Carbon recovery (%)	99.7 ± 1.1 ^{b}	105.2 ± 0.7 ^{a}	104.4 ± 0.6 ^{a}	99.7 ± 1.5 ^{b}	99.3 ± 0.8 ^{b}	99.2 ± 0.2 ^{b}	95.7 ± 2.7 ^{c}	96.9 ± 1.1 ^{c}
2-Methyl-1-propanol (mg l^{-1})	8.7 ± 2.0 ^{c}	13.0 ± 0.7 ^{ab}	13.7 ± 3.9 ^{a}	10.7 ± 0.3 ^{ac}	9.3 ± 2.9 ^{c}	9.9 ± 1.7 ^{bc}	8.3 ± 0.3 ^{c}	9.4 ± 1.1 ^{c}
Isoamyl alcohol (mg l^{-1})	40.0 ± 4.1 ^{cd}	50.1 ± 1.3 ^{ab}	51.2 ± 8.0 ^{a}	48.4 ± 1.7 ^{ac}	40.0 ± 8.4 ^{cd}	44.6 ± 7.5 ^{ad}	36.5 ± 4.4 ^{d}	40.8 ± 1.6 ^{bcd}
Ethyl acetate (mg l^{-1})	13.1 ± 0.9 ^{c}	19.4 ± 1.6 ^{ab}	23.6 ± 8.2 ^{a}	12.9 ± 1.7 ^{c}	13.3 ± 1.3 ^{c}	13.6 ± 3.4 ^{bc}	12.7 ± 1.0 ^{c}	14.9 ± 1.8 ^{bc}
Isoamyl acetate (mg l^{-1})	0.11 ± 0.02	0.15 ± 0.02	0.17 ± 0.04	0.15 ± 0.01	0.13 ± 0.04	0.14 ± 0.02	0.13 ± 0.01	0.14 ± 0.01
Ethyl hexanoate (mg l^{-1})	0.29 ± 0.05	0.38 ± 0.13	0.37 ± 0.07	0.43 ± 0.02	0.40 ± 0.14	0.33 ± 0.05	0.31 ± 0.01	0.34 ± 0.03
Ethyl octanoate (mg l^{-1})	0.33 ± 0.06 ^{de}	0.58 ± 0.01 ^{ab}	0.69 ± 0.19 ^{a}	0.49 ± 0.10 ^{bc}	0.35 ± 0.11 ^{cde}	0.43 ± 0.03 ^{bd}	0.27 ± 0.04 ^{e}	0.32 ± 0.03 ^{de}

At the end of backslopping step 1, the concentrations of total lactic acid correlated positively with the concentrations of acetic acid (0.534; $p < 0.014$), but not with the pH (-0.162; $p = 0.480$). At the end of backslopping step 8, the concentrations of total lactic acid correlated positively with the concentrations of acetic acid (0.532; $p = 0.014$) and negatively with the pH (-0.436; $p = 0.049$).

At the end of backslopping step 1, the concentrations of acetic acid correlated positively with the pH (0.514; $p = 0.018$) and the concentrations of mannitol (0.486; $p = 0.027$), but not with the concentrations of glycerol (0.143; $p = 0.535$). At the end of backslopping step 8, the concentrations of acetic acid correlated positively with the concentrations of mannitol (0.564; $p = 0.009$), but not with the pH (-0.073; $p = 0.754$) or the concentrations of glycerol (0.027; $p = 0.908$). The concentrations of glycerol and mannitol did not correlate at the end of backslopping steps 1 (-0.096; $p = 0.678$) and 8 (-0.106; $p = 0.645$).

Overall, a carbon recovery of approximately 100 % was found in all fermentation series at the end of backslopping steps 1 (Table 1) and 8 (Table 5), but the carbon recovery correlated negatively with the water kefir grain growth at the end of backslopping step 8 (-0.890; $p < 0.001$).

3.3 Microbial enumerations

The buffer capacity of the water did not correlate with the viable counts of the LAB on the water kefir grains, correlated negatively with those of the yeasts and positively with those of the AAB (Table 2). This resulted in a positive correlation between the buffer capacity of the water and the ratios of the viable counts of the LAB to the yeasts on the water kefir grains. The calcium concentration had no significant influence on the viable counts of the water kefir microorganisms on the water kefir grains.

The water kefir grain growth correlated negatively with the viable counts of the yeasts (-0.797; $p < 0.001$) and LAB (-0.528; $p = 0.014$) on the water kefir grains, and positively with those of the AAB (0.690; $p = 0.001$). Further, the water kefir grain growth correlated positively with the ratios of the viable counts of the LAB to yeasts (0.592; $p = 0.005$) on the water kefir grains. The total residual carbohydrate concentrations correlated negatively with the viable counts of the yeasts (-0.578, $p = 0.007$) and LAB (-0.670, $p = 0.001$), and positively with those of the AAB (0.578, $p = 0.007$) on the water kefir grains.

The viable counts of the yeasts on the water kefir grains correlated positively with the concentrations of ethanol (0.845, $p < 0.001$), but not with those of acetic acid (0.123, $p = 0.593$). The viable counts of the LAB on the water kefir grains correlated positively with the concentrations of total lactic acid (0.821, $p < 0.001$), but not with those of acetic acid (0.335, $p = 0.138$). The viable counts of the AAB on the water kefir grains did not correlate with the concentrations of acetic acid (0.132, $p = 0.566$) either. The ratios of the viable counts of the LAB to the yeasts on the water kefir grains ranged from 7 to 14 (Table 5), and correlated positively with the ratios of the concentrations of lactic acid to ethanol (0.690; $p = 0.001$) and acetic acid to ethanol (0.483; $p = 0.028$), but not with those of the concentrations of acetic acid to lactic acid (0.158; $p = 0.491$).

3.4 Culture-dependent microbial species diversity

The main LAB species found culture-dependently in the water kefir grain inoculum were *Lb. paracasei*, *Lb. higardii*, and *Lb. nagelii* (Figure 2). At the end of backslopping step 8, *Lb. paracasei* and *Lb. nagelii* remained the main LAB species in all fermentation series, whereas *Lb. hilgardii* was not found anymore. Additionally, at the end of backslopping step 8, *Lb.*

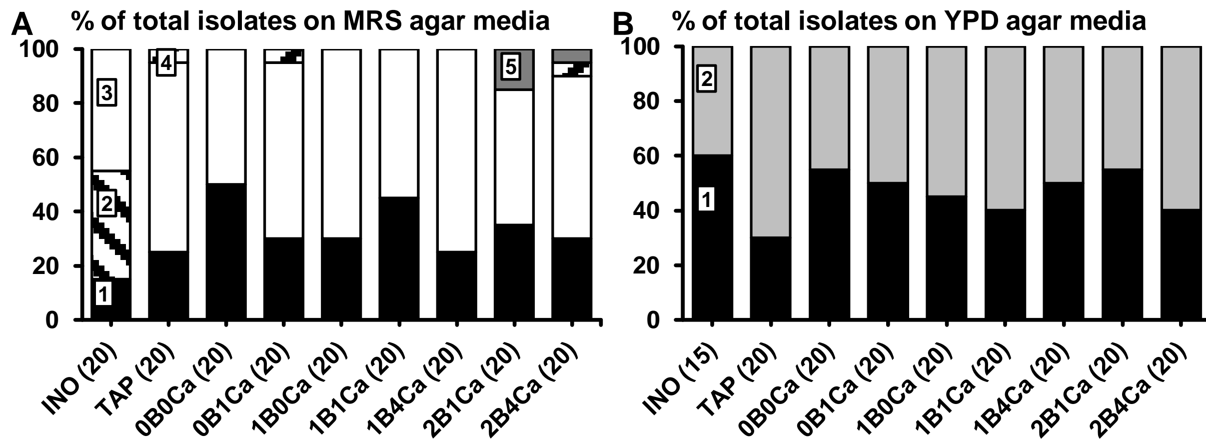

Figure 2. Culture-dependent species diversity on the water kefir grains of the inoculum (INO) and eight fermentation series differing in the buffer capacity and calcium concentration of the water at the end of backslopping step 8. The closest known type strains of the sequenced fragments are given. (A) Isolates from MRS agar media: 1, *Lactobacillus paracasei* (99 % identity; GenBank accession no. AP012541); 2, *Lactobacillus hilgardii* (100 % identity; accession no. LC064898); 3, *Lactobacillus nagelii* (99 % identity; accession no. NR112754); 4, *Lactobacillus harbinensis* (100 % identity; accession no. NR113969); and 5, *Leuconostoc pseudomesenteroides* (99 % identity; accession no. LC096220). (B) Isolates from YPD agar media: 1, *Saccharomyces cerevisiae* [LSU (99 % identity; accession no. KC881066) and ITS (99 % identity; accession no. KC881067)]; and 2, *Dekkera bruxellensis* [LSU (99 % identity; accession no. AY969049) and ITS (99 % identity; accession no. NR111030)]. LSU, large subunit rRNA gene; ITS, internal transcribed spacer.

harbinensis was found in fermentation series TAP, 0B1Ca, and 2B4Ca, and *Leuconostoc pseudomesenteroides* was found in fermentation series 2B1Ca and 2B4Ca. EPS production was found for all the *Leuc. pseudomesenteroides* strains and for 63 % of the *Lb. hilgardii* strains.

The main yeast species found culture-dependently in the water kefir grain inoculum were *S. cerevisiae* and *Dekkera bruxellensis*. They remained the main yeast species until the end of backslopping step 8 in the eight fermentation series (Figure 2).

3.5 Culture-independent microbial species diversity

At the end of backslopping step 8, the rRNA-PCR-DGGE community profiles obtained with the four different primer pairs (V3, LAC, Bif, and Yeast) for the three independent biological replicates performed for each fermentation series were similar (data not shown).

The main bands in the community profiles obtained with the V3 primer pair for the water kefir liquors and grains of the inoculum and the eight fermentation series at the end of backslopping step 8 were attributed to *Lb. hilgardii*, *Lb. mali/hordei*, *Lb. nagelii*, *Lb. paracasei*, *Leuc. pseudomesenteroides*, *Bifidobacterium aquikefiri*, and a non-identified *Oencoccus* species, the latter in particular in fermentation series TAP, 0B0Ca, and 0B1Ca (Figure 3). The partial 16S rRNA gene sequence of the non-identified *Oenococcus* species (213 bp) was deposited in the NCBI nucleotide database (GenBank accession no. LT220205). The relative intensities of the bands attributed to *Lb. nagelii*, *Lb. mali/hordei*, *Leuc. pseudomesenteroides*, and the non-identified *Oencoccus* species where higher for the water kefir liquors than for the water kefir grains, whereas those attributed to *Lb. hilgardii* were

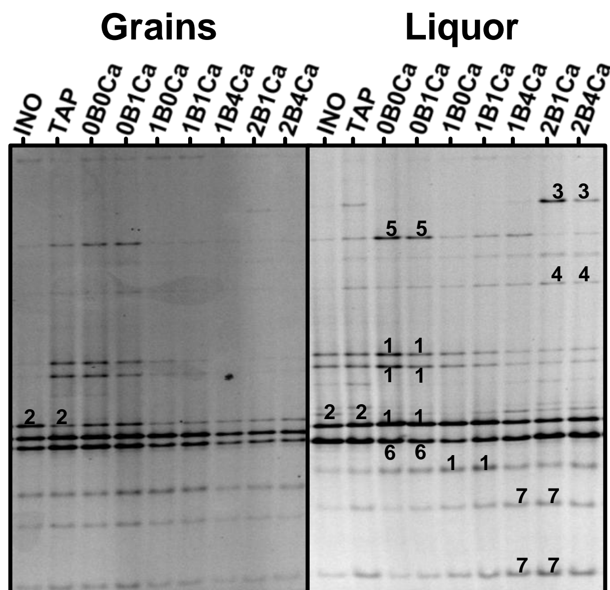

Figure 3. Community profiles obtained with the V3 primer pair for the water kefir grains (left) and water kefir liquors (right) of the inoculum (INO) and eight fermentation series differing in the buffer capacity and calcium concentration of the water, at the end of backslopping step 8. The numbers indicate the bands that were sequenced and the closest known type strains of the sequenced fragments are given. With the V3 primer pair: 1, *Lactobacillus nagelii*/*ghanensis* (99 % identity for both species; GenBank accession no. NR112754/NR043896); 2, *Lactobacillus hilgardii*/*diolivorans* (100 % identity; accession no. LC064898/NR037004); 3, *Leuconostoc pseudomesenteroides* (99 % identity; accession no. LC096220); 4, *Lactobacillus mali/hordei* (100 % identity; accession no. NR112691/NR044394); 5, *Oenococcus kitaharae* (97 % identity; accession no. NR041312); 6, *Bifidobacterium aquikefiri* (100 % identity; accession no. LN849254); and 7, *Lactobacillus paracasei/casei/zeae/rhamnosus* (99 % identity; accession no. AP012541/AP012544/ NR037122/JQ58098).

higher for the grains than for the liquors. When the buffer capacity of the water increased, the relative intensities of the bands attributed to *Leuc*. *pseudomesenteroides* and *Lb*. *paracasei* increased, but those of the bands attributed to *Lb*. *hilgardii* and *Lb*. *nagelii* decreased. The relative intensities of the bands attributed to *Lb*. *mali*/*hordei* were always low and those attributed to *B*. *aquikefiri* were always high for the water kefir liquors and grains of the inoculum and the eight fermentation series at the end of backslopping step 8. The community profiles obtained with the LAC primer pair confirmed the results for the LAB species obtained with the V3 primer pair. The more or less stable presence of bands attributed to *B. aquikefiri* was confirmed by the community profiles obtained with the Bif primer pair (100 % identity; accession no. LN849254).

The main bands in the community profiles obtained with the Yeast primer pair for the water kefir liquors and grains of the inoculum and the eight fermentation series at the end of backslopping step 8 were attributed to *S. cerevisiae* (100 % identity; accession no. KC881066) and *D. bruxellensis* (100 % identity; accession no. AY969049). In the case of the liquors, the relative intensities of the bands attributed to the two species mentioned above were similar. In the case of the grains, the relative intensities of the bands attributed to *S. cerevisiae* were always higher than those of the bands attributed to *D. bruxellensis*.

4 Discussion

The present study revealed that the buffer capacity and the calcium concentration of the water used for water kefir fermentation had an impact on the water kefir grain growth, microbial species diversity, and metabolite production during the water kefir fermentation process. A high buffer capacity and a high calcium concentration of the water resulted in high and low pH values at the end of the fermentations, respectively. When the buffer capacity and/or calcium concentration of the water were below certain minima, the water kefir grain growth decreased gradually over multiple backslopping steps.

Excessive acidic stress decreased the water kefir grain growth during fermentation. This decrease could not be attributed to the disappearance of the EPS-producing *Lb. hilgardii*, as this LAB species was also present when the water kefir grain growth was low. Glucansucrases produced by LAB, which are responsible for the water kefir grain growth, are extracellular enzymes, whose activity is optimal at pH 4.0-5.5 and decreases toward lower pH values (Waldherr *et al.*, 2010; Côté & Skory, 2012). Similarly, EPS production by *Lactobacillus delbrueckii* subsp. *bulgaricus* and kefiran production by *Lactobacillus kefiranofaciens* is optimal around pH 4.5-5.5 (Kimmel *et al.*, 1998; Cheirsilp *et al.*, 2001). However, the water kefir grain growth remained high during the first two backslopping steps of the fermentation series without added buffer, despite their immediate low pH values. This indicated that it was more likely that low pH values compromised the water kefir grain growth by inhibiting the production of glucansucrases during fermentation than by inhibiting the glucansucrase activity itself.

The present study also revealed that an insufficient calcium concentration of the water can cause a decrease of the water kefir grain growth during fermentation. The supply of approximately 51 mg l^{-1} of calcium by adding dried figs, as determined by the national nutrient database for standard reference (release 26, http://ndb.nal.usda.gov/), was not sufficient to sustain good water kefir grain growth. A large part of this calcium was probably not available for the water kefir microorganisms and their enzymes. The calcium concentration of the water necessary for good water kefir grain growth depended on the buffer capacity of the water, as a higher calcium concentration was required at a lower buffer capacity. Further, a higher calcium concentration of the water resulted in a lower pH value, which was associated with lower water grain growth. This indicated that the higher water kefir grain growth at a higher calcium concentration was not mediated by the pH. A high calcium concentration indeed increases the activity of reuteransucrase GTFA-ΔN from *Lb. reuteri* (Kralj *et al.*, 2004), glucansucrase GTF180-ΔN from *Lb. reuteri* (Vujičić-Žagar *et al.*, 2010), and dextransucrase from *Leuc. mesenteroides* (Lopez & Monsan, 1980), and increases the production of kefiran by a *Lactobacillus* sp. from milk kefir grains (Yokoi & Watanabe, 1992).

Further, a high buffer capacity of the water seemed to be advantageous for the growth and metabolism of the LAB compared to the yeasts and resulted in high ratios of LAB to yeasts on the grains, which were reflected in high ratios of the concentrations of lactic acid to ethanol. A high buffer capacity of the water also resulted in high ratios of glycerol to ethanol, and high ratios of acetic acid to lactic acid. Indeed, yeasts grow optimally under acidic conditions, whereas glycerol production by yeasts is optimal around pH 6.0 (Yalcin & Ozbas, 2008).

Low water kefir grain growth was associated with small water kefir grains, high viable counts on the water kefir grains, low total residual carbohydrate concentrations, and high metabolite concentrations, confirming previous results (Chapter 4). When the water kefir grain growth is low, the water kefir grains become small, as they are brittle and break easily during sieving and handling. This increases the viable counts of the microorganisms on the

water kefir grains, as they reside mostly on their surface, resulting in a fast fermentation (Moinas *et al.*, 1980; Neve & Heller, 2002; Chapter 4). Additionally, low water kefir grain growth leaves more glucose available for metabolite production, further resulting in high metabolite concentrations, confirming previous results (Chapter 4).

Lactobacillus hilgardii, *Lb. nagelii*, *Lb. paracasei*, and *S. cerevisiae*, were present both in the inoculum and at the end of all fermentation series, confirming their key role during water kefir fermentation (Chapter 4). Furthermore, *Leuc. pseudomesenteroides, Lb. harbinensis*, *Lb. mali/hordei, B. aquikefiri*, *D. bruxellensis*, and a non-identified *Oenococcus* species were found. These species have been found in water kefir before (Gulitz *et al.*, 2011, 2013; Laureys & De Vuyst, 2014; Laureys *et al.*, 2016; Chapters 3, 4, 5, and 6). The presence of *Lb. hilgardii* strains was not sufficient for good water kefir grain growth, confirming previous results (Chapter 4). Further, *Leuc. pseudomesenteroides* was only present when the buffer capacity was high, which is consistent with its low acid tolerance compared to other LAB species (Axelsson, 2004; Ludwig *et al.*, 2009). This microorganism also produced EPS from sucrose, but probably did not play a role in water kefir gain growth, as it was not always present, preferred the water kefir liquor over the water kefir grains, and did not influence the water kefir grain growth when it was present. This microorganism produces mainly D-lactic acid (Ludwig *et al.*, 2009), and the proportions of D-lactic acid were indeed higher when the buffer capacity of the water was higher. The *Oenococcus* species found might represent a novel species, as its partial 16S rRNA gene sequence was only 97 % identical to that of the closest known *Oenococcus* type strains (Mattarelli *et al.*, 2014). Its relative abundance was high at low pH values, which was in accordance with the acidophilic nature of this genus that occurs naturally in wine, cider, and related habitats (Ludwig *et al.*, 2009).

In conclusion, this study revealed that the buffer capacity and calcium concentration of the water used for water kefir fermentation had an impact on the pH and the water kefir grain growth during fermentation. Furthermore, the buffer capacity of the water impacted the microbial communities and their metabolite production during water kefir fermentation. These data will contribute to the development and upscaling of a stable water kefir production process.

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CHAPTER 8

The presence of oxygen, nutrient concentration, and nutrient source influence the water kefir fermentation process

David Laureys, Maarten Aerts, Peter Vandamme, and Luc De Vuyst

SUMMARY

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*, and high nutrient concentrations favored the growth of *Lactobacillus nagelii* and *Saccharomyces cerevisiae*. Dried figs, dried apricots, and raisins resulted in stable water kefir fermentation, whereby water kefir fermentation with dried apricots resulted in the highest pH and water kefir grain growth, and water kefir fermentation with raisins resulted in the lowest pH and water kefir grain growth. Further, water kefir fermentation with raisins resembled fermentation with low nutrient concentrations, water kefir fermentation with dried apricots resembled fermentation with normal nutrient concentrations, and water kefir fermentation with fresh figs or a mixture of yeast extract and peptone resembled fermentation 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; Marsh *et al.*, 2013b; Stadie *et al.*, 2013; Laureys & De Vuyst, 2014; Chapters 3 and 4). 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, 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 (Waldherr *et al.*, 2010; Laureys & De Vuyst, 2014; Chapters 3 and 4). 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 & De Vuyst, 2014; Chapters 3 and 4). 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 & De Vuyst, 2014; Chapters 3 and 4).

The contents of the vessel wherein water kefir fermentation takes place are usually separated from the atmosphere by a rubber sealing or water lock (Pidoux, 1989; Gulitz *et al.*, 2011; Stadie *et al.*, 2013; Laureys & De Vuyst, 2014; Chapters 3 and 4). 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 microorganisms, such as yeasts (Aceituno *et al.*, 2012) and acetic acid bacteria (AAB) (Guillamón & Mas, 2009), suggesting that the presence of oxygen 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 (Chapter 7). 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 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 water kefir fermentation. Dried figs are the most common fruits used for water kefir fermentation (Pidoux, 1989; Gulitz *et al.*, 2011; Stadie *et al.*, 2013; Laureys & De Vuyst, 2014; Chapters 3, 4, 5, and 7), but raisins, plums, or dates have also been used (Reiß, 1990).

This chapter 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

The water kefir grain inoculum was prepared by means of prefermentations through backslopping as described in Chapter 7.

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 during 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 glass 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 bottles were equipped with a PTFE water lock for fermentation 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 fermentation 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 days, 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 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 and water kefir grain wet and dry mass 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 in Chapter 7.

2.5 Microbial enumerations

The viable counts of the presumptive LAB were determined on de Man-Rogosa-Sharpe (MRS) agar medium, those of the presumptive AAB on modified deoxycholate mannitol sorbitol (mDMS) agar medium, and those of presumptive yeasts on yeast extract peptone dextrose (YPD) agar medium, as described in Chapter 7.

2.6 Culture-dependent microbial species diversity analyses

The culture-dependent microbial species diversity analyses of the LAB, AAB, and yeasts on the water kefir grains were determined by randomly picking up 10 to 20 % of the total number of colonies from the respective agar media with 30 to 300 colonies. Isolates were subcultivated on their respective agar media until the third generation and used for dereplication by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) fingerprinting, as described in Chapter 7. The fingerprint peptide patterns obtained were clustered numerically by means of the BioNumerics software version 7.50 (Applied Maths, Sint-Martens-Latem, Belgium). Representative bacterial isolates within each cluster were identified by sequencing part of their 16S rRNA gene from genomic DNA, as described in Chapter 3. Representative yeast isolates within each cluster were identified by sequencing part of their 26S large subunit (LSU) rRNA gene and internal transcribed spacer (ITS) region from genomic DNA, as described in Chapter 3.

2.7 Exopolysaccharide production

All bacterial isolates were grown on MRS agar medium supplemented with 10 g l^{-1} of sucrose at 30 °C for 7 days to visually assess their EPS production capacity.

2.8 Culture-independent microbial species diversity analyses

The culture-independent microbial species diversity analyses of the bacteria and yeasts in the water kefir liquors and on the water kefir grains were determined after preparing total DNA extracts from the cell pellets of the water kefir liquors and 0.2 g of crushed water kefir grains, respectively, as described in Chapter 7. The culture-independent microbial community profiles were obtained by amplifying selected genomic fragments in the total DNA with the universal prokaryotic primer pair (V3), the LAB-specific primer pair (LAC), the *Bifidobacterium*-specific primer pair (Bif), and the universal eukaryotic primer pair (Yeast); and separating the PCR amplicons through denaturing gradient gel electrophoresis (DGGE), as described in Chapter 3. Selected bands in the community profiles were cut from the gels and identified through sequencing, as described in Chapter 3.

2.9 Substrate and metabolite concentration determinations

The preparation of samples and the determination of the concentrations of sucrose, glucose, fructose, glycerol, and mannitol (high-performance anion exchange chromatography with pulsed amperometric detection, HPAEC-PAD), of those of D- and L-lactic acid and acetic acid (high-performance liquid chromatography with ultraviolet detection, HPLC-UV), of those of ethanol (gas chromatography with flame ionization detection, GC-FID), and of those of the aroma compounds (static headspace gas chromatography with mass spectrometry detection, SH-GC-MS) were carried out as described in Chapter 7.

2.10 Carbon recovery

The carbon recovery at the end of a backslopping step was calculated as described in Chapter 3, whereby the total amount of carbon including that of the fruits added to the fermentation was taken into account. The mono- and disaccharide contents ($m\ m^{-1}$) of dried figs (48 %), dried apricots (53 %), dried raisins (59 %), and fresh figs (16 %) were obtained from the national nutrient database for standard reference (release 26, http://ndb.nal.usda.gov/). Those of the YP solution were assumed to be 0 %.

2.11 Statistics

An ANOVA was performed in R 3.2.0 to test for differences between the eight water kefir fermentation series, followed by a series of post-hoc pairwise comparisons with Fisher's least significant difference (LSD) test, as described in Chapter 7. All statistical tests were performed with a significance level of 0.05.

3 Results

3.1 Influence of oxygen

3.1.1 pH and water kefir grain wet and dry mass

The pH and the water kefir grain growth (based on wet mass) were similar in the aerobic and anaerobic fermentation series at the end of backslopping step 1 (Table 1). Over the course of the eight backslopping steps, their values decreased slightly in the aerobic fermentation series (Figure 1; Tables 2 and 3). The water kefir grain dry mass was similar in the aerobic and anaerobic fermentation series at the end of bacslopping step 8 (Table 4).

3.1.2 Microbial enumerations

The viable counts of the LAB and yeasts, and the ratios of the viable counts of the LAB to the yeasts were similar in the anaerobic and aerobic fermentation series (Table 4). The viable counts of the AAB were significantly higher in the aerobic fermentation series than in the anaerobic ones.

3.1.3 Culture-dependent microbial species diversity

The culture-dependent species diversity of the yeasts, LAB, and AAB in the aerobic and anaerobic fermentation series was similar and more or less comparable to the inoculum (Figure 2). Two yeast species were found, whereby the relative abundances of *S. cerevisiae* were always higher than those of *Dekkera bruxellensis*. The main LAB species were

Table 1. Characteristics of eight water kefir fermentation series differing in the presence of oxygen, nutrient concentration, and nutrient source [anaerobic control fermentation with dried figs (1DF-An); aerobic fermentation with dried figs (1DF-Ae); anaerobic fermentation with low (0DF-An) and high (2DF-An) amounts of dried figs; and anaerobic fermentation with dried apricots (1DA-An), dried raisins (1DR-An), fresh figs (1FF-An), and a mixture of yeast extract and peptone (YP-An)] at the end of backslopping step 1. Significant differences ($p < 0.05$) between the series are indicated with different superscripts (a, b, c, d, e, and f).

Characteristic	0DF-An	1DF-An	1DF-Ae	2DF-An	1DA-An	1DR-An	1FF-An	YP-An
Water kefir grain growth (%)	65.9 ± 2.8 ^{ab}	63.4 ± 1.9 ^{b}	62.7 ± 0.4 ^{b}	58.0 ± 2.5 ^{c}	69.2 ± 1.1 ^{a}	51.7 ± 4.0 ^{d}	56.0 ± 3.3 ^{c}	55.3 ± 1.4 ^{cd}
pH	3.54 ± 0.01 ^{b}	3.46 ± 0.05 ^{cd}	3.43 ± 0.01 ^{ce}	3.47 ± 0.03 ^{c}	3.64 ± 0.04 ^{a}	3.41 ± 0.01 ^{de}	3.39 ± 0.02 ^{e}	3.34 ± 0.01 ^{f}
Sucrose (g l^{-1})	1.0 ± 0.1 ^{bc}	1.1 ± 0.2 ^{ab}	1.1 ± 0.1 ^{ab}	1.2 ± 0.1 ^{a}	1.1 ± 0.1 ^{ab}	0.9 ± 0.1 ^{cd}	0.8 ± 0.1 ^{d}	0.5 ± 0.1 ^{e}
Glucose (g l^{-1})	0.2 ± 0.1 ^{bc}	0.3 ± 0.3 ^{b}	0.1 ± 0.1 ^{bc}	0.3 ± 0.3 ^{bc}	0.0 ± 0.1 ^{bc}	1.0 ± 0.4 ^{a}	0.1 ± 0.1 ^{bc}	0.0 ± 0.1 ^{c}
Fructose (g l^{-1})	13.0 ± 0.1 ^{a}	7.8 ± 4.0 ^{bc}	5.6 ± 2.5 ^{cd}	7.3 ± 3.1 ^{c}	2.6 ± 4.1 ^{de}	12.2 ± 1.5 ^{ab}	3.7 ± 2.3 ^{ce}	0.0 ± 0.1 ^{e}
Total residual carbohydrates (g l^{-1})	14.1 ± 0.2 ^{a}	9.3 ± 4.2 ^{b}	6.7 ± 2.5 ^{bc}	8.7 ± 3.3 ^{b}	3.8 ± 4.1 ^{cd}	14.1 ± 1.9 ^{a}	4.6 ± 2.4 ^{bd}	0.6 ± 0.1 ^{d}
Ethanol (g l^{-1})	7.65 ± 0.08 ^{c}	15.23 ± 1.76 ^{b}	15.78 ± 1.31 ^{b}	20.60 ± 1.87 ^{a}	15.51 ± 0.55 ^{b}	16.09 ± 1.27 ^{b}	14.08 ± 0.81 ^{b}	16.01 ± 0.04 ^{b}
Lactic acid (g l^{-1})	1.32 ± 0.04 ^{d}	2.67 ± 0.14 ^{bc}	2.50 ± 0.09 ^{c}	3.47 ± 0.19 ^{a}	2.89 ± 0.10 ^{b}	2.45 ± 0.19 ^{c}	2.67 ± 0.35 ^{bc}	2.44 ± 0.12 ^{c}
Acetic acid (g l^{-1})	0.58 ± 0.07 ^{e}	1.18 ± 0.31 ^{ac}	1.46 ± 0.34 ^{a}	1.41 ± 0.24 ^{a}	1.26 ± 0.21 ^{ab}	0.98 ± 0.19 ^{bcd}	0.75 ± 0.21 ^{de}	0.79 ± 0.18 ^{ce}
Glycerol (g l^{-1})	1.12 ± 0.06 ^{f}	2.07 ± 0.17 ^{bc}	1.94 ± 0.06 ^{c}	2.73 ± 0.15 ^{a}	2.06 ± 0.07 ^{bc}	2.14 ± 0.15 ^{b}	1.71 ± 0.11 ^{d}	1.50 ± 0.01 ^{e}
Mannitol (g l^{-1})	0.75 ± 0.03 ^{de}	0.95 ± 0.06 ^{bc}	0.90 ± 0.06 ^{c}	1.06 ± 0.01 ^{ab}	0.83 ± 0.05 ^{cd}	1.19 ± 0.06 ^{a}	0.67 ± 0.18 ^{e}	0.45 ± 0.01 ^{f}
2-Methyl-1-propanol (mg l^{-1})	4.59 ± 0.30 ^{d}	9.23 ± 1.53 ^{bc}	11.05 ± 1.92 ^{b}	13.28 ± 1.75 ^{a}	9.03 ± 1.15 ^{bc}	9.89 ± 1.23 ^{bc}	8.69 ± 0.41 ^{c}	14.91 ± 0.46 ^{a}
Isoamyl alcohol (mg l^{-1})	31.97 ± 2.73 ^{e}	50.65 ± 2.86 ^{c}	55.65 ± 3.62 ^{bc}	64.48 ± 5.71 ^{a}	54.79 ± 5.62 ^{bc}	50.52 ± 3.67 ^{c}	42.24 ± 2.26 ^{d}	57.61 ± 1.43 ^{b}
2-Phenylethanol (mg l^{-1})	8.03 ± 1.87	8.33 ± 3.80	8.91 ± 1.49	8.83 ± 4.01	7.70 ± 1.28	7.32 ± 1.87	11.27 ± 0.30	12.67 ± 1.66
Ethyl acetate (mg l^{-1})	6.86 ± 0.83 ^{e}	13.77 ± 1.49 ^{bc}	17.74 ± 1.22 ^{a}	16.87 ± 1.05 ^{a}	14.65 ± 0.59 ^{b}	14.23 ± 1.00 ^{bc}	6.74 ± 0.87 ^{d}	13.76 ± 0.19 ^{cd}
Isoamyl acetate (mg l^{-1})	0.019 ± 0.002 ^{e}	0.075 ± 0.026 ^{cd}	0.064 ± 0.016 ^{cd}	0.120 ± 0.028 ^{b}	0.094 ± 0.029 ^{bc}	0.053 ± 0.008 ^{d}	0.056 ± 0.007 ^{d}	0.204 ± 0.010 ^{a}
Ethyl hexanoate (mg l^{-1})	0.036 ± 0.029 ^{f}	0.085 ± 0.024 ^{bcd}	0.058 ± 0.017 ^{def}	0.126 ± 0.019 ^{a}	0.097 ± 0.027 ^{ac}	0.076 ± 0.010 ^{ce}	0.050 ± 0.004 ^{ef}	0.113 ± 0.013 ^{ab}
Ethyl octanoate (mg l^{-1})	0.230 ± 0.062 ^{d}	0.540 ± 0.054 ^{c}	0.568 ± 0.075 ^{c}	0.823 ± 0.184 ^{b}	0.651 ± 0.189 ^{bc}	0.765 ± 0.060 ^{b}	0.537 ± 0.060 ^{c}	1.173 ± 0.049 ^{a}
Ethyl decanoate (mg l^{-1})	0.025 ± 0.016 ^{c}	0.344 ± 0.231 ^{bc}	0.233 ± 0.141 ^{bc}	0.668 ± 0.197 ^{b}	0.537 ± 0.245 ^{bc}	0.570 ± 0.048 ^{b}	0.525 ± 0.288 ^{bc}	3.456 ± 0.714 ^{a}
Glycerol/ethanol (mmol/mol)	110 ± 4 ^{a}	103 ± 13 ^{ab}	93 ± 6 ^{b}	100 ± 12 ^{ab}	100 ± 7 ^{ab}	100 ± 5 ^{ab}	91 ± 1 ^{b}	70 ± 1 ^{c}
Lactic acid/ethanol (mmol/mol)	133 ± 4 ^{abc}	135 ± 13 ^{abc}	122 ± 7 ^{cd}	129 ± 5 ^{bd}	143 ± 11 ^{ab}	117 ± 6 ^{d}	145 ± 11 ^{a}	117 ± 6 ^{d}
Acetic acid/ethanol (mmol/mol)	58 ± 7 ^{ad}	60 ± 18 ^{abc}	71 ± 17 ^{a}	52 ± 5 ^{ad}	63 ± 14 ^{ab}	47 ± 12 ^{bd}	41 ± 10 ^{cd}	38 ± 9 ^{d}
Acetic acid/lactic acid (mmol/mol)	439 ± 41 ^{b}	439 ± 95 ^{b}	580 ± 117 ^{a}	405 ± 50 ^{bc}	436 ± 69 ^{b}	401 ± 83 ^{bc}	279 ± 49 ^{c}	322 ± 59 ^{bc}
D-lactic acid (% of total)	45.0 ± 1.1	44.6 ± 0.5	45.1 ± 0.4	45.2 ± 0.6	45.6 ± 1.1	44.1 ± 0.6	46.4 ± 0.9	44.8 ± 0.7
Carbon recovery (%)	97.4 ± 1.0 ^{c}	99.1 ± 0.8 ^{bc}	98.5 ± 1.3 ^{bc}	97.2 ± 1.8 ^{c}	98.1 ± 0.2 ^{bc}	100.1 ± 1.6 ^{b}	90.4 ± 1.2 ^{d}	103.2 ± 0.9 ^{a}

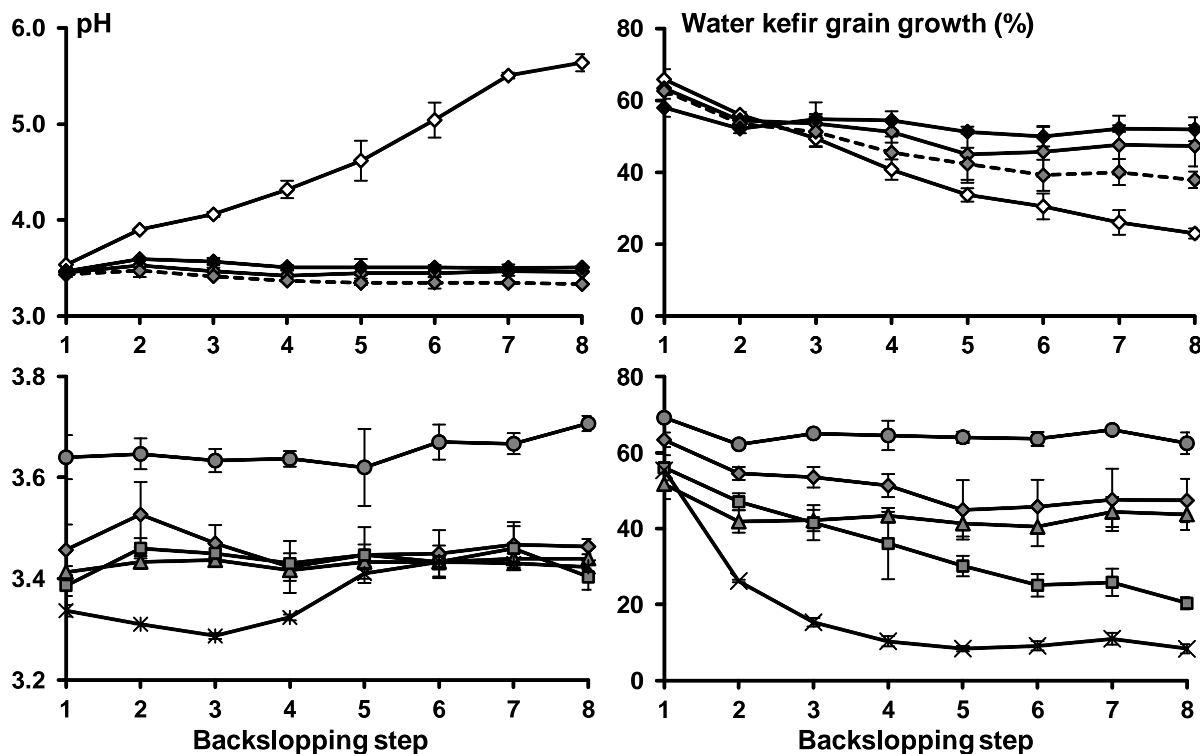

Figure 1. The pH and water kefir grain growth for eight water kefir fermentation series differing in the presence of oxygen, nutrient concentration, and nutrient source (at the end of each backslopping step): anaerobic [1DF-An (♦——)] and aerobic [1DF-Ae (♦- - -)] fermentation series with dried figs (top); anaerobic fermentation series with low [0DF-An (◊——)], normal [1DF-An (♦——)], or high [2DF-An (♦——)] nutrient concentration (top); and anaerobic fermentation series with dried figs [1DF-An (♦——)], dried apricots [1DA-An (●——)], raisins [1DR-An (▲——)], fresh figs [1FF-An (■——)], and a mixture of yeast extract and peptone [YP-An (X——)] (bottom). For differences of significance, see Tables 2 and 3.

Lb. paracasei, *Lb. hilgardii* (67 % of the strains produced EPS), and *Lb. nagelii*. Additionally, *Lactobacillus harbinensis* strains were found in the water kefir grain inoculum and in the anaerobic fermentation series. The main AAB species in the inoculum and anaerobic fermentation series were *Gluconobacter roseus*/*oxydans* and *Acetobacter indonesiensis*, and the main AAB species in the aerobic fermentation series was *Acetobacter fabarum*.

3.1.4 Culture-independent microbial species diversity

At the end of backslopping step 8, the rRNA-PCR-DGGE community profiles obtained with the four different primer pairs used (V3, LAC, Bif, and Yeast) were similar for the three independent biological replicates performed for each fermentation series (data not shown).

The main bands in the community profiles of the inoculum, the aerobic fermentation series, and the anaerobic fermentation series obtained with the Yeast primer pair were attributed to *S. cerevisiae* and *D. bruxellensis* (Figure 3). The relative intensities of the bands attributed to *S. cerevisiae* were higher than those of the bands attributed to *D. bruxellensis*. The relative intensities of the bands attributed to *D. bruxellensis* were higher in the liquors than on the grains. Additionally, a band with weak relative intensity, which was attributed to *Candida smithsonii*, was detected in the liquors of the aerobic and anaerobic fermentation series.

Table 2. The pH in eight water kefir fermentation series differing in the presence of oxygen, nutrient concentration, and nutrient source [anaerobic control fermentation with dried figs (1DF-An); aerobic fermentation with dried figs (1DF-Ae); anaerobic fermentations with low (0DF-An) and high (2DF-An) amounts of dried figs; and anaerobic fermentation with dried apricots (1DA-An), dried raisins (1DR-An), fresh figs (1FF-An), and a mixture of yeast extract and peptone (YP-An)], at the end of backslopping steps 1-8. Significant differences ($p < 0.05$) between the series are indicated with different superscripts (a, b, c, d, e, and f).

Backslopping step	0DF-An	1DF-An	1DF-Ae	2DF-An	1DA-An	1DR-An	1FF-An	YP-An
1	3.54 ± 0.01 ^{b}	3.46 ± 0.05 ^{cd}	3.43 ± 0.01 ^{ce}	3.47 ± 0.03 ^{c}	3.64 ± 0.04 ^{a}	3.41 ± 0.01 ^{de}	3.39 ± 0.02 ^{e}	3.34 ± 0.01 ^{f}
2	3.90 ± 0.01 ^{a}	3.53 ± 0.06 ^{c}	3.47 ± 0.07 ^{cd}	3.59 ± 0.01 ^{b}	3.65 ± 0.03 ^{b}	3.43 ± 0.01 ^{d}	3.46 ± 0.02 ^{d}	3.31 ± 0.01 ^{e}
3	4.06 ± 0.03 ^{a}	3.47 ± 0.04 ^{d}	3.42 ± 0.01 ^{e}	3.57 ± 0.04 ^{c}	3.63 ± 0.02 ^{b}	3.44 ± 0.01 ^{de}	3.45 ± 0.02 ^{de}	3.29 ± 0.01 ^{f}
4	4.32 ± 0.09 ^{a}	3.42 ± 0.05 ^{d}	3.37 ± 0.01 ^{de}	3.51 ± 0.02 ^{c}	3.64 ± 0.02 ^{b}	3.42 ± 0.02 ^{d}	3.43 ± 0.02 ^{d}	3.32 ± 0.01 ^{e}
5	4.62 ± 0.21 ^{a}	3.45 ± 0.06 ^{cd}	3.35 ± 0.02 ^{d}	3.51 ± 0.08 ^{bc}	3.62 ± 0.08 ^{b}	3.43 ± 0.02 ^{cd}	3.45 ± 0.02 ^{cd}	3.41 ± 0.01 ^{cd}
6	5.04 ± 0.18 ^{a}	3.45 ± 0.05 ^{cd}	3.35 ± 0.06 ^{d}	3.51 ± 0.03 ^{c}	3.67 ± 0.03 ^{b}	3.43 ± 0.01 ^{cd}	3.43 ± 0.03 ^{cd}	3.43 ± 0.02 ^{cd}
7	5.51 ± 0.03 ^{a}	3.47 ± 0.05 ^{cd}	3.35 ± 0.02 ^{e}	3.50 ± 0.04 ^{c}	3.67 ± 0.02 ^{b}	3.44 ± 0.01 ^{d}	3.46 ± 0.04 ^{cd}	3.43 ± 0.01 ^{d}
8	5.64 ± 0.09 ^{a}	3.46 ± 0.02 ^{cd}	3.33 ± 0.01 ^{e}	3.51 ± 0.02 ^{c}	3.71 ± 0.02 ^{b}	3.44 ± 0.02 ^{d}	3.40 ± 0.03 ^{d}	3.42 ± 0.01 ^{d}

Table 3. The water kefir grain growth of eight water kefir fermentation series differing in the presence of oxygen, nutrient concentration, and nutrient source [anaerobic control fermentation with dried figs (1DF-An); aerobic fermentation with dried figs (1DF-Ae); anaerobic fermentations with low (0DF-An) and high (2DF-An) amounts of dried figs; and anaerobic fermentation with dried apricots (1DA-An), dried raisins (1DR-An), fresh figs (1FF-An), and a mixture of yeast extract and peptone (YP-An)], at the end of backslopping steps 1-8. Significant differences ($p < 0.05$) between the series are indicated with different superscripts (a, b, c, d, e, and f).

Backslopping step	0DF-An	1DF-An	1DF-Ae	2DF-An	1DA-An	1DR-An	1FF-An	YP-An
1	65.9 ± 2.8 ^{ab}	63.4 ± 1.9 ^{b}	62.7 ± 0.4 ^{b}	58.0 ± 2.5 ^{c}	69.2 ± 1.1 ^{a}	51.7 ± 4.0 ^{d}	56.0 ± 3.3 ^{c}	55.3 ± 1.4 ^{cd}
2	56.1 ± 0.8 ^{b}	54.5 ± 1.7 ^{bc}	53.5 ± 1.5 ^{bc}	52.2 ± 1.3 ^{c}	62.2 ± 1.0 ^{a}	41.9 ± 2.9 ^{e}	47.1 ± 2.2 ^{d}	26.2 ± 0.3 ^{f}
3	49.4 ± 2.1 ^{b}	53.5 ± 2.7 ^{b}	51.3 ± 4.3 ^{b}	54.9 ± 4.6 ^{b}	65.1 ± 0.6 ^{a}	42.1 ± 2.8 ^{c}	41.5 ± 4.6 ^{c}	15.3 ± 1.1 ^{d}
4	40.8 ± 2.8 ^{de}	51.3 ± 3.0 ^{bc}	45.5 ± 4.5 ^{cd}	54.4 ± 2.6 ^{b}	64.5 ± 3.9 ^{a}	43.4 ± 1.0 ^{de}	36.1 ± 9.4 ^{e}	10.3 ± 1.4 ^{f}
5	33.7 ± 1.8 ^{d}	44.9 ± 7.8 ^{c}	42.4 ± 4.5 ^{c}	51.3 ± 0.6 ^{b}	64.1 ± 1.5 ^{a}	41.3 ± 3.4 ^{c}	30.1 ± 2.7 ^{d}	8.4 ± 0.7 ^{e}
6	30.5 ± 3.6 ^{d}	45.8 ± 7.1 ^{bc}	39.2 ± 4.3 ^{c}	49.9 ± 2.7 ^{b}	63.6 ± 1.8 ^{a}	40.5 ± 5.1 ^{c}	25.1 ± 3.0 ^{d}	9.1 ± 1.2 ^{e}
7	26.1 ± 3.4 ^{e}	47.6 ± 8.2 ^{bc}	40.1 ± 3.6 ^{d}	52.2 ± 0.9 ^{b}	66.0 ± 0.2 ^{a}	44.4 ± 3.9 ^{cd}	25.9 ± 3.6 ^{e}	11.0 ± 1.6 ^{f}
8	23.0 ± 1.4 ^{e}	47.4 ± 5.7 ^{bc}	37.9 ± 2.3 ^{d}	52.0 ± 3.3 ^{b}	62.5 ± 2.9 ^{a}	43.7 ± 4.1 ^{c}	20.3 ± 1.6 ^{e}	8.3 ± 1.2 ^{f}

Table 4. Characteristics of eight water kefir fermentation series differing in the presence of oxygen, nutrient concentration, and nutrient source [anaerobic control fermentation with dried figs (1DF-An); aerobic fermentation with dried figs (1DF-Ae); anaerobic fermentation with low (0DF-An) and high (2DF-An) amounts of dried figs; and anaerobic fermentation with dried apricots (1DA-An), dried raisins (1DR-An), fresh figs (1FF-An), and a mixture of yeast extract and peptone (YP-An)] at the end of backslopping step 8. Significant differences between the series are indicated with different superscripts (a, b, c, d, e, and f).

Characteristic	0DF-An	1DF-An	1DF-Ae	2DF-An	1DA-An	1DR-An	1FF-An	YP-An
Yeasts (log cfu g^{-1})	6.8 ± 0.1 ^{f}	7.5 ± 0.1 ^{de}	7.4 ± 0.1 ^{de}	7.7 ± 0.1 ^{bc}	7.3 ± 0.1 ^{e}	7.6 ± 0.1 ^{cd}	7.8 ± 0.1 ^{b}	8.2 ± 0.2 ^{a}
Lactic acid bacteria (log cfu g^{-1})	8.2 ± 0.1 ^{bc}	8.6 ± 0.1 ^{a}	8.6 ± 0.1 ^{a}	8.5 ± 0.1 ^{a}	8.0 ± 0.2 ^{cd}	7.9 ± 0.1 ^{de}	8.2 ± 0.1 ^{b}	7.8 ± 0.1 ^{e}
Acetic acid bacteria (log cfu g^{-1})	4.8 ± 0.3 ^{b}	3.3 ± 0.7 ^{ef}	6.3 ± 0.4 ^{a}	3.6 ± 0.1 ^{de}	4.5 ± 0.2 ^{bc}	4.0 ± 0.2 ^{cd}	2.8 ± 0.2 ^{fg}	2.3 ± 0.3 ^{g}
Lactic acid bacteria/yeasts (cfu/cfu)	25.9 ± 1.2 ^{a}	12.6 ± 1.2 ^{b}	13.4 ± 1.2 ^{b}	6.3 ± 1.3 ^{c}	5.0 ± 1.7 ^{cd}	1.9 ± 1.4 ^{de}	2.9 ± 1.3 ^{ce}	0.3 ± 1.5 ^{e}
Water kefir grain growth (%)	23.0 ± 1.4 ^{e}	47.4 ± 5.7 ^{bc}	37.9 ± 2.3 ^{d}	52.0 ± 3.3 ^{b}	62.5 ± 2.9 ^{a}	43.7 ± 4.1 ^{c}	20.3 ± 1.6 ^{e}	8.3 ± 1.2 ^{f}
Water kefir grain dry mass (%)	17.1 ± 0.2 ^{a}	14.1 ± 0.1 ^{bd}	14.1 ± 0.3 ^{cd}	13.9 ± 0.2 ^{cd}	14.3 ± 0.2 ^{bc}	14.5 ± 0.3 ^{b}	14.1 ± 0.2 ^{cd}	13.8 ± 0.2 ^{d}
pH	5.64 ± 0.09 ^{a}	3.46 ± 0.02 ^{cd}	3.33 ± 0.01 ^{e}	3.51 ± 0.02 ^{c}	3.71 ± 0.0 ^{b}	3.44 ± 0.02 ^{d}	3.40 ± 0.03 ^{d}	3.42 ± 0.01 ^{d}
Sucrose (g l^{-1})	27.1 ± 0.6 ^{a}	0.8 ± 0.2 ^{b}	0.6 ± 0.2 ^{b}	0.6 ± 0.1 ^{b}	1.0 ± 0.1 ^{b}	0.8 ± 0.1 ^{b}	0.1 ± 0.1 ^{c}	0.1 ± 0.1 ^{c}
Glucose (g l^{-1})	3.0 ± 0.1 ^{a}	0.1 ± 0.1 ^{c}	0.1 ± 0.1 ^{c}	0.1 ± 0.1 ^{c}	0.1 ± 0.1 ^{c}	0.5 ± 0.3 ^{b}	0.0 ± 0.0 ^{c}	0.0 ± 0.0 ^{c}
Fructose (g l^{-1})	12.7 ± 0.6 ^{a}	2.9 ± 4.6 ^{c}	2.8 ± 2.0 ^{c}	0.1 ± 0.1 ^{c}	3.4 ± 3.3 ^{c}	7.5 ± 2.9 ^{b}	0.1 ± 0.1 ^{c}	0.0 ± 0.1 ^{c}
Total residual carbohydrates (g l^{-1})	42.8 ± 0.1 ^{a}	3.8 ± 5.0 ^{c}	3.5 ± 2.3 ^{c}	0.8 ± 0.2 ^{c}	4.4 ± 3.4 ^{bc}	8.8 ± 3.2 ^{b}	0.3 ± 0.2 ^{c}	0.1 ± 0.0 ^{c}
Ethanol (g l^{-1})	0.3 ± 0.02 ^{e}	21.3 ± 3.37 ^{c}	18.8 ± 1.67 ^{cd}	28.2 ± 0.70 ^{a}	16.2 ± 1.81 ^{d}	20.0 ± 0.64 ^{c}	24.5 ± 0.99 ^{b}	26.1 ± 0.39 ^{ab}
Lactic acid (g l^{-1})	0.11 ± 0.01 ^{e}	2.83 ± 0.41 ^{c}	2.26 ± 0.15 ^{d}	3.99 ± 0.18 ^{a}	2.63 ± 0.20 ^{c}	2.23 ± 0.12 ^{d}	3.35 ± 0.13 ^{b}	2.06 ± 0.01 ^{d}
Acetic acid (g l^{-1})	0.11 ± 0.01 ^{d}	1.19 ± 0.08 ^{bc}	7.88 ± 1.47 ^{a}	1.24 ± 0.06 ^{bc}	1.16 ± 0.10 ^{bc}	1.52 ± 0.31 ^{b}	0.79 ± 0.11 ^{bd}	0.47 ± 0.03 ^{cd}
Glycerol (g l^{-1})	0.11 ± 0.01 ^{e}	1.96 ± 0.06 ^{b}	1.61 ± 0.18 ^{d}	2.42 ± 0.05 ^{a}	1.89 ± 0.08 ^{bc}	2.42 ± 0.12 ^{a}	1.87 ± 0.24 ^{bc}	1.72 ± 0.05 ^{cd}
Mannitol (g l^{-1})	0.10 ± 0.01 ^{c}	0.42 ± 0.12 ^{bc}	0.58 ± 0.32 ^{b}	0.69 ± 0.07 ^{b}	0.47 ± 0.07 ^{bc}	2.34 ± 0.29 ^{a}	0.76 ± 0.62 ^{b}	0.00 ± 0.00 ^{c}
2-Methyl-1-propanol (mg l^{-1})	0.24 ± 0.02 ^{d}	13.51 ± 3.14 ^{bc}	12.66 ± 0.80 ^{bc}	17.56 ± 0.55 ^{a}	10.82 ± 2.60 ^{c}	11.60 ± 0.76 ^{c}	17.75 ± 1.61 ^{a}	15.19 ± 0.38 ^{ab}
Isoamyl alcohol (mg l^{-1})	1.25 ± 0.18 ^{e}	56.12 ± 4.90 ^{c}	42.95 ± 1.79 ^{d}	68.06 ± 2.63 ^{ab}	62.65 ± 5.79 ^{bc}	44.99 ± 1.55 ^{d}	62.66 ± 6.51 ^{bc}	70.71 ± 1.83 ^{a}
2-Phenylethanol (mg l^{-1})	0.08 ± 0.04 ^{c}	7.72 ± 1.80 ^{ab}	4.94 ± 1.19 ^{b}	7.58 ± 2.19 ^{ab}	10.24 ± 2.51 ^{a}	5.91 ± 2.49 ^{b}	6.71 ± 0.61 ^{b}	9.98 ± 0.92 ^{a}
Ethyl acetate (mg l^{-1})	0.02 ± 0.00 ^{e}	16.07 ± 1.36 ^{c}	19.64 ± 2.62 ^{ab}	14.88 ± 0.90 ^{c}	17.38 ± 2.92 ^{bc}	22.40 ± 1.27 ^{a}	15.99 ± 2.80 ^{c}	10.83 ± 0.44 ^{d}
Isoamyl acetate (mg l^{-1})	0.001 ± 0.000 ^{f}	0.170 ± 0.081 ^{cd}	0.080 ± 0.007 ^{df}	0.387 ± 0.081 ^{b}	0.114 ± 0.035 ^{de}	0.060 ± 0.005 ^{ef}	0.226 ± 0.096 ^{c}	0.573 ± 0.034 ^{a}
Ethyl hexanoate (mg l^{-1})	0.001 ± 0.000 ^{e}	0.163 ± 0.049 ^{c}	0.051 ± 0.009 ^{de}	0.264 ± 0.027 ^{b}	0.146 ± 0.044 ^{c}	0.092 ± 0.009 ^{d}	0.222 ± 0.035 ^{b}	0.371 ± 0.019 ^{a}
Ethyl octanoate (mg l^{-1})	0.005 ± 0.001 ^{e}	1.570 ± 0.650 ^{c}	1.304 ± 0.38 ^{cd}	2.252 ± 0.314 ^{b}	0.841 ± 0.221 ^{d}	0.920 ± 0.039 ^{d}	1.819 ± 0.315 ^{bc}	3.057 ± 0.102 ^{a}
Ethyl decanoate (mg l^{-1})	0.015 ± 0.008 ^{e}	1.345 ± 0.999 ^{cd}	1.400 ± 0.075 ^{cd}	2.429 ± 0.588 ^{b}	0.756 ± 0.464 ^{de}	0.438 ± 0.108 ^{e}	1.945 ± 0.196 ^{bc}	4.502 ± 0.682 ^{a}
Glycerol/ethanol (mmol/mol)	321 ± 23 ^{a}	71 ± 11 ^{c}	64 ± 4 ^{cd}	64 ± 2 ^{cd}	88 ± 7 ^{b}	91 ± 2 ^{b}	57 ± 9 ^{cd}	49 ± 1 ^{d}
Lactic acid/ethanol (mmol/mol)	331 ± 37 ^{a}	103 ± 7 ^{bc}	92 ± 3 ^{c}	108 ± 2 ^{bc}	126 ± 14 ^{b}	85 ± 3 ^{c}	105 ± 2 ^{bc}	60 ± 1 ^{d}
Acetic acid/ethanol (mmol/mol)	337 ± 32 ^{a}	43 ± 5 ^{b}	325 ± 78 ^{a}	34 ± 2 ^{b}	56 ± 8 ^{b}	58 ± 10 ^{b}	25 ± 3 ^{b}	14 ± 1 ^{b}
Acetic acid/lactic acid (mmol/mol)	1022 ± 30 ^{b}	422 ± 37 ^{c}	3511 ± 799 ^{a}	310 ± 16 ^{c}	441 ± 22 ^{c}	677 ± 99 ^{bc}	234 ± 26 ^{c}	227 ± 15 ^{c}
D-lactic acid (% of total)	46.4 ± 2.9	45.0 ± 1.2	44.2 ± 2.6	42.4 ± 1.2	44.3 ± 1.1	42.7 ± 0.3	43.1 ± 2.5	42.8 ± 0.6
Carbon recovery (%)	98.6 ± 0.5 ^{d}	101.8 ± 2.8 ^{c}	103.2 ± 1.0 ^{bc}	102.9 ± 0.8 ^{bc}	97.4 ± 0.7 ^{d}	104.7 ± 0.7 ^{ab}	96.7 ± 2.3 ^{d}	105.7 ± 0.2 ^{a}

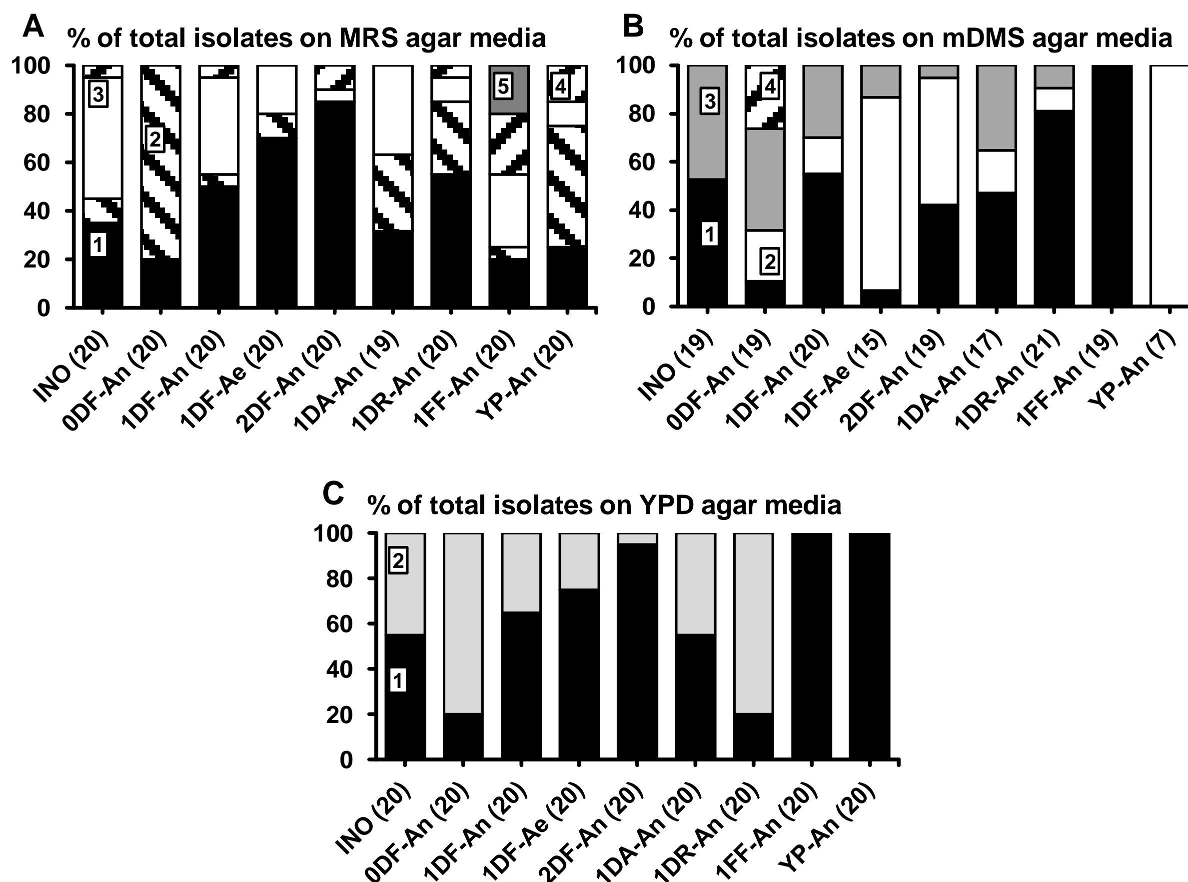

Figure 2. Culture-dependent species diversity of bacteria and yeasts of the water kefir grains of the inoculum (INO) and the eight fermentation series differing in the presence of oxygen, nutrient concentration, and nutrient source, at the end of backslopping step 8. The closest known type strains of the sequenced fragments are given. (A) Isolates from MRS agar media: 1, *Lactobacillus paracasei* (100 % identity; accession no. AP012541); 2, *Lactobacillus hilgardii* (100 % identity; accession no. LC064898); 3, *Lactobacillus nagelii* (99 % identity; accession no. NR112754); 4, *Lactobacillus harbinensis* (100 % identity; accession no. NR113969); and 5, *Leuconostoc mesenteroides* (99 % identity; accession no. LC071839). (B) Isolates from mDMS agar media: 1, *Gluconobacter roseus*/*oxydans* (100 % identity for both species; accession no. NR041049/NR026118); 2, *Acetobacter fabarum* (100 % identity; accession no. NR113556); 3, *Acetobacter indonesiensis* (99 % identity; accession no. NR113847); and 4, *Gluconobacter japonicus*/*frateurii* (100 % identity; accession no. NR041445/NR112239). (C) Isolates from YPD agar media: 1, *Saccharomyces cerevisiae* [LSU (99% identity; accession no. CP011558) and ITS (99% identity; accession no. KC515374)]; and 2, *Dekkera bruxellensis* [LSU (99% identity; accession no. GU291284) and ITS (99% identity; accession no. FJ545249)]. LSU, large subunit rRNA gene; ITS, internal transcribed spacer.

The main bands in the community profiles of the inoculum, the aerobic fermentation series, and the anaerobic fermentation series obtained with the V3 and LAC primer pairs were attributed to *Lb. paracasei*, *Lb. hilgardii*, and *Lb. nagelii*. Further, a band in the community profiles obtained with the V3 and Bif primer pairs of the water kefir liquors and water kefir grains of the inoculum and the aerobic and anaerobic fermentation series, was attributed to *Bifidobacterium aquikefiri*. The relative intensities of these bands were similar for the inoculum and the anaerobic fermentation series, but lower for the aerobic ones. In the

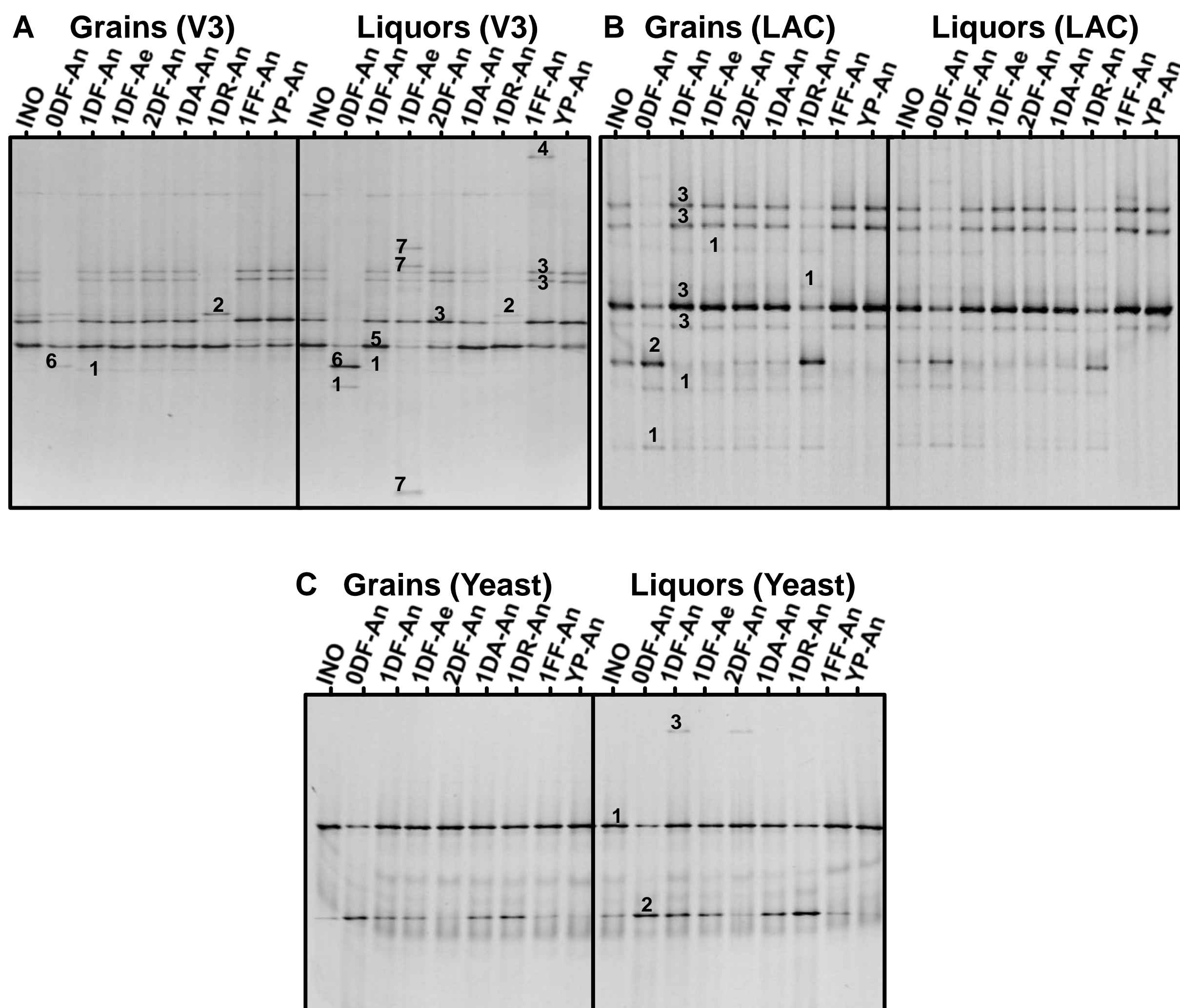

Figure 3. Community profiles of the bacteria and yeasts on the water kefir grains and in the water kefir liquors of the inoculum (INO) and the eight fermentation series differing in the presence of oxygen, nutrient concentration, and nutrient source, at the end of backslopping step 8. The numbers indicate the bands that were sequenced and the closest known type strains of the sequenced fragments are given. (A) With the V3 primer pair: 1, *Lactobacillus casei*/*paracasei*/*zeae*/*rhamnosus* (99% identity for the four species; accession no. LC064894/AB289229/AB289313/JQ580982); 2, *Lactobacillus hilgardii* (100 % identity; accession no. LC064898); 3, *Lactobacillus nagelii*/*ghanensis* (99% identity; accession no. NR112754/NR043896); 4, *Leuconostoc mesenteroides*/*pseudomesenteroides* (99 % identity; accession no. LC071839/LC096220); 5, *Bifidobacterium aquikefiri* (100 % identity; accession no. LN849254); 6, *Comamonas testosteroni*/*thiooxydans* (100 % identity; accession no. NR113709/NR115741); and 7, *Acetobacteraceae* (100 % identity). (B) With the LAC primer pair: 1, *Lactobacillus casei*/*paracasei*/*zeae* (99 % identity; accession no. LC064894/AB289229/AB289313); 2, *Lactobacillus hilgardii*/*diolivorans* (100 % identity; accession no. LC064898/NR037004); and 3, *Lactobacillus nagelii* (99 % identity; accession no. NR119275). (C) With the Yeast primer pair: 1, *Saccharomyces cerevisiae* (100 % identity; accession no. NG042623); 2, *Dekkera bruxellensis* (100 % identity; accession no. AY969049); and 3, *Candida smithsonii* (99 % identity; accession no. AY518525).

community profiles obtained with the V3 primer pair, several bands that were attributed to the taxon *Acetobacteraceae*, were detected in the water kefir liquors but not in the water kefir grains of the aerobic fermentation series. These bands were not detected in the liquors and grains of the inoculum or the anaerobic fermentation series. The limited length of the amplified 16S rRNA gene fragments (± 210 bp) from these bands did not allow their species level identification.

3.1.5 Substrate consumption and metabolite production

At the end of backslopping step 1, the total residual carbohydrate and metabolite concentrations were similar in the aerobic and anaerobic fermentation series. The total residual carbohydrate concentrations remained similar at the end of backslopping step 8, but the concentrations of acetic acid were higher, and those of ethanol, lactic acid, and glycerol were lower in the aerobic fermentation series than in the anaerobic ones (Table 4). Furthermore, the concentrations of ethyl acetate were higher and those of the higher esters were lower in the aerobic fermentation series than in the anaerobic ones.

3.2 Influence of the nutrient concentration

3.2.1 pH and water kefir grain wet and dry mass

At the end of backslopping step 1, the water kefir grain growth (based on wet mass) was similar for the fermentation series 0DF-An, 1DF-An, and 2DF-An, and it decreased over the course of the eight backslopping steps in the fermentation series 0DF-An (Figure 1 and Table 3). At the end of backslopping step 8, the water kefir grains of fermentation series 0DF-An were larger and their dry mass was higher than those of fermentation series 1DF-An and 2DF-An (Table 4).

At the end of backslopping step 1, the pH value of fermentation series 0DF-An was already significantly higher than the pH values of fermentation series 1DF-An and 2DF-An, and further increased over the course of the eight backslopping steps (Figure 1 and Table 2). The pH in fermentation series 2DF-An was always slightly higher than in 1DF-An.

3.2.2 Microbial enumerations

The viable counts of the yeasts and LAB on the water kefir grains were higher when the amounts of dried figs added to the fermentation series were higher (Table 4). Further, the ratios of the LAB to the yeasts decreased when the amount of dried figs added to the fermentation series increased.

3.2.3 Culture-dependent microbial species diversity

The main yeast species were *S. cerevisiae* and *D. bruxellensis*, whereby the relative abundances of *S. cerevisiae* increased and those of *D. bruxellensis* decreased when the amount of dried figs added to the fermentation series increased (Figure 2).

The main LAB species in all three fermentation series were *Lb. paracasei* and *Lb. hilgardii* (82 % of the strains produced EPS), whereby the relative abundances of *Lb. hilgardii* were higher in fermentation series 0DF-An than in fermentation series 1DF-An and 2DF-An (Figure 2). Further, *Lb. nagelii* and *Lb. harbinensis* were only isolated from fermentation series 1DF-An and 2DF-An.

The main AAB species in the three fermentation series were *G. oxydans/roseus*, *A. fabarum*, and *A. indonesiensis*, whereby the relative abundances of *A. fabarum* increased when the amount of dried figs added to the fermentation series increased (Figure 2). *Gluconobacter japonicus/frateurii* was only isolated from fermentation series 0DF-An.

3.2.4 Culture-independent microbial species diversity

The main bands in the rRNA-PCR-DGGE community profiles obtained with the Yeast primer pair for the water kefir liquors and the water kefir grains of fermentation series 0DF-An, 1DF-An, and 2DF-An were attributed to *S. cerevisiae* and *D. bruxellensis*. The relative intensities of the bands attributed to *S. cerevisiae* increased and those of the bands attributed to *D. bruxellensis* decreased when the amount of dried figs added increased (Figure 3). Further, a band with weak relative intensity in the community profiles of the liquors of fermentation series 1DF-An and 2DF-An was attributed to *C. smithsonii*.

The main bands in the community profiles obtained with the V3 and LAC primer pairs for the water kefir liquors and water kefir grains of fermentation series 0DF-An, 1DF-An, and 2DF-An were attributed to *Lb. hilgardii*, *Lb. paracasei*, and *Lb. nagelii* (Figure 3). The relative intensities of the bands attributed to *Lb. nagelii* increased and those of the bands attributed to *Lb. hilgardii* decreased when the amount of dried figs added increased. In the community profiles obtained with the V3 and Bif primer pairs, a band attributed to *B. aquikefiri* was detected in the three fermentation series, whereby the relative intensities of the bands were highest in the community profiles of the fermentation series 1DF-An. Additionally, in the community profiles obtained with the V3 primer pair, a band attributed to *Comamonas testosteroni*/*thiooxydans* was detected in the water kefir liquors and the water kefir grains of the fermentation series 0DF-An, with higher relative intensities in the liquors than on the grains. This band was not detected in the water kefir liquors or the water kefir grains of fermentation series 1DF-An and 2DF-An.

3.2.5 Substrate consumption and metabolite production

The concentrations of the total residual carbohydrates at the end of backslopping steps 1 and 8 were higher and those of the metabolites were lower when the amount of dried figs added to the fermentation series were lower (Tables 1 and 4).

3.3 Influence of the nutrient source

3.3.1 pH and water kefir grain wet and dry mass

The water kefir grain growth (based on wet mass) was around 60 % for all fermentation series at the end of backslopping step 1, and remained more or less stable over the course of the eight backslopping steps for fermentation series 1DF-An, 1DA-An, and 1 DR-An, but decreased slowly in the fermentation series 1FF-An and fast in the fermentation series YP-An (Figure 1). The water kefir grain growth was highest in fermentation series 1DA-An, followed by 1DF-An, and 1DR-An (Figure 1 and Table 3). The water kefir grain dry mass was similar for all fermentation series. The water kefir grains were largest in the fermentation series 1DA-An, smaller in 1FF-An, and smallest in YP-An.

The pH at the end of backslopping step 1 was comparable in the fermentation series 1DF-An, 1DR-An, and 1FF-An (approximately 3.45), significantly higher in the fermentation series 1DA-An (approximately 3.65), and significantly lower in the fermentation series YP-An (approximately 3.35) (Table 1). The pH values of fermentation series 1DF-An, 1DA-An, 1DR-An, and 1FF-An remained stable over the course of the eight backslopping steps, whereas the pH of the series YP-An increased after backslopping step 4 to become similar to the pH in fermentation series 1DF-An, 1DR-An, and 1FF-An (Figure 1 and Table 2).

3.3.2 Microbial enumerations

The viable counts of the yeasts were highest in fermentation series YP-An and 1FF-An, lower in 1DF-An and 1DR-An, and lowest in the fermentation series 1DA-An (Table 4). The

viable counts of the LAB were highest in the fermentation series 1DF-An, lower in 1FF-An, 1DA-An, and 1DR-An, and lowest in YP-An. The ratios of the viable counts of the LAB to the yeasts were highest in the fermentation series 1DF-An, lower in 1DA-An, 1FF-An, and 1DR-An, and lowest in YP-An.

3.3.3 Culture-dependent microbial species diversity

The yeast species *S. cerevisiae* and *D. bruxellensis* were isolated from all fermentation series, whereby the relative abundances of *D. bruxellensis* were highest in the fermentation series 1DR-An, lower in 1DA-An and 1DF-An, and lowest in 1FF-An and YP-An (Figure 2).

The LAB species *Lb. paracasei*, *Lb. nagelii*, and *Lb. hilgardii* (79 % of these strains produced EPS) were isolated from fermentation series 1DF-An, 1DA-An, 1DR-An, 1FF-An, and YP-An; *Lb. harbinensis* was isolated from fermentation series 1DF-An, 1DR-An, and YP-An; and *Leuc. mesenteroides* was isolated from the fermentation series 1FF-An (Figure 2).

The AAB species *G. roseus*/*oxydans*, *A. fabarum*, and *A. indonesiensis* were isolated from fermentation series 1DF-An, 1DA-An, and 1DR-An; *G. roseus*/*oxydans* was the only AAB species isolated from fermentation series 1FF-An; and *A. fabarum* was the only one isolated from fermentation series YP-An (Figure 2).

3.3.4 Culture-independent microbial species diversity

The main bands in the rRNA-PCR-DGGE community profiles obtained with the Yeast primer pair of the water kefir liquors and the water kefir grains of all fermentation series were attributed to *S. cerevisiae* and *D. bruxellensis*. The relative intensities of the bands attributed to *D. bruxellensis* were highest in the fermentation series 1DR-An, lower in 1DA-An and 1DF-An, and lowest in 1FF-An and YP-An (Figure 3).

The main bands in the community profiles obtained with the V3 and LAC primer pairs of the water kefir liquors and the water kefir grains of all fermentation series were attributed to *Lb. nagelii*, *Lb. hilgardii*, and *Lb. paracasei*. The relative intensities of the bands attributed to *Lb. nagelii* were highest in fermentation series 1FF-An and YP-An, lower in 1DF-An and 1DA-An, and lowest in 1DR-An (Figure 3). The relative intensities of the bands attributed to *Lb. hilgardii* were highest in the fermentation series 1DR-An, lower in 1DA-An and 1DF-An, and lowest in 1FF-An and YP-An. The relative intensities of the bands attributed to *Lb. paracasei* were lower in fermentation series 1FF-An and 1YP-An than in the other ones. In the community profiles obtained with the V3 primer pair, a band attributed to *Leuc. mesenteroides* was detected in the fermentation series 1FF-An. In the community profiles with the V3 and Bif primer pairs, a band attributed to *B. aquikefiri* was detected in all fermentation series, whereby the relative intensities of these bands were lowest in fermentation series 1FF-An and YP-An.

3.3.5 Substrate consumption and metabolite production

The total residual carbohydrate concentrations were always lowest in fermentation series YP-An and 1FF-An, higher in 1DF-An and 1DA-An, and highest in 1DR-An (Tables 1 and 4). The ethanol concentrations at the end of backslopping step 1 were approximately 15 g l^{-1} for all fermentation series. At the end of backslopping step 8, the ethanol concentrations remained at approximately 16 g l^{-1} for the fermentation series 1DA-An, increased to approximately 20 g l^{-1} in 1DF-An and 1DR-An, and increased to approximately 25 g l^{-1} in fermentation series 1FF-An and YP-An. The concentrations of glycerol were always highest in the fermentation series 1DR-An. At the end of backslopping step 8, the ratios of the

concentrations of glycerol to ethanol were higher in fermentation series 1DA-An, 1DR-An, and 1DF-An than in fermentation series 1FF-An and YP-An.

The lactic acid concentrations were always higher in fermentation series 1DF-An, 1DA-An, and 1FF-An than in fermentation series 1DR-An and YP-An (Tables 1 and 4). The acetic acid concentrations were always lower in fermentation series 1FF-An and YP-An than in fermentation series 1DF-An and 1DA-An, and were highest in the fermentation series 1DR-An at the end of backslopping step 8. The ratios of the concentrations of lactic acid to ethanol were always higher in fermentation series 1DF-An, 1DA-An, and 1FF-An than in fermentation series 1DR-An and YP-An, and those of acetic acid to ethanol and acetic acid to lactic acid were always higher in fermentation series 1DF-An, 1DA-An, and 1DR-An than in fermentation series 1FF-An and YP-An. At the end of backslopping step 8, the concentrations of the higher alcohols and higher esters were lowest in the fermentation series 1DR-An, higher in fermentation series 1DF-An and 1DA-An, and highest in fermentation series 1FF-An and YP-An, whereas the concentrations of ethyl acetate were opposite.

4 Discussion

This chapter showed that the presence of oxygen, the nutrient concentration, and the nutrient source influenced the water kefir grain growth, microbial species diversity, substrate consumption, and metabolite production during water kefir fermentation.

The most characteristic effect of the presence of oxygen during water kefir fermentation was the proliferation of the AAB. These obligately aerobic microorganisms are often present in water kefir (Laureys & De Vuyst, 2014; Chapter 3). Their viable counts vary widely (Franzetti *et al.*, 1998; Gulitz *et al.*, 2011; Laureys & De Vuyst, 2014; Chapters 3, 5, and 7), but are usually low during water kefir fermentation, because oxygen is only periodically available at the start of each backslopping step, whereas ethanol (an energy source for AAB) is only available at the end of a water kefir fermentation process. However, AAB are known to survive low-oxygen conditions, even for long periods of time (Bartowsky & Henschke, 2008; Moens *et al.*, 2014). The main AAB found in the present study were *A. fabarum*, *G. roseus*/*oxydans*, and *A. indonesiensis*. The former two AAB species were reported in water kefir before (Gulitz *et al.*, 2011; Laureys & De Vuyst, 2014; Chapters 3 and 5). To our knowledge, this is the first time that *A. indonesiensis* was isolated from water kefir. Further, *G. roseus*/*oxydans* and *A. indonesiensis* were more abundant under anaerobic fermentation conditions, whereas *A. fabarum* was more abundant under aerobic fermentation conditions. Also, the AAB species were more abundant in the water kefir liquors than on the water kefir grains, indicating that the liquor was their preferred niche.

The proliferation of AAB in the aerobic fermentation series resulted in high acetic acid concentrations and thus low pH values. This probably caused a slow but gradual decrease of the water kefir grain growth upon backslopping of the aerobic fermentation processes, as excessive acidic stress can decrease the water kefir grain growth during fermentation (Chapter 7). The lower concentrations of ethanol and lactic acid in the aerobic fermentations probably resulted from their consumption by the AAB species (Moens *et al.*, 2014). Further, there were no indications that *S. cerevisiae* and/or *D. bruxellensis* switched to respirational metabolism in the presence of oxygen (Schifferdecker *et al.*, 2014). The proliferation of AAB species in the aerobic water kefir fermentations coincided with higher concentrations of ethyl acetate and lower concentrations of fruity esters than in the anaerobic ones. Similarly, the proliferation of AAB species in wine results in higher concentrations of ethyl acetate and lower overall fruitiness (Bartowsky *et al.*, 2003; Bartowsky & Henschke, 2008). Furthermore, the high concentrations of acetic acid may have caused lower relative abundances of *B.*

aquikefiri in the aerobic fermentation series, as this bifidobacterial species is not inhibited by aerobic conditions (Laureys *et al.*, 2016, Chapter 6) or low pH values during water kefir fermentation (Chapter 7). Indeed, high concentrations of acetic acid may inhibit the growth of certain microorganisms; for instance, *D. bruxellensis* is sensitive to acetic acid concentrations higher than 1 g l^{-1} (Yahara *et al.*, 2007).

Low nutrient concentrations caused a slow fermentation, resulting in high total residual carbohydrate concentrations, low metabolite concentrations, and high pH values. In contrast, high nutrient concentrations caused a fast fermentation, resulting in high metabolite concentrations without a decrease of the total residual carbohydrate concentrations or pH values. The latter showed that dried figs supplied both carbohydrates and buffer compounds to the water kefir fermentation mixtures, allowing high metabolite production without a decrease of the total residual carbohydrate concentrations or pH.

The water kefir grain growth was initially not affected by the nutrient concentrations, but insufficient nutrient concentrations resulted in a gradual decrease of the water kefir grain growth upon backslopping. This was caused by a lack of nutrients, as high pH values at low nutrient concentrations excluded its decrease due to acidic stress (Chapter 7). Nutrient concentrations in excess of a certain threshold value did not further increase the water kefir grain growth.

Low nutrient concentrations resulted in high viable counts of AAB species, which were probably caused by the limited expulsion of oxygen due to the low metabolic activity of the microorganisms in this fermentation series. High nutrient concentrations favored the growth of yeasts at the expense of the LAB species, and this was reflected in the ratios of the metabolite concentrations of the yeasts to those of the LAB. The relative abundances of *Lb. nagelii* and *S. cerevisiae* were high at high nutrient concentrations, whereas those of *Lb. hilgardii* and *D. bruxellensis* were high at low nutrient concentrations. This is in line with the low nutrient requirements of *D. bruxellensis* (Uscanga *et al.*, 2000). Furthermore, high nutrient concentrations resulted in low ratios of the concentrations of acetic acid to ethanol and acetic acid to lactic acid, which may be related to the shift in microbial species diversity. Indeed, *Lb. hilgardii* (obligately heterofermentative) produces more acetate than *Lb. nagelii* (obligately homofermentative), and *D. bruxellensis* produces more acetate than *S. cerevisiae* (Oelofse *et al.*, 2008; Ludwig *et al.*, 2009). Finally, *B. aquikefiri*, which produces acetic acid upon fermentation of hexoses (Laureys *et al.*, 2016, Chapter 6), thrived best under moderate nutrient concentrations.

Low nutrient concentrations allowed the growth of *C. testosteroni*/*thiooxydans* during water kefir fermentation. This environmental microorganism is widely present in soil and water and on plants, but has not yet been reported for water kefir (Bayhan *et al.*, 2013). It is a motile, obligately aerobic *β*-proteobacterium that grows at pH 6.0-8.5 (Narayan *et al.*, 2010; Bayhan *et al.*, 2013). Its growth during water kefir fermentation with low nutrient concentrations was likely caused by the high pH values and prolonged presence of oxygen. Further, this microorganism preferred the water kefir liquors above the water kefir grains, reflecting its obligate aerobic and mobile nature. Under normal water kefir fermentation conditions, *C. testosteroni*/*thiooxydans* is not expected, as water kefir fermentation normally proceeds under anaerobic conditions whereby the pH decreases fast below 4.0 (Laureys & De Vuyst, 2014; Chapters 3, 4, 5, and 7).

Dried figs are the most commonly used source of nutrients during water kefir fermentation. Yet, stable water kefir fermentation was also possible with dried apricots and dried raisins, but not with fresh figs or a mixture of yeast extract and peptone (YP solution), as this resulted in a gradually decreasing water kefir grain growth. The high and low water

kefir grain growth in the fermentation series with dried apricots and raisins, respectively, was probably caused by the high pH values when dried apricots were added and the low pH values when raisins were added (Chapter 7). Low pH values in the fermentation series with YP solution probably caused a fast decrease of the water kefir grain growth during the first backslopping steps. However, the low pH values in these fermentation series were not caused by high acid concentrations, underlining that the nutrient source influenced the pH during water kefir fermentation via the release of buffer compounds, as mentioned above. After the fast initial decrease, the water kefir grain growth in the fermentation series with YP solution remained low, despite the presence of EPS-producing *Lb. hilgardii* strains. This showed that excessive acidic stress caused low water kefir grain growth (Chapter 7) and that the presence of EPS-producing *Lb. hilgardii* strains was not sufficient for water kefir grain growth (Chapter 4). Further, high relative abundances of *Lb. hilgardii* in the fermentation series with dried raisins or low nutrient concentrations were not reflected in a high water kefir grain growth, confirming that the relative abundance of *Lb. hilgardii* during water kefir fermentation did not determine water kefir grain growth (Chapter 7). Low water kefir grain growth resulted in small water kefir grains with high viable counts of microorganisms, resulting in a fast fermentation with low total residual carbohydrate and high metabolite concentrations, as was shown previously (Chapters 4 and 7).

The nutrient source had an immediate impact on the substrate consumption and metabolite production during the water kefir fermentation processes, and this impact became even more pronounced upon backslopping, probably due to the shift in the microbial communities. Indeed, high relative abundances of the obligately heterofermentative *Lb. hilgardii* coincided with high acetate concentrations and high ratios of the concentrations of acetate to ethanol and acetate to lactic acid (Ludwig *et al.*, 2009). The concentrations of glycerol did not parallel those of ethanol, and the ratios of the concentrations of glycerol to ethanol were higher when *D. bruxellensis* was more abundant and *S. cerevisiae* less. This was in contrast with literature data, which indicate that *S. cerevisiae* produces more glycerol than *D. bruxellensis* (Blomqvist *et al.*, 2010). The concentrations of ethyl acetate were highest when raisins were added to the water kefir fermentation process and coincided with high relative abundances of *Lb. hilgardii*.

The fermentations with dried raisins resulted in high relative abundances of *Lb. hilgardii* and *D. bruxellensis*, low relative abundances of *Lb. nagelii* and *S. cerevisiae*, high total residual carbohydrate concentrations, and low metabolite concentrations, thus resembling the fermentations with low nutrient concentrations described above. The fermentations with fresh figs or with a YP solution resulted in high relative abundances of *Lb. nagelii* and *S. cerevisiae*, low relative abundances of *Lb. hilgardii* and *D. bruxellensis*, low total residual carbohydrate concentrations, and high metabolite concentrations, thus resembling the fermentations with high nutrient concentrations described above. High relative abundances of *D. bruxellensis* resulted in high concentrations of ethyl acetate, whereas high relative abundances of *S. cerevisiae* resulted in high concentrations of higher alcohols and higher esters.

In conclusion, the presence of oxygen allowed the proliferation of AAB species during water kefir fermentation, resulting in high acetic acid concentrations, and decreased the relative abundances of *B. aquikefiri*. The nutrient concentrations had an immediate impact on the metabolism of the water kefir microorganisms and influenced the microbial species diversity upon backslopping, which in turn influenced the substrate consumption and metabolite production. The influence of the nutrient source was similar to that of the nutrient concentration, indicating that different nutrient sources supplied different (amounts of) nutrients to the water kefir fermentation mixtures.

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CHAPTER 9

The type and concentration of the inoculum and substrate influence the grain growth and fermentation rate during water kefir fermentation

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SUMMARY

Eleven series of water kefir fermentations were inoculated with a grain or liquor inoculum from the same origin. They were followed as a function of time to investigate the influence of the presence of oxygen and the type and concentration of the inoculum and substrate on the kinetics of the water kefir fermentation process through a modelling approach. *Lactobacillus paracasei*, *Lactobacillus hilgardii*, *Lactobacillus nagelii*, *Saccharomyces cerevisiae*, and *Dekkera bruxellensis* were the main microorganisms present. Visualization of the water kefir grains with scanning electron microscopy revealed that the majority of the microorganisms was attached onto their surface. The lactic acid bacteria and yeasts were predominantly associated with the grains, whereas the acetic acid bacteria were predominantly associated with the liquor. Acetic acid bacteria were present in low abundances under anaerobic conditions and only proliferated under aerobic conditions. The metabolic activity during water kefir fermentation was mainly associated with the grains. Increasing concentrations of the water kefir grain inoculum increased the water kefir fermentation rate. Partial substitution of sucrose with glucose and/or fructose reduced the grain growth, whereby glucose was fermented faster than fructose. Water kefir liquor could be used as an alternative means of inoculation, whereby the production of water kefir grain mass was absent. However, the resulting fermentation process progressed slower than the one inoculated with water kefir grains.

1 Introduction

Water kefir is a naturally fermented beverage that is mainly produced at household level (Pothakos *et al.*, 2016). Its fermentation process is usually started with water kefir grains (Laureys & De Vuyst, 2014; Chapters 3, 4, 5, and 7). Water kefir grains contain around 14 % (m m^{-1}) dextran exopolysaccharides (EPS), are translucent, have a brittle structure, and are insoluble in water (Horisberger, 1969; Laureys & De Vuyst, 2014; Chapters 3, 4, 5, and 7). The microorganisms responsible for the water kefir fermentation process are thought to reside on the surface of the grains (Moinas *et al.*, 1980; Laureys & De Vuyst, 2014). The microbial colonization of the water kefir grains encompasses bacterial and yeast cells, and is influenced by the fermentation substrate (Moinas *et al.*, 1980; Neve & Heller, 2002; Hsieh *et al.*, 2012). The main water kefir microorganisms are lactic acid bacteria (LAB), yeasts, acetic acid bacteria (AAB), and bifidobacteria (Laureys & De Vuyst, 2014; Chapters 3, 4, 5, 6, 7, and 8). The key microorganisms were defined as *Lactobacillus hilgardii*, *Lactobacillus nagelii*, *Lactobacillus paracasei*, and *Saccharomyces cerevisiae* (Chapter 4).

Water kefir fermentation is usually carried out anaerobically but may be performed aerobically too (Chapters 5 and 8). In the long term, the presence of oxygen allows the proliferation of AAB, which results in the production of high concentrations of acetic acid (Chapter 8). Sucrose is usually the main substrate during water kefir fermentation and is metabolized by the microorganisms into ethanol, glycerol, lactic acid, acetic acid, mannitol, and a variety of aroma compounds (Laureys & De Vuyst, 2014; Chapters 3 and 4). Additionally, sucrose is converted into water kefir grain dextran EPS by glucansucrases of *Lb. hilgardii* (Pidoux, 1989; Waldherr *et al.*, 2010), resulting in an increase of the water kefir grain mass during fermentation (Laureys & De Vuyst, 2014; Chapters 3, 4, 7, and 8). The production of water kefir grain mass can be considered as a waste stream, because the usual goal of water kefir fermentation is the production of liquor for its use as beverage. Nevertheless, the production of grains is sometimes desirable, for example to scale up a water kefir production process. To reduce the water kefir grain growth during fermentation, sucrose may be (partially) substituted with glucose and/or fructose, as sucrose is necessary for dextran EPS production (Monsan *et al.*, 2001). However, the influence of these alternative substrates on the water kefir fermentation process has not been investigated yet. Additionally, decreasing the sucrose concentration could increase the water kefir grain growth as well, as glucansucrases suffer from substrate inhibition (Hehre, 1946). Furthermore, the water kefir grain growth may depend on the concentration of the grain inoculum, as the activity of dextran sucrase shifts from sucrose hydrolysis to dextran biosynthesis when the concentration of dextran increases (Mooser *et al.*, 1985). Investigation of the influence of the type and concentration of the substrate and of the concentration of the grain inoculum on the water kefir grain growth will allow more control over the water kefir fermentation process.

Part of the microorganisms of the grain inoculum detach from the water kefir grains into the liquor at the start of a fermentation process, but the majority of the microorganisms remains always associated with the grains (Laureys & De Vuyst, 2014; Chapters 3 and 4). This suggests that the majority of the microbial metabolism during water kefir fermentation is associated with the grains, and that the fermentation rate will be determined by the concentration of the grain inoculum. Modeling and quantification of this effect may allow greater control over the water kefir fermentation rate.

Water kefir liquor may be used as alternative inoculum to start a fermentation process, as it contains a substantial amount of microorganisms with a species diversity more or less similar to that on the water kefir grains (Laureys & De Vuyst, 2014; Chapters 3 and 4). Such an innovative inoculation strategy may remove the need for water kefir grain mass altogether.

However, the metabolic and kinetic implications of this inoculation strategy have not been investigated yet. The lack of such fundamental insights into the water kefir fermentation process hampers its further industrial exploitation.

This chapter aimed to elucidate the influence of the presence of oxygen and of the type and concentration of the inoculum and substrate on the kinetics of the water kefir grain growth, substrate consumption, and metabolite production during water kefir fermentation. For process quantifications, rather than performing a mere qualitative analysis, mathematical models were fitted to the experimental data to allow the comparison of the biokinetic parameters involved.

2 Materials and methods

2.1 Prefermentations

An inoculum of approximately 100 g of water kefir grains was obtained from the household water kefir fermentation process described in Chapter 3. To obtain the necessary amount of water kefir grains, the inoculum was cultivated through a series of consecutive prefermentations through backslopping until > 2,500 g of water kefir grain wet mass was produced. The prefermentations were performed in glass bottles (1, 2, 5, and 10 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 recultivated in fresh medium under the same conditions as described above.

2.2 Fermentations

The water kefir grain mass and the water kefir liquor, obtained through the series of prefermentations mentioned above, were used to start eleven series of water kefir fermentations differing in the presence of oxygen, and the type and concentration of the inoculum and the substrate (Table 1). Each fermentation series was performed in independent biological triplicates. The fermentations were performed in 100-ml glass bottles. Each fermentation bottle contained 85 ml of autoclaved (121 °C, 2.1 bar, 20 min) water kefir simulation medium (WKSM). The WKSM was composed of 65 ml of tap water (Brussels, Belgium) and 20 ml of fig extract, supplemented with 3 (fermentation series 1S-2G-An), 6 (2S-2L-An, 2S-2G-An, 2S-2G-Ae, and 2S-3G-An), or 9 g (3S-2G-An) of sucrose (Merck, Darmstadt, Germany); 6 g of glucose (Merck; 2G-2G-An); 6 g of fructose (Merck; 2F-2G-An); 3 g of sucrose, 1.5 g of glucose, and 1.5 g of fructose (2SGF-2G-An); or 3 g of glucose and 3 g of fructose (2GF-2G-An). The fig extract was prepared as described in Chapter 3. To start the fermentations, 15.0 ml of liquor inoculum (2S-2L-An); or 7.5 (2S-1G-An), 15 (2S-2G-An, 2S-2G-Ae, 1S-2G-An, 3S-2G-An, 2SGF-2G-An, 2GF-2G-An, 2G-2G-An, and 2F-2G-An), or 22.5 g (2S-3G-An) of rinsed grain inoculum was added to the fermentation bottles. Rinsing of the grains was performed with 2 l of tap water per 50 g of water kefir grains. The fermentation bottles were equipped with a PTFE water lock for fermentation under anaerobic conditions (2S-2L-An, 2S-2G-An, 1S-2G-An, 3S-2G-An, 2S-1G-An, 2S-3G-An, 2SGF-2G-An, 2GF-2G-An, 2G-2G-An, and 2F-2G-An) or were covered with a muslin cloth for fermentation under aerobic conditions (2S-2G-Ae). All fermentation bottles were incubated in an air-conditioned room at 21 °C. The contents of the fermentation bottles were

Table 1. Composition of the water kefir simulation media and atmospheric conditions used for eleven series of water kefir fermentations.

Fermentation series	Sucrose (g l^{-1})	Glucose (g l^{-1})	Fructose (g l^{-1})	Inoculum	Oxygen conditions
2S-2G-An	71	0	0	15 g of grains	Anaerobic
2S-2L-An	71	0	0	15 ml of liquor	Anaerobic
2S-2G-Ae	71	0	0	15 g of grains	Aerobic
2S-1G-An	71	0	0	7.5 g of grains	Anaerobic
2S-3G-An	71	0	0	22.5 g of grains	Anaerobic
1S-2G-An	35	0	0	15 g of grains	Anaerobic
3S-2G-An	106	0	0	15 g of grains	Anaerobic
2SGF-2G-An	35	18	18	15 g of grains	Anaerobic
2GF-2G-An	0	35	35	15 g of grains	Anaerobic
2G-2G-An	0	71	0	15 g of grains	Anaerobic
2F-2G-An	0	0	71	15 g of grains	Anaerobic

mixed by gently turning the bottles at the start of the fermentation process and before their sampling.

2.3 Visualization of the water kefir grains

To study the microbial colonization of the water kefir grains, grain samples of a household water kefir fermentation process were brought into tubes, rinsed twice with 1 ml of 0.05 M phosphate buffer (PB) at pH 7.2, and incubated at room temperature for 10 min, after which the supernatants were removed. The samples were fixated with 1 ml of 2.5 % (m v^{-1}) glutaraldehyde solution in PB and incubated for 10 min, after which the supernatants were removed. This fixating procedure was repeated with an incubation time of 18 h. Afterwards, the samples were rinsed twice with PB as described above. The samples were dehydrated by consecutively adding 1 ml of 50, 70, 90, and twice 100 % (v v^{-1}) of ethanol in ultrapure water and incubated for 20 min, after which the supernatants were removed. The samples were dried by adding 500 µl of hexamethyldisilazane (Sigma-Aldrich, Saint Louis, MO, USA) and incubated for 1 h, after which the supernatants were removed. This drying procedure was repeated, after which the water kefir grain samples were dried under vacuum for 12 h.

The water kefir grain sample was fixed on the sample holder with carbon tape and coated with 3.0 nm of gold with a Cressington 208hr sputter coater (Cressington Scientific Instruments, Watford, UK). Afterwards, the sample was loaded under high vacuum in a JSM-IT300 scanning electron microscope for visualization (Jeol Europe, Nieuw-Vennep, The Netherlands).

2.4 Analyses

After 0, 1, 2, 3, and 4 d of fermentation for all fermentation series, as well as after 6 d of fermentation for fermentation series 2S-2L-An, 2S-2G-Ae, 2S-1G-An, and 3S-2G-An, three fermentation bottles (representing three independent biological replicates) were removed and their contents were analyzed. The pH, the water kefir grain wet mass, and the concentrations of the substrates and metabolites were determined at every sampling time. The viable counts of the LAB, yeasts, and AAB were determined in the water kefir liquor inoculum, on the non-rinsed grains of the water kefir grain inoculum, and in the liquors and on the non-rinsed water

kefir grains of fermentation series 2S-2G-An, 2S-2L-An, and 2S-2G-Ae after 4 d of fermentation. The culture-dependent microbial species diversity and community dynamics of the LAB, yeasts, and AAB were determined in the water kefir liquor inoculum and on the non-rinsed water kefir grains of the grain inoculum. Those of the AAB were also determined in the water kefir liquors of fermentation series 2S-2G-An, 2S-2L-An, and 2S-2G-Ae after 4 d of fermentation. The culture-independent microbial species diversity and community dynamics were determined in the water kefir liquor inoculum, on the non-rinsed water kefir grains of the grain inoculum, and in the water kefir liquors and on the non-rinsed water kefir grains of fermentation series 2S-2G-An, 2S-2L-An, 2S-2G-Ae, 2GF-2G-An, 2G-2G-An, and 2F-2G-An after 4 d of fermentation. The results are presented as the mean ± standard deviation of the three independent biological replicates performed for each fermentation series at each sampling point.

2.5 pH, water kefir grain wet mass, and water kefir grain density determinations

The pH, the water kefir grain wet mass, and the water kefir grain growth were determined as described in Chapter 7. The density of the water kefir grains was determined in triplicate with a volumetric flask of 1.00 l. Therefore, its exact volume was determined by weighing the volumetric flask when empty and when it was filled with ultrapure water at 21 °C. Approximately 280 g of water kefir grains were brought into the empty flask, which was then filled with ultrapure water at 21 °C. The water kefir grain density was calculated based on the volume of the flask, the mass of the water kefir grains, and the mass of ultrapure water that was needed to fill the flask containing water kefir grain mass.

2.6 Microbial enumerations

The viable counts of the presumptive LAB were determined on de Man-Rogosa-Sharpe (MRS) agar medium, those of the presumptive AAB on modified deoxycholate-mannitol-sorbitol (mDMS) agar medium, and those of presumptive yeasts on yeast extract-peptone-dextrose (YPD) agar medium, as described in Chapter 7.

2.7 Culture-dependent microbial species diversity and community dynamics analyses

The culture-dependent microbial species diversitiy and community dynamics analyses of the LAB, yeasts, and AAB in the water kefir liquors and on the water kefir grains were determined by randomly picking up 10 to 20 % of the total number of colonies from the respective agar media with 30 to 300 colonies. The isolates were subcultivated on their respective agar media until the third generation, which was used for dereplication via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) fingerprinting, as described in Chapter 7. The peptide fingerprint patterns obtained were clustered numerically by means of the BioNumerics software version 7.50 (Applied Maths, Sint-Martens-Latem, Belgium). Representative bacterial isolates within each cluster were identified by sequencing part of their 16S rRNA gene from genomic DNA, and representative yeast isolates within each cluster were identified by sequencing part of their 26S large subunit (LSU) rRNA gene and internal transcribed spacer (ITS) region from genomic DNA, as described in Chapter 3.

2.8 Exopolysaccharide production

All bacterial isolates were grown on MRS agar medium supplemented with 10 g l^{-1} of sucrose at 30 °C for 7 d to visually assess their EPS production capacity. The turbidity of the water kefir liquors was assessed visually during all water kefir fermentation processes, as an indication for the production of EPS that were suspended in the liquors.

2.9 Culture-independent microbial species diversity and community dynamics analyses

The culture-independent microbial species diversity and community dynamics of bacteria and yeasts in the water kefir liquors and on the water kefir grains were determined after preparing total DNA extracts from the cell pellets of the water kefir liquors and 0.2 g of crushed water kefir grains, respectively, as described in Chapter 7. The culture-independent microbial community profiles were obtained by amplifying selected genomic fragments in the total DNA with the universal prokaryotic primer pair (V3), the LAB-specific primer pair (LAC), the *Bifidobacterium*-specific primer pair (Bif), and the universal eukaryotic primer pair (Yeast); and separating the PCR amplicons through denaturing gradient gel electrophoresis (DGGE), as described in Chapter 3. Selected bands of the community profiles were cut from the gels and identities were assigned through sequencing, as described in Chapter 3.

2.10 Substrate and metabolite concentration determinations

Samples for substrate and metabolite concentration analyses were prepared as described in Chapter 3. The concentrations of sucrose, glucose, fructose, glycerol, and mannitol were determined through high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), those of D- and L-lactic acid and acetic acid through high-performance liquid chromatography with ultraviolet detection (HPLC-UV), those of ethanol through gas chromatography with flame ionization detection (GC-FID), and those of the aroma compounds through static headspace gas chromatography with mass spectrometry detection (SH-GC-MS), as described in Chapter 3.

2.11 Statistics

An ANOVA was performed to test for differences between the eleven fermentation series, followed by a series of post-hoc pairwise comparisons with Fisher's least significant difference (LSD) test, as described in Chapter 7. All statistical tests were performed in R 3.2.0 with a significance level of 0.05.

3 Kinetic model development

3.1 Model equations

To compare the kinetics of different water kefir fermentation processes, a mathematical model was developed as follows. During water kefir fermentation, sucrose could be converted into glucose and fructose by invertases, or could be converted into fructose and suspended EPS (EPS_{Liquor}) or grain EPS (EPS_{Grains}) by glucansucrases. The production of EPS_{Liquor} was indicated by the higher turbidity of the liquors of the fermentation series with sucrose than of those without sucrose. The concentration of EPS_{Grains}, expressed as the water kefir grain wet

mass (g l^{-1}), is a measure for the amount of water kefir grains. The production of water kefir grain wet mass as a function of time during water kefir fermentation could be described by a logistic model with a maximum specific water kefir grain production rate k_{EPS_Grains} (h^{-1}; g of grain wet mass per liter per hour per g of grain wet mass per liter) and a maximal water kefir grain wet mass concentration $[EPS_{Grains_max}]$ (g l^{-1}), in analogy with a report on milk kefir grain growth (Zajšek & Goršek, 2010a):

$$d[EPS_{Grains}]/dt = k_{EPS_Grains} (1 - [EPS_{Grains}]/[EPS_{Grains_max}]) [EPS_{Grains}] \quad (1)$$

This differential equation was solved with $[EPS_{Grains}] = [EPS_{Grains_0}]$ when t = 0 h, resulting in a non-linear model.

The concentrations of ethanol (Eth), glycerol (Gly), lactic acid (LA), acetic acid (AA), and mannitol (Mtl) (g l^{-1}) were described as a function of time with their initial concentrations $[Eth_0]$, $[Gly_0]$, $[LA_0]$, $[AA_0]$, and $[Mtl_0]$ (g l^{-1}), and their volumetric production rates k_{Eth}, k_{Gly}, k_{LA}, k_{AA}, and k_{Mtl} (g l^{-1} h^{-1}). This could be illustrated via a general expression for each metabolite (P), as follows:

$$[P] = [P_0] + k_P t \quad (2)$$

To estimate the initial concentrations and volumetric production rates for all fermentation series, a linear model was developed, whereby the initial concentrations depended on the inoculum (Inoculum) and the volumetric production rates on the fermentation series (Time:Series):

$$P \sim Inoculum + Time:Series \quad (3)$$

The consumption of glucose and fructose as a function of time was only described for the fermentation series without sucrose. Experimental data from fermentation series containing sucrose were not modelled, due to the complexity related to the release of either fructose (glucansucrase) or glucose and fructose (invertase) from sucrose. The consumption of glucose and/or fructose for the production of each metabolite was described by a conversion factor, which represented the mass of glucose (or fructose) consumed for the production of a certain mass of metabolite (g g^{-1}). The production of ethanol and acetic acid due to yeast and LAB metabolism, respectively, were assumed to release equimolar amounts of carbon dioxide. The consumption of glucose and/or fructose for the production of metabolites and products that were not measured, such as biomass, was described with a volumetric production rate k_{Rest} (g l^{-1} h^{-1}). When the initial concentrations of glucose and fructose were similar, glucose was consumed faster than fructose (see Results). To describe the faster consumption of glucose (Glc) compared to fructose (Fru), a dimensionless glucose preference factor (P_{Glc}) was introduced.

$$d[Glc]/dt = - (1.96\ k_{Eth} + 0.98\ k_{Gly} + 1.00\ k_{LA} + 1.50\ k_{AA} + 0.99\ k_{Mtl} + k_{Rest})\ P_{Glc} [Glc] / (P_{Glc} [Glc] + [Fru]) \quad (4)$$

$$d[Fru]/dt = - (1.96\ k_{Eth} + 0.98\ k_{Gly} + 1.00\ k_{LA} + 1.50\ k_{AA} + 0.99\ k_{Mtl} + k_{Rest})\ [Fru] / (P_{Glc} [Glc] + [Fru]) \quad (5)$$

3.2 Fitting of the models to the experimental data

The parameters for the production kinetics of the water kefir grain mass and the metabolites were estimated by fitting the above described non-linear and linear models, respectively, to the experimental data. The volumetric production rates for the production of metabolites and products that were not measured and the glucose preference factor were estimated by solving the above mentioned set of differential equations. All calculations were

performed in R 3.2.0. The estimations of the biokinetic parameters are presented as the mean ± standard error.

The model parameters of equation 1, describing the production of EPS during the water kefir fermentations, were estimated by fitting a non-linear model to the experimental data obtained after 0, 24, 48, 72, and 96 h of fermentation for all fermentation series containing sucrose. The values of $[P_0]$ and k_P were estimated for each metabolite by fitting a linear model to the linear portions of the experimental data, which was from 0 to 72 h (see Results), for all fermentation series. The values of k_{Rest} were estimated by fitting the set of differential equations to the experimental data of fermentation series 2GF-2G-An, 2G-2G-An, and 2F-2G-An after 0, 24, 48, and 72 h of fermentation. The value of P_{Glc} was estimated by fitting the set of differential equations to the experimental data of the fermentation series 2GF-2G-An obtained after 0, 24, 48, and 72 h of fermentation.

4 Results

4.1 Water kefir grain density and visualization of the water kefir grains

The density of the water kefir grains was 1.0495 ± 0.0004 g ml^{-1}. Visualization of the water kefir grains via scanning electron microscopy revealed that their surface was covered with microorganisms (Figures 1a and 1b). Yeasts and LAB were found as mixed consortia. Some areas were occupied by either LAB (Figure 1c) or yeasts (Figure 1d). When a water kefir grain was cut with a sterile scalpel, no discernible microorganisms were found inside the grains (Figures 1e and 1f).

4.2 Microbial enumerations

The viable counts of the yeasts on the grain inoculum were similar to those on the grains of the anaerobic (2S-2G-An) and aerobic fermentation series (2S-2G-Ae) after 4 d of fermentation (Table 2). The viable counts of the yeasts in the liquor inoculum were similar to those in the liquors of the anaerobic (2S-2G-An) and aerobic (2S-2G-Ae) fermentation series, and to those in the liquors of the fermentation series performed with a liquor inoculum (2S-L-An) after 4 d of fermentation. Likewise, the viable counts of the LAB on the grain inoculum were similar to those on the grains of the anaerobic (2S-2G-An) and aerobic (2S-2G-Ae) fermentation series. The viable counts of the LAB in the liquor inoculum and in the liquors of the fermentation series performed with a liquor inoculum (2S-2L-An) were higher than those in the liquors of the anaerobic (2S-2G-An) and aerobic (2S-2G-Ae) fermentation series. The viable counts of the AAB were higher on the grains of the aerobic fermentation series (2S-2G-Ae) than on those of the grain inoculum and the anaerobic fermentation series (2S-2G-An). The viable counts of the AAB were highest in the liquors of the aerobic fermentation series (2S-2G-Ae), lower in the liquors of the fermentation series inoculated with a liquor inoculum (2S-2L-An), and lowest in the liquor inoculum and in the liquors of the anaerobic fermentation series (2S-2G-An).

The ratios of the viable counts of the yeasts on the water kefir grains to those in the liquors were always around 3 and this was also true for the LAB. In contrast, the ratios of the AAB on the water kefir grains to those in the liquors were always below 1. The ratios of the LAB to the yeasts were always between 2 and 10.

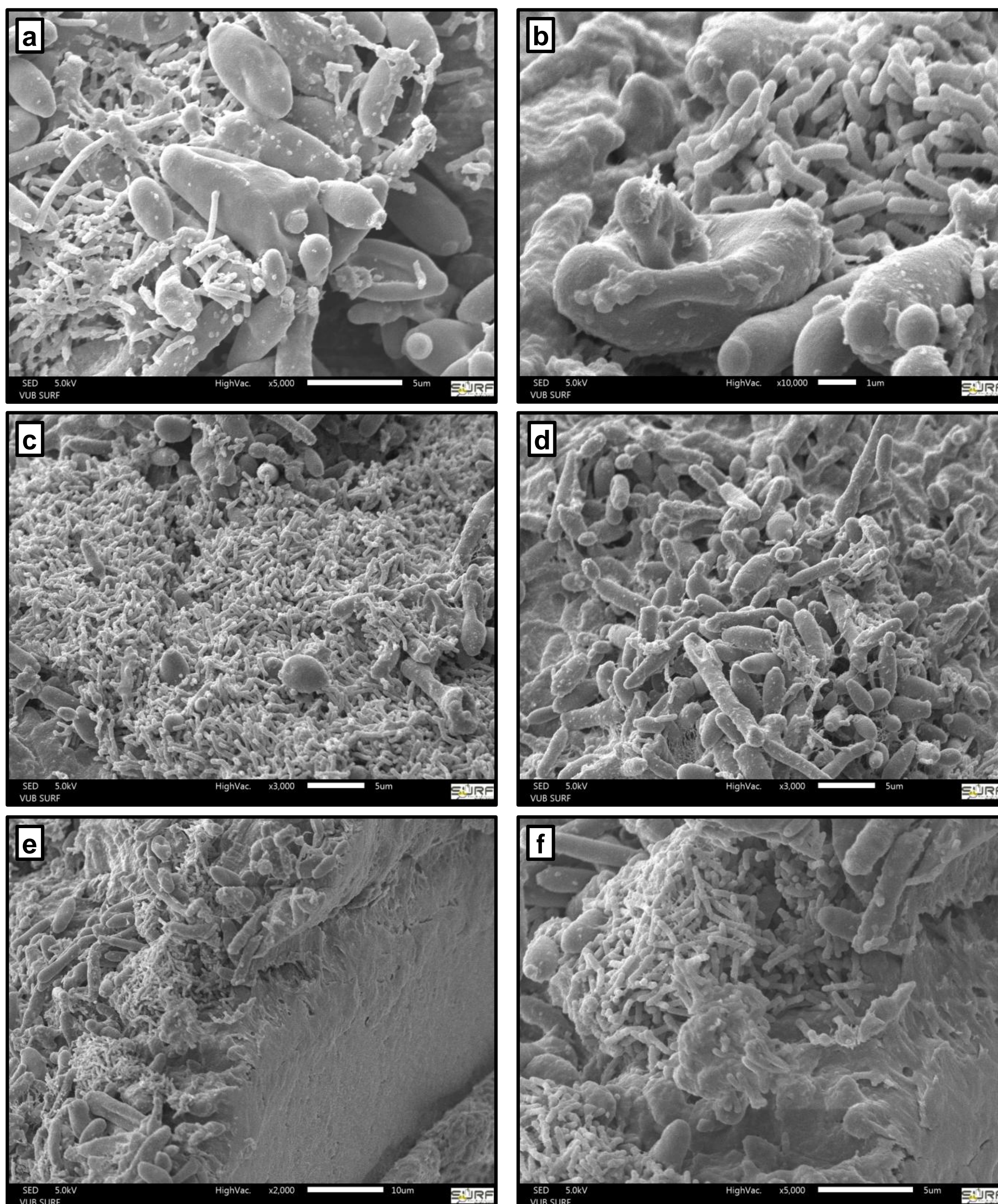

Figure 1. Scanning electron microscopy images of water kefir grains. Visualization of two different locations on the water kefir grain surface with a magnification level of 5,000 (a), 10,000 (b), and 3,000 (c and d), and visualization of the inside of a water kefir grain with a magnification level of 2,000 (e) and 5,000 (f).

Table 2. Viable counts of the yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) in the liquor (log cfu ml^{-1}) and grain inocula (log cfu g^{-1}), and in the liquors (log cfu ml^{-1}) and on the grains (log cfu g^{-1}) of fermentation series 2S-2G-An, 2S-2L-An, and 2S-2G-Ae after 4 d of fermentation, as well as the ratios between these values. The results are presented as the mean ± standard deviation; significant differences ($p < 0.05$) between the series are indicated with superscripts a, b, and c. Abbreviations are as in Table 1.

Viable counts or ratio		Inoculum	2S-2G-An	2S-2L-An	2S-2G-Ae
Yeasts	Liquors	7.0 ± 0.1^{a}	6.8 ± 0.1^{b}	7.0 ± 0.1^{a}	6.7 ± 0.1^{b}
	Grains	7.5 ± 0.1^{a}	7.3 ± 0.1^{b}	NA	7.5 ± 0.1^{a}
LAB	Liquors	8.0 ± 0.1^{a}	7.2 ± 0.1^{c}	7.8 ± 0.1^{b}	7.1 ± 0.1^{c}
	Grains	8.3 ± 0.1^{a}	8.0 ± 0.1^{b}	NA	7.9 ± 0.1^{b}
AAB	Liquors	3.4 ± 0.1^{c}	3.2 ± 0.3^{c}	4.9 ± 0.1^{b}	5.8 ± 0.1^{a}
	Grains	2.9 ± 0.2^{b}	2.5 ± 0.3^{c}	NA	5.3 ± 0.1^{a}
LAB/yeasts	Liquors	10.0 ± 2.2^{a}	2.3 ± 0.3^{c}	6.1 ± 0.5^{b}	2.3 ± 0.2^{c}
	Grains	6.3 ± 1.4^{a}	4.2 ± 0.7^{b}	NA	2.7 ± 0.1^{c}
Grains/liquor	Yeasts	3.0 ± 0.5^{b}	3.6 ± 0.9^{b}	NA	6.2 ± 1.6^{a}
	LAB	1.9 ± 0.6^{b}	6.5 ± 0.7^{a}	NA	7.3 ± 1.3^{a}
	AAB	0.32 ± 0.13^{a}	0.17 ± 0.03^{b}	NA	0.31 ± 0.04^{a}

NA, not available.

4.3 Culture-dependent microbial species diversity and community dynamics

Saccharomyces cerevisiae and *D. bruxellensis* were the only yeast species found culture-dependently in the grain and liquor inocula, whereby the relative abundance of *D. bruxellensis* was higher in the liquor than on the grains (Figure 2). *Lactobacillus paracasei* and *Lb. nagelii* were the main LAB species found culture-dependently in the liquor and grain inocula, whereas *Lb. hilgardii* (of which 50% of the isolates produced EPS) was only found in the grain inoculum.

Gluconobacter roseus/*oxydans*, *Acetobacter fabarum*, and *Acetobacter indonesiensis* were found culture-dependently in the grain and liquor inocula, whereby the relative abundances of *A. fabarum* were higher in the liquors and those of *A. indonesiensis* were higher on the grains. After 4 d of fermentation, *G. roseus*/*oxydans* and *A. fabarum* were found in the liquors of fermentation series 2S-2G-An, 2S-2L-An, and 2S-2G-Ae; and *A. indonesiensis* was found in the liquors of fermentation series 2S-2G-An and 2S-2G-Ae. The relative abundances of *A. fabarum* were higher in the fermentation series 2S-2L-An than in 2S-2G-An and 2S-2G-Ae, and those of *G. roseus*/*oxydans* were higher in fermentation series 2S-2G-An and 2S-2L-An than in 2S-2G-Ae.

4.4 Culture-independent microbial species diversity and community dynamics

The main bands in the rRNA-PCR-DGGE community profiles obtained with the Yeast primer pair for the liquor and grain inocula were attributed to *S. cerevisiae* and *D. bruxellensis*, whereby the relative intensities of the bands attributed to *D. bruxellensis* were higher for the liquor inoculum than for the grain inoculum (Figure 3). Furthermore, the community profiles obtained with the Yeast primer pair for the water kefir liquors of fermentation series 2S-2G-An, 2S-2L-An, 2S-2G-Ae, 2GF-2G-An, 2G-2G-An, and 2F-2G-An were similar to those for the liquor inoculum.

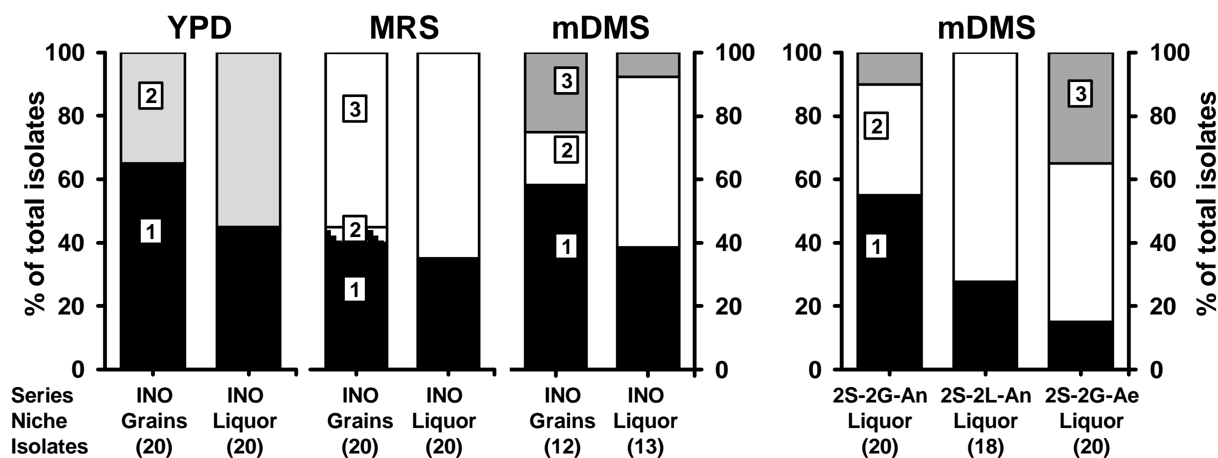

Figure 2. Culture-dependent species diversity for the water kefir grain and liquor inocula (INO), and for the water kefir liquors of fermentation series 2S-2G-An, 2S-2L-An, and 2S-2G-Ae after 4 d of fermentation. The number of isolates are indicated between brackets. Isolates from YPD agar medium: 1, *Saccharomyces cerevisiae* [LSU (99 % identity; GenBank accession no. CP011558) and ITS (99 % identity; accession no. KC515374)]; and 2, *Dekkera bruxellensis* [LSU (99 % identity; accession no. GU291284) and ITS (99 % identity; accession no. FJ545249)]. Isolates from MRS agar media: 1, *Lactobacillus paracasei* (100 % identity; accession no. AP012541); 2, *Lactobacillus hilgardii* (100 % identity; accession no. LC064898); and 3, *Lactobacillus nagelii* (99 % identity; accession no. NR112754). Isolates from mDMS agar media: 1, *Gluconobacter roseus*/*oxydans* (100 % identity for both species; accession no. NR041049/NR026118); 2, *Acetobacter fabarum* (100 % identity; accession no. NR113556); and 3, *Acetobacter indonesiensis* (99 % identity; accession no. NR113847). LSU, large subunit rRNA gene; ITS, internal transcribed spacer. Abbreviations are as in Table 1.

The main bands in the community profiles obtained with the V3 and LAC primer pairs for the liquor and grain inocula were attributed to *Lb. paracasei*, *Lb. nagelii*, and *Lb. hilgardii*, whereby the relative intensities of the bands attributed to *Lb. hilgardii* were higher for the grain inoculum than for the liquor inoculum (Figure 3). Furthermore, the relative intensities of the bands attributed to *Lb. hilgardii* were higher for the liquors of fermentation series 2S-2G-An and 2S-2G-Ae than for those of fermentation series 2S-2L-An, 2GF-2G-An, 2G-2G-An, and 2F-2G-An. Bands with low relative intensities attributed to a non-identified *Oenococcus* species, and bands with high relative intensities attributed to *Bifidobacterium aquikefiri* were present in the community profiles obtained with the V3 primer pair for the liquor and grain inocula, and for the liquors of fermentation series 2S-2G-An, 2S-2L-An, 2S-2G-Ae, 2GF-2G-An, 2G-2G-An, and 2F-2G-An. The partial 16S rRNA gene sequence of the non-identified *Oenococcus* species was 100 % identical to the sequence of an *Oenococcus* species (accession no. LT220205) found in water kefirs before (Chapters 4 and 7). A band with low relative intensity attributed to the taxon *Acetobacteraceae* was found in the community profiles obtained with the V3 primer pair for the liquors of the fermentation series 2S-2G-Ae, but not for the liquor inoculum and the liquors of the other fermentation series.

4.5 Substrate consumption and metabolite production profiles

The concentrations of the water kefir grain wet mass (Figure 4), the substrates (Figure 5), and the metabolites (Figure 6) as a function of time during the eleven series of water kefir fermentations were fitted by the kinetic models described above. The pH (Figure 4) followed

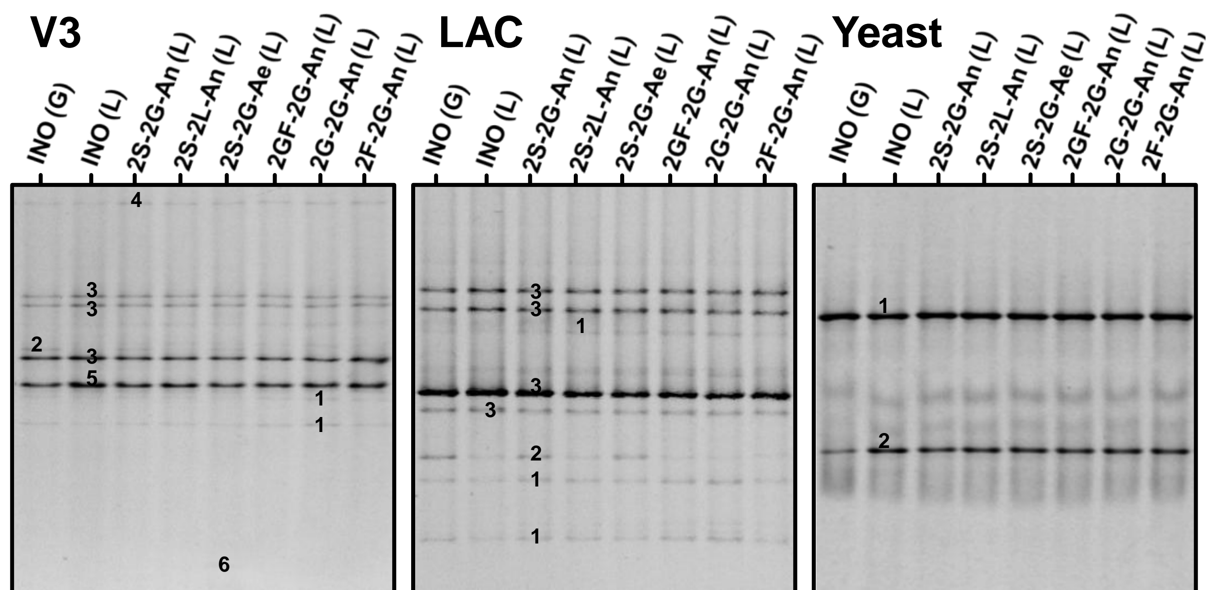

Figure 3. Culture-independent species diversity for the grain (G) and liquor (L) inocula (INO), and for the water kefir liquors (L) of fermentation series 2S-2G-An, 2S-2L-An, 2S-2G-Ae, 2GF-2G-An, 2G-2G-An, and 2F-2G-An after 4 d of fermentation. The numbers indicate the bands that were sequenced and the closest known type strains of the sequenced fragments are given. With the V3 primer pair: 1, *Lactobacillus casei*/*paracasei*/*zeae*/*rhamnosus* (99 % identity for all species; GenBank accession no. LC064894/AB289229/AB289313/JQ580982); 2, *Lactobacillus hilgardii*/*diolivorans* (100 % identity; accession no. LC064898/NR037004); 3, *Lactobacillus nagelii*/*ghanensis* (99 % identity; accession no. NR119275/NR043896); 4, *Oenococcus kitaharae* (97 % identity; accession no. NR041312); 5, *Bifidobacterium aquikefiri* (100 % identity; accession no. LN849254); 6, *Acetobacteraceae* sp. (100 % identity). With the LAC primer pair: 1, *Lb. casei*/*paracasei*/*zeae* (99 % identity; accession no. LC064894/AB289229/AB289313); 2, *Lb. hilgardii* (100 % identity; accession no. LC064898); and 3, *Lb. nagelii* (99 % identity; accession no. NR119275). With the Yeast primer pair: 1, *Saccharomyces cerevisiae* (100 % identity; accession no. NG042623); and 2, *Dekkera bruxellensis* (100 % identity; accession no. AY969049). Abbreviations are as in Table 1.

always a similar pattern and was mainly influenced by the type and concentration of the inoculum.

The model describing the production of EPS during the water kefir fermentation is illustrated for the fermentation series 2S-2G-An (Figure 7). When the concentrations of the grain inoculum increased, k_{EPS_Grains} and $[EPS_{Grains_max}]$ increased (Table 3), whereas the water kefir grain growth at the end of the fermentation decreased (Figure 4). When the concentrations of sucrose decreased or when sucrose was partially substituted with glucose and fructose, k_{EPS_Grains} increased and $[EPS_{Grains_max}]$ decreased, and the water kefir grain growth (%) at the end of the fermentation decreased. When the fermentations were performed aerobically, the water kefir grain growth was similar to that of the fermentations under anaerobic conditions. When sucrose was substituted completely, the water kefir grain growth was zero.

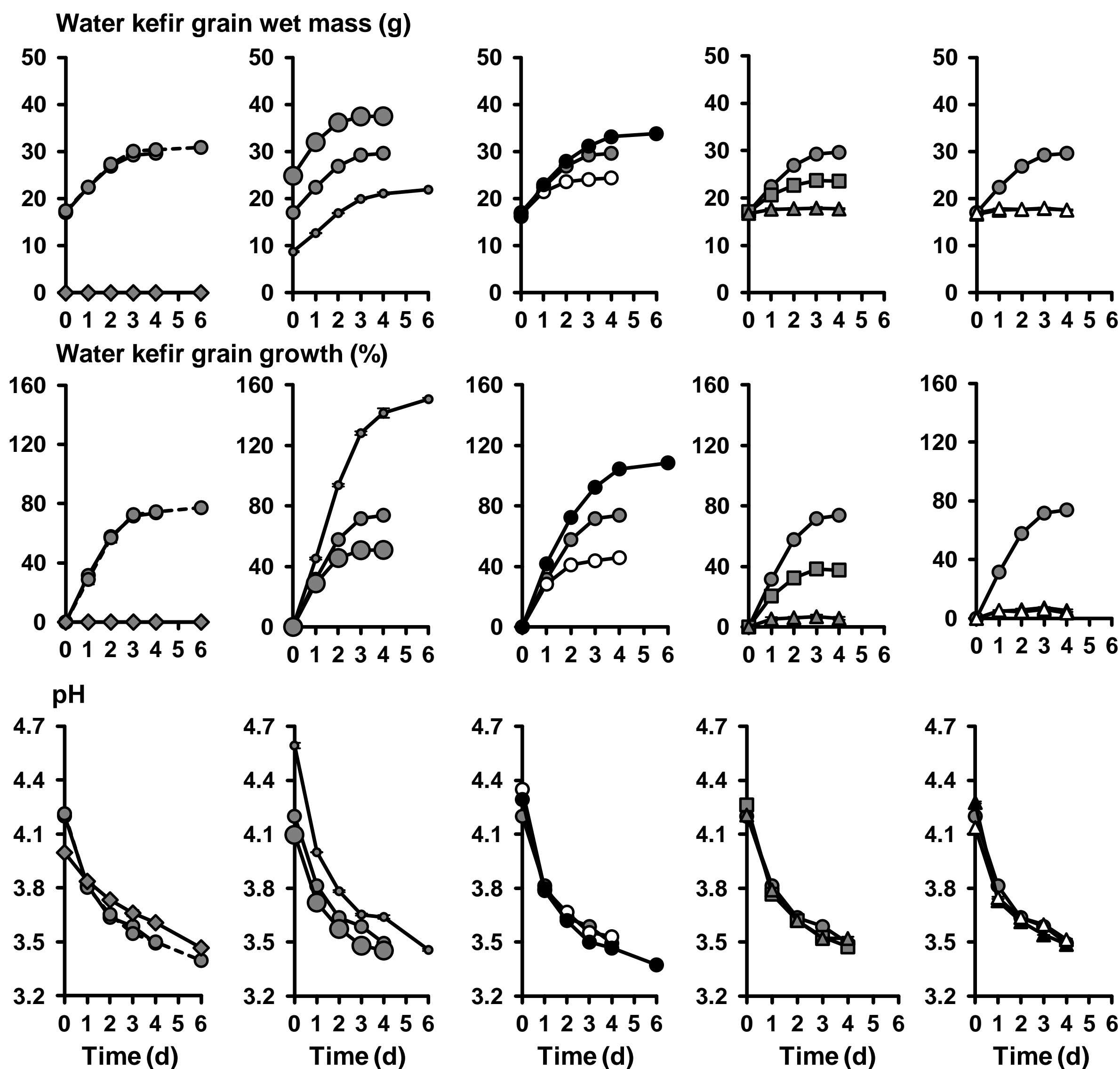

Figure 4. Water kefir grain wet mass (g), water kefir grain growth (%), and pH as a function of time during water kefir fermentation series 2S-2G-An (●——), 2S-2G-Ae (●- - -), 2S-2L-An (♦——), 2S-1G-An (•——), 2S-3G-An (●——), 1S-2G-An (○——), 3S-2G-An (●——), 2SGF-2G-An (■——), 2GF-2G-An (▲——), 2G-2G-An (▲——), and 2F-2G-An (Δ——). Abbreviations are as in Table 1.

The models describing the production of metabolites during water kefir fermentation are illustrated for fermentation series 2S-2G-An and 2S-2L-An (Figure 7). The volumetric production rates of ethanol (k_{Eth}), glycerol (k_{Gly}), lactic acid (k_{LA}), and acetic acid (k_{AA}) increased with the concentration of the grain inoculum, but less than expected from the increases in the concentrations of the grain inoculum (Table 3). The volumetric metabolite production rates in the fermentation series 2S-2L-An were around half of those in the fermentation series 2S-2G-An, except for the volumetric production rate of mannitol, which was almost zero in 2S-2L-An. They increased with the concentrations of the water kefir grain inoculum added. The concentrations of sucrose did not substantially impact the production of metabolites. When sucrose was substituted with glucose and fructose in fermentation series

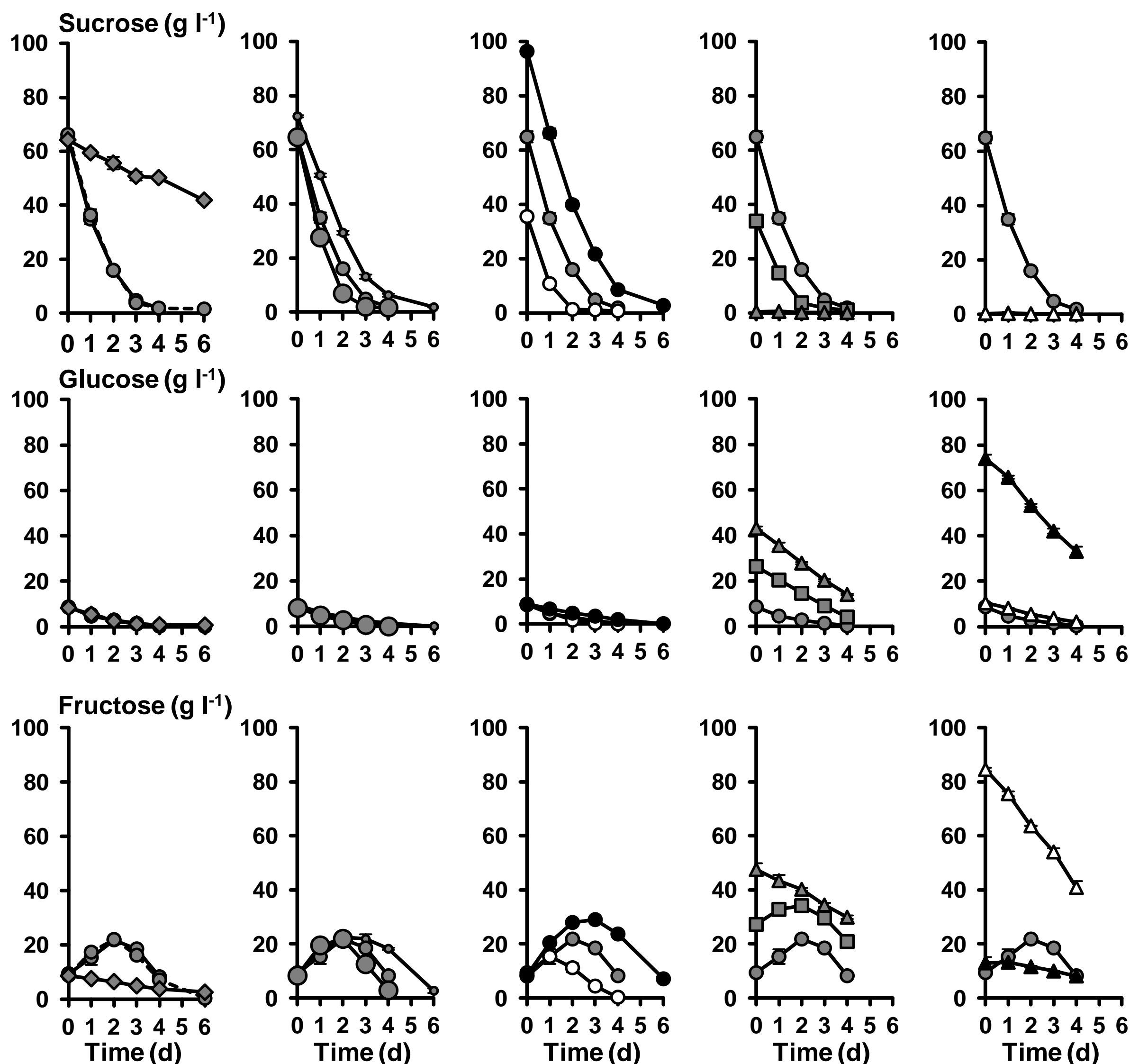

Figure 5. Concentrations of sucrose, glucose, and fructose as a function of time during water kefir fermentation series 2S-2G-An (●——), 2S-2G-Ae (●- - -), 2S-2L-An (♦——), 2S-1G-An (•——), 2S-3G-An (●——), 1S-2G-An (○——), 3S-2G-An (●——), 2SGF-2G-An (■——), 2GF-2G-An (▲——), 2G-2G-An (▲——), and 2F-2G-An (Δ——). Abbreviations are as in Table 1.

2S-2G-An, 2SGF-2G-An, and 2GF-2G-An, the volumetric production rates of ethanol, glycerol, lactic acid, and acetic acid decreased. Furthermore, the volumetric production rates of ethanol, glycerol, lactic acid, and acetic acid were higher with glucose (2G-2G-An) than with fructose (2F-2G-An), whereas the volumetric production rate of mannitol was higher with fructose (2F-2G-An). The volumetric production rates of ethanol, glycerol, and acetic acid were higher under aerobic fermentation conditions (2S-2G-Ae), whereas those for lactic acid and mannitol were higher under anaerobic fermentation conditions (2S-2G-An). The fermentation series inoculated with a liquor inoculum had the lowest ratios of the volumetric production rates of glycerol to ethanol, and the highest ones of lactic acid to ethanol and acetic acid to ethanol. The highest ratios of the volumetric production rates of acetic acid to lactic acid were found for the aerobic fermentation series.

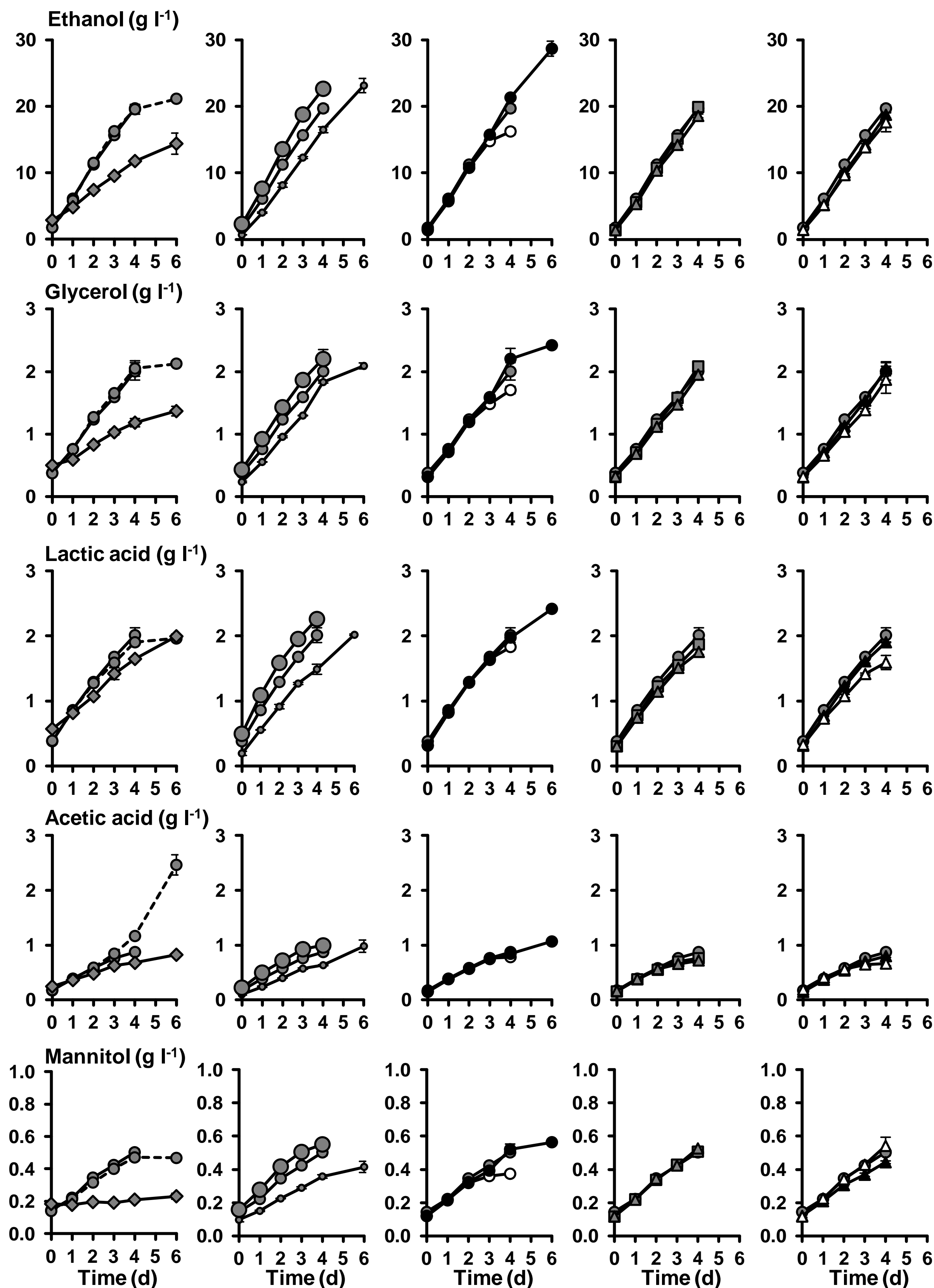

Figure 6. Concentrations of ethanol, glycerol, lactic acid, acetic acid, and mannitol as a function of time during water kefir fermentation series 2S-2G-An (●——), 2S-2G-Ae (●- - -), 2S-2L-An (♦——), 2S-1G-An (•——), 2S-3G-An (●——), 1S-2G-An (○——), 3S-2G-An (●——), 2SGF-2G-An (■——), 2GF-2G-An (▲——), 2G-2G-An (▲——), and 2F-2G-An (Δ——). Abbreviations are as in Table 1.

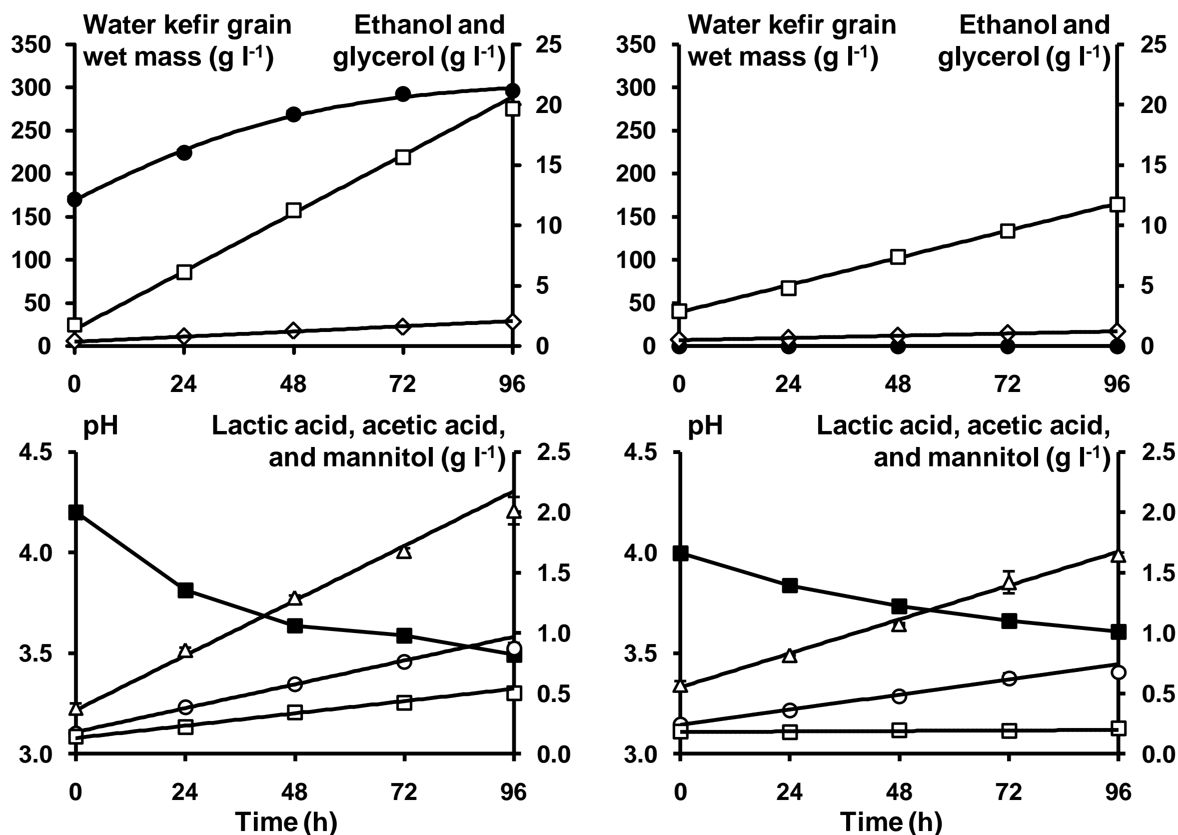

Figure 7. The pH (■) and concentrations of water kefir grain wet mass (●), ethanol (□), glycerol (◊), lactic acid (Δ), acetic acid (○), and mannitol (□) as a function of time for the anaerobic water kefir fermentation series 2S-2G-An with sucrose as substrate and started with a grain inoculum (left), and for the anaerobic water kefir fermentation series 2S-2L-An with sucrose as substrate and started with a liquor inoculum (right). The model lines (solid lines) describe the modelled concentrations of water kefir grain wet mass, ethanol, glycerol, lactic acid, acetic acid, and mannitol during the first 72 h of fermentation. Abbreviations are as in Table 1.

The fraction of the total metabolism used for the production of metabolites and products that were not measured was higher with fructose (2F-2G-An) than with glucose (2G-2G-An) or glucose and fructose (2GF-2G-An) (Table 3). When the concentrations of glucose and fructose were similar (2GF-2G-An), glucose was consumed approximately 2.19 times faster than fructose.

4.6 Aroma compounds

The main higher alcohols found via SH-GC-MS were 2-methyl-1-propanol, isoamyl alcohol, and 2-phenylethanol, and the main esters were ethyl acetate, isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate (Figures 8 and 9). The production profiles of 2-methyl-1-propanol and isoamyl alcohol followed those of ethanol. In contrast, the concentrations of ethyl acetate, ethyl decanoate, and to a lesser extent ethyl hexanoate increased only slowly during the first 24 to 48 h of all fermentation series, after which their concentrations increased faster than before. The concentrations of ethyl octanoate increased

Table 3. Estimated values of the model parameters during eleven series of water kefir fermentations differing in the presence of oxygen and the type and concentration of the inoculum and substrate: initial concentrations of water kefir grain wet mass ($[EPS_{Grains_0}]$), maximum concentrations of water kefir grain wet mass ($[EPS_{Grains_max}]$), and maximum specific water kefir grain production rates (k_{EPS_Grains}) for the logistic models describing the concentrations of the water kefir grain wet mass as a function of time; initial concentrations and volumetric production rates of ethanol ($[Eth_0]$ and k_{Eth}), glycerol ($[Gly_0]$ and k_{Gly}), lactic acid ($[LA_0]$ and k_{LA}), acetic acid ($[AA_0]$ and k_{AA}), and mannitol ($[Mtl_0]$ and k_{Mtl}) for the linear models describing their concentrations as a function of time; volumetric production rates of the metabolites and products that were not measured (k_{Rest}) for the models describing their production as a function of time; estimated value for the glucose preference factor (P_{Glc}) describing the consumption of glucose and fructose as a function of time; and the ratios of the volumetric production rates for the production of glycerol to ethanol, lactic acid to ethanol, acetic acid to ethanol, and acetic acid to lactic acid. The results are presented as the mean ± standard error and significant differences ($p < 0.05$) between different fermentation series are indicated with different superscripts a, b, c, d, e, f, g, h, and i. Abbreviations are as in Table 1.

Parameter	2S-2G-An	2S-2L-An	2S-2G-Ae	2S-3G-An	2S-1G-An	1S-2G-An	3S-2G-An	2SGF-2G-An	2GF-2G-An	2G-2G-An	2F-2G-An
$[EPS_{Grains_0}]$ ($g\ l^{-1}$)	169 ± 1 ^{b}	NA	169 ± 1 ^{b}	231 ± 2 ^{a}	93 ± 1 ^{c}	169 ± 1 ^{b}	169 ± 1 ^{b}	169 ± 1 ^{b}	NA	NA	NA
$[EPS_{Grains_max}]$ ($g\ l^{-1}$)	308 ± 3 ^{c}	NA	318 ± 4 ^{b}	354 ± 2 ^{a}	247 ± 3 ^{d}	245 ± 2 ^{d}	352 ± 4 ^{a}	240 ± 2 ^{d}	NA	NA	NA
k_{EPS_Grains} ($10^{-3}\ h^{-1}$)	35 ± 2 ^{cd}	NA	34 ± 2 ^{d}	46 ± 2 ^{ab}	32 ± 1 ^{d}	50 ± 4 ^{a}	30 ± 1 ^{d}	41 ± 4 ^{bc}	NA	NA	NA
$[Eth_0]$ ($g\ l^{-1}$)	1.35 ± 0.05 ^{c}	2.78 ± 0.16 ^{a}	1.35 ± 0.05 ^{c}	2.26 ± 0.16 ^{b}	0.44 ± 0.16 ^{d}	1.35 ± 0.05 ^{c}	1.35 ± 0.05 ^{c}	1.35 ± 0.05 ^{c}	1.35 ± 0.05 ^{c}	1.35 ± 0.05 ^{c}	1.35 ± 0.05 ^{c}
$[Gly_0]$ ($g\ l^{-1}$)	0.33 ± 0.01 ^{b}	0.47 ± 0.02 ^{a}	0.33 ± 0.01 ^{b}	0.44 ± 0.02 ^{a}	0.22 ± 0.02 ^{c}	0.33 ± 0.01 ^{b}	0.33 ± 0.01 ^{b}	0.33 ± 0.01 ^{b}	0.33 ± 0.01 ^{b}	0.33 ± 0.01 ^{b}	0.33 ± 0.01 ^{b}
$[LA_0]$ ($g\ l^{-1}$)	0.36 ± 0.01 ^{b}	0.55 ± 0.02 ^{a}	0.36 ± 0.01 ^{b}	0.55 ± 0.02 ^{a}	0.19 ± 0.02 ^{c}	0.36 ± 0.01 ^{b}	0.36 ± 0.01 ^{b}	0.36 ± 0.01 ^{b}	0.36 ± 0.01 ^{b}	0.36 ± 0.01 ^{b}	0.36 ± 0.01 ^{b}
$[AA_0]$ ($g\ l^{-1}$)	0.18 ± 0.01 ^{b}	0.24 ± 0.01 ^{a}	0.18 ± 0.01 ^{b}	0.24 ± 0.01 ^{a}	0.09 ± 0.01 ^{c}	0.18 ± 0.01 ^{b}	0.18 ± 0.01 ^{b}	0.18 ± 0.01 ^{b}	0.18 ± 0.01 ^{b}	0.18 ± 0.01 ^{b}	0.18 ± 0.01 ^{b}
$[Mtl_0]$ ($g\ l^{-1}$)	0.13 ± 0.01 ^{b}	0.18 ± 0.01 ^{a}	0.13 ± 0.01 ^{b}	0.16 ± 0.01 ^{a}	0.09 ± 0.01 ^{c}	0.13 ± 0.01 ^{b}	0.13 ± 0.01 ^{b}	0.13 ± 0.01 ^{b}	0.13 ± 0.01 ^{b}	0.13 ± 0.01 ^{b}	0.13 ± 0.01 ^{b}
k_{Eth} ($mg\ l^{-1}\ h^{-1}$)	201 ± 2 ^{bc}	94 ± 3 ^{i}	207 ± 2 ^{b}	231 ± 3 ^{a}	162 ± 3 ^{h}	189 ± 3 ^{e}	198 ± 2 ^{cd}	192 ± 2 ^{de}	180 ± 2 ^{f}	178 ± 2 ^{fg}	172 ± 2 ^{g}
k_{Gly} ($mg\ l^{-1}\ h^{-1}$)	17.9 ± 0.1 ^{bc}	7.6 ± 0.4 ^{g}	18.7 ± 0.2 ^{b}	20.0 ± 0.4 ^{a}	14.9 ± 0.4 ^{f}	16.7 ± 0.2 ^{de}	17.4 ± 0.2 ^{cd}	17.2 ± 0.2 ^{cd}	16.0 ± 0.2 ^{e}	16.7 ± 0.2 ^{de}	14.6 ± 0.2 ^{f}
k_{LA} ($mg\ l^{-1}\ h^{-1}$)	18.9 ± 0.3 ^{b}	11.7 ± 0.5 ^{g}	18.0 ± 0.3 ^{bcd}	20.3 ± 0.5 ^{a}	15.0 ± 0.5 ^{f}	18.5 ± 0.3 ^{bc}	18.3 ± 0.3 ^{bc}	17.1 ± 0.3 ^{de}	16.1 ± 0.3 ^{e}	17.6 ± 0.3 ^{cd}	14.8 ± 0.3 ^{f}
k_{AA} ($mg\ l^{-1}\ h^{-1}$)	8.2 ± 0.2 ^{c}	5.2 ± 0.3 ^{g}	9.0 ± 0.2 ^{b}	9.7 ± 0.3 ^{a}	6.6 ± 0.3 ^{f}	8.2 ± 0.2 ^{c}	8.0 ± 0.2 ^{cd}	7.4 ± 0.2 ^{de}	7.2 ± 0.2 ^{e}	7.4 ± 0.2 ^{e}	7.1 ± 0.2 ^{ef}
k_{Mtl} ($mg\ l^{-1}\ h^{-1}$)	4.25 ± 0.09 ^{b}	0.17 ± 0.13 ^{f}	3.84 ± 0.09 ^{c}	4.91 ± 0.13 ^{a}	2.72 ± 0.13 ^{e}	3.50 ± 0.09 ^{d}	3.81 ± 0.13 ^{c}	4.23 ± 0.09 ^{b}	4.23 ± 0.09 ^{b}	3.47 ± 0.09 ^{d}	4.26 ± 0.09 ^{b}
k_{Rest} ($mg\ l^{-1}\ h^{-1}$)	NA	NA	NA	NA	NA	NA	NA	NA	111 ± 8	114 ± 8	145 ± 6
P_{Glc}	NA	NA	NA	NA	NA	NA	NA	NA	2.19 ± 0.08	NA	NA
Glycerol/ethanol ($mmol\ mol^{-1}$)	45 ± 1 ^{abc}	40 ± 2 ^{d}	45 ± 1 ^{abc}	43 ± 1 ^{bcd}	46 ± 1 ^{ab}	44 ± 1 ^{abc}	44 ± 1 ^{abc}	45 ± 1 ^{abc}	44 ± 1 ^{abc}	47 ± 1 ^{a}	42 ± 1 ^{cd}
Lactic acid/ethanol ($mmol\ mol^{-1}$)	48 ± 1 ^{bcd}	64 ± 4 ^{a}	44 ± 1 ^{d}	45 ± 1 ^{d}	47 ± 2 ^{bcd}	50 ± 1 ^{bc}	47 ± 1 ^{bcd}	46 ± 1 ^{cd}	46 ± 1 ^{cd}	51 ± 1 ^{b}	44 ± 1 ^{d}
Acetic acid/ethanol ($mmol\ mol^{-1}$)	31 ± 1 ^{bc}	43 ± 3 ^{a}	33 ± 1 ^{b}	32 ± 1 ^{bc}	31 ± 1 ^{bc}	33 ± 1 ^{bc}	31 ± 1 ^{bc}	30 ± 1 ^{c}	31 ± 1 ^{bc}	32 ± 1 ^{bc}	32 ± 1 ^{bc}
Acetic acid/lactic acid ($mmol\ mol^{-1}$)	652 ± 19 ^{bc}	673 ± 46 ^{bc}	751 ± 21 ^{a}	715 ± 27 ^{ab}	657 ± 35 ^{bc}	665 ± 19 ^{bc}	653 ± 19 ^{bc}	652 ± 20 ^{bc}	667 ± 22 ^{bc}	626 ± 19 ^{c}	721 ± 25 ^{ab}

NA, not available.

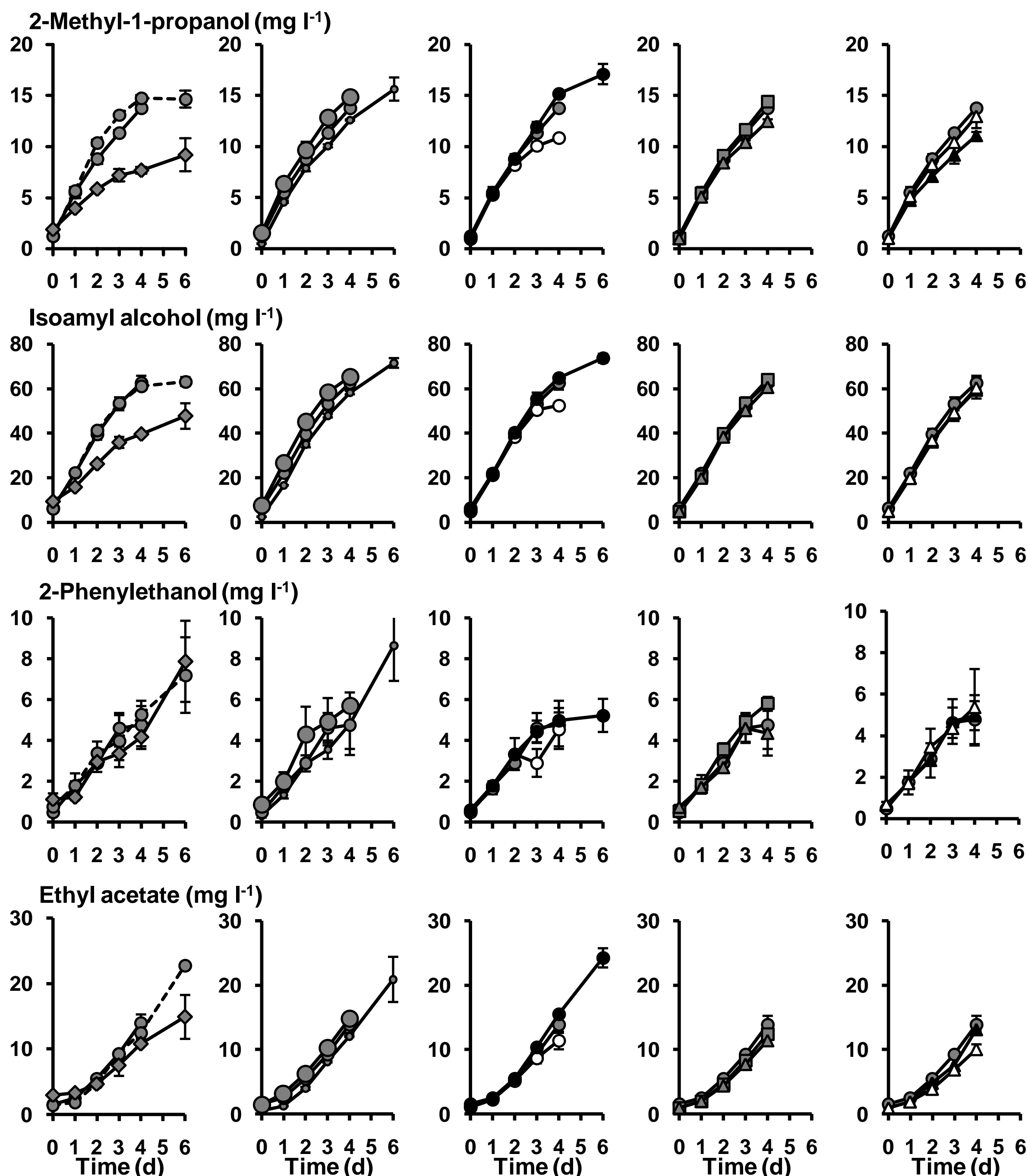

Figure 8. Concentrations of 2-methyl-1-propanol, isoamyl alcohol, 2-phenylethanol, and ethyl acetate as a function of time during water kefir fermentation series 2S-2G-An (●——), 2S-2G-Ae (●- - -), 2S-2L-An (♦——), 2S-1G-An (•——), 2S-3G-An (●——), 1S-2G-An (○——), 3S-2G-An (●——), 2SGF-2G-An (■——), 2GF-2G-An (▲——), 2G-2G-An (▲——), and 2F-2G-An (Δ——). Abbreviations are as in Table 1.

fast in all fermentation series and decreased after 72 h of fermentation, whereby the decrease was most pronounced in the fermentation series 2S-2G-Ae. Also, the concentrations of ethyl hexanoate decreased noticeably in the fermentation series 2S-2G-Ae after 72 h of fermentation. The production of ethyl decanoate increased with the time of fermentation, and

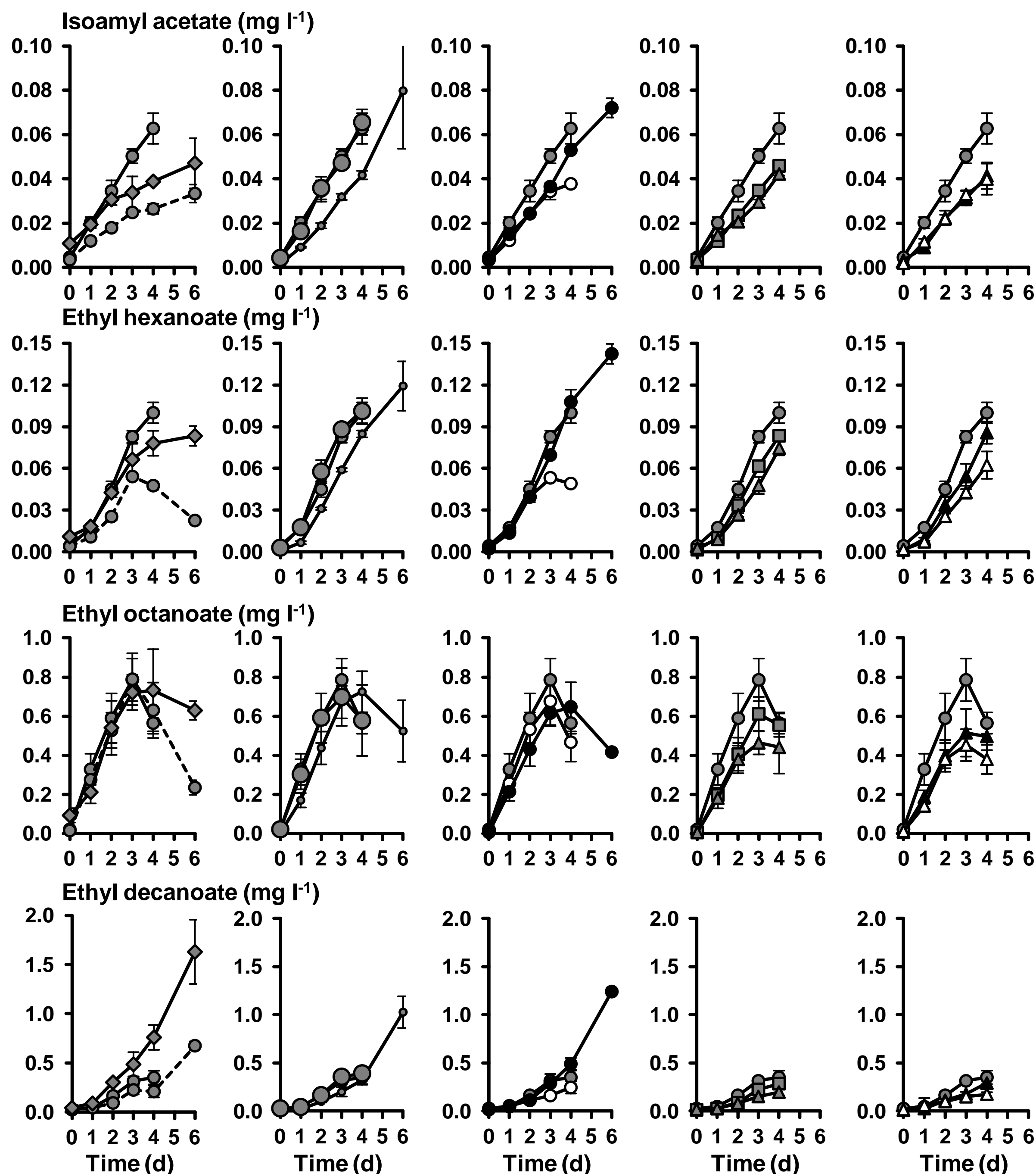

Figure 9. Concentrations of isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate as a function of time during water kefir fermentation series 2S-2G-An (●——), 2S-2G-Ae (●- - -), 2S-2L-An (♦——), 2S-1G-An (●——), 2S-3G-An (●——), 1S-2G-An (○——), 3S-2G-An (●——), 2SGF-2G-An (■——), 2GF-2G-An (▲——), 2G-2G-An (▲——), and 2F-2G-An (Δ——). Abbreviations are as in Table 1.

its concentrations after 96 h of fermentation were higher in the fermentation series 2S-2L-An than in 2S-2G-An. Overall, the production of esters was lower in the fermentation series 2F-2G-An compared to 2G-2G-An and 2S-2G-An.

5 Discussion

Water kefir fermentation is commonly started with water kefir grains as inoculum (Waldherr *et al.*, 2010; Gulitz *et al.*, 2011; Laureys & De Vuyst, 2014; Chapters 3, 4, 5, 7, and 8). Yet, several questions about the exact nature and role of these grains during water kefir fermentation remained to be addressed. This chapter contributed to a better characterization of the properties of the water kefir grains by determining their density and microbial colonization, and of their function during water kefir fermentation. The latter was realized by applying a modeling strategy to describe the production of water kefir grain wet mass (expressed as EPS produced) as a function of time during the fermentation process.

The state-of-the-art equipment used in the present study allowed to show that the water kefir microorganisms were predominantly attached onto the surface of the water kefir grains, whereby the yeasts and LAB were not structurally arranged around each other. These results were in line with a previous report (Moinas *et al.*, 1980). The main yeasts and LAB species were *S. cerevisiae*, *D. bruxellensis*, *Lb. nagelii, Lb. paracasei,* and *Lb. hilgardii*, whereby the relative abundances of *S. cerevisiae* and *Lb. hilgardii* were higher on the grains than in the liquors. The main AAB species were *Gl. roseus*/*oxydans*, *A. fabarum*, and *A. indonesiensis*, which were all found in water kefir before (Gulitz *et al.*, 2011; Gulitz, 2013; Laureys & De Vuyst, 2014; Chapters 3, 5, and 8).

The LAB and yeasts were always the most prevalent microorganisms during the water kefir fermentation processes studied, and were predominantly associated with the water kefir grains, confirming previous data (Laureys & De Vuyst, 2014; Chapters 3 and 4). In contrast, the AAB only proliferated under aerobic fermentation conditions and were always predominantly associated with the water kefir liquors. The proliferation of AAB resulted in high concentrations of acetic acid and ethyl acetate, and low concentrations of higher esters, confirming previous results (Chapter 8). Similarly, the proliferation of AAB in wine results in high concentrations of ethyl acetate and loss of fruity aromas (Bartowsky *et al.*, 2003). High concentrations of acetic acid or ethyl acetate are probably not desired in water kefir, as acetic acid can contribute a harsh acidic taste and aroma, and ethyl acetate a solvent-like aroma. In contrast, higher esters will be desirable in water kefir, as they can contribute fruity aromas (Lambrechts & Pretorius, 2000; Laureys & De Vuyst, 2014; Chapter 3).

The majority of the metabolic activity of the microorganisms was associated with the grains, and the water kefir fermentation rate increased with the concentration of the water kefir grain inoculum. However, the increase in fermentation rate was less than expected from the increase in the concentration of the water kefir grain inoculum. Indeed, substantial metabolic activity was also found in the water kefir liquors. As an innovative approach, water kefir liquor could be used as alternative inoculum to start a water kefir fermentation process, without the production of water kefir grain mass. However, the volumetric production rates for ethanol, glycerol, lactic acid, and acetic acid during a water kefir fermentation process inoculated with liquor were only half of those during a comparable fermentation process inoculated with grains. The production of mannitol was mainly associated with the grains and was negligible in the liquors. This corresponded with the higher relative abundance of *Lb. hilgardii* on the grains than in the liquors. Furthermore, starting a water kefir fermentation process with liquor instead of grains resulted in high viable counts of AAB, as these microorganisms were predominantly associated with the liquors, and this was reflected in high ratios of acetic acid to ethanol.

The water kefir grain growth could be decreased by substituting sucrose (partly) with glucose and/or fructose. Glucose was the preferred alternative substrate, as it was fermented faster than fructose. Indeed, *S. cerevisiae* and most LAB ferment glucose faster than fructose

(Berthels *et al.*, 2004; Endo, 2012), although the growth and metabolism of *Lb. hilgardii* was reported to be faster with fructose than with sucrose or glucose as substrates (Leroi & Pidoux, 1993). Furthermore, when fructose was the substrate during water kefir fermentation, the production of non-measured metabolites and/or products was higher than with sucrose or glucose. Complete substitution of sucrose with glucose and/or fructose resulted in the absence of water kefir grain growth and in lower relative abundances of *Lb. hilgardii* in the water kefir liquors. The latter may be undesirable on the long term, as it might compromise the potential for water kefir grain growth. Low water kefir grain growth decreases the size of the water kefir grains, as they are brittle and break easily (Chapters 4 and 7). This makes them more difficult to sieve and increases their viable counts of microorganisms, resulting in an unstable production process (Chapters 4, 5, 7, and 8).

Sometimes the fast production of water kefir grain wet mass is desirable, for example to scale up a water kefir production process. The specific water kefir grain production rate increased with increasing concentrations of the grain inoculum, which was probably caused by a shift of the dextran sucrase activity from sucrose hydrolysis towards dextran biosynthesis at higher dextran concentrations (Mooser *et al.*, 1985). The specific water kefir grain production rate decreased slightly with increasing sucrose concentrations, which was probably caused by substrate inhibition of the dextran sucrases by sucrose concentrations above 36 g l^{-1} (Hehre, 1946). The highest water kefir grain growth was obtained when the concentration of the grain inoculum was lowest, as this minimized acidic stress, substrate inhibition, and substrate depletion (Chapter 7). The water kefir grain growth may thus be maximized with moderate sucrose concentrations and low concentrations of grain inoculum.

In conclusion, yeasts and LAB were always the most prevalent microorganisms during water kefir fermentation. They were mainly found on the surface of the water kefir grains. In contrast, AAB proliferated only under aerobic fermentation conditions and were mainly found in the water kefir liquors. The water kefir fermentation rate could be increased by increasing the concentration of the grain inoculum, as the majority of the microbial metabolic activity was associated with the water kefir grains. Nevertheless, substantial microbial metabolic activity was also found in the water kefir liquors. Moreover, the water kefir liquor could be used as an alternative inoculum to start a water kefir fermentation process, whereby no water kefir grain wet mass was produced. However, the volumetric production rates of most metabolites (and especially mannitol) were lower when the fermentation processes were inoculated with liquors instead of grains. The production of water kefir grains could be controlled by (partly) substituting sucrose with glucose and/or fructose, whereby glucose was fermented faster than fructose.

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CHAPTER 10

Backslopping time, rinsing of the grains during backslopping, and incubation temperature influence the water kefir fermentation process

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SUMMARY

Eight series of water kefir fermentations differing in backslopping time and rinsing of the grains before each backslopping step, and eight series of fermentations differing in incubation temperature and backslopping time were investigated during eight backslopping steps. A kinetic modelling approach was applied to determine the influences of these process conditions on the water kefir fermentation process. Short backslopping times resulted in high relative abundances of *Lactobacillus nagelii* and *Saccharomyces cerevisiae*, intermediate backslopping times in high relative abundances of *Leuconostoc pseudomesenteroides*, and long backslopping times in high relative abundances of a non-identified *Oenococcus* species and *Dekkera bruxellensis*. When the grains were rinsed before each backslopping step, the relative abundances of *Lactobacillus hilgardii* and *Leuc*. *pseudomesenteroides* increased and those of *D*. *bruxellensis* and *Lb*. *nagelii* decreased. Further, rinsing of the grains before each backslopping step resulted in a slightly higher water kefir grain growth and lower metabolite concentrations. The relative abundances of *Lactobacillus mali* were highest at 18 °C, those of *Leuc*. *pseudomesenteroides* at 21 and 25 °C, and those of *Lb*. *nagelii* at 29 °C. The metabolite volumetric production rates were mainly influenced by the incubation temperature and the viable counts of the LAB and yeasts in the grain inoculum, whereby the latter were not influenced by rinsing of the grains.

1 Introduction

Water kefir is a naturally fermented beverage with a fruity, slightly sweet, alcoholic, and acidic flavor (Gulitz *et al.*, 2013; Marsh *et al.*, 2013b; Laureys & De Vuyst, 2014; Chapter 3). The water kefir fermentation process is started by inoculating a mixture of water, sugar, and (dried) fruits with water kefir grains, followed by anaerobic incubation at room temperature, which usually lasts two to four days. At the end of the water kefir fermentation process, the water kefir grains are separated from the liquor by sieving. The liquor is used as a refreshing beverage. Part of the grains are reused to start the next fermentation process. The key microorganisms during water kefir fermentation are *Lactobacillus paracasei*, *Lactobacillus hilgardii*, *Lactobacillus nagelii*, and *Saccharomyces cerevisiae*, but other species of lactic acid bacteria (LAB), yeasts, bifidobacteria, and/or acetic acid bacteria (AAB) can be present (Laureys & De Vuyst, 2014; Chapters 3, 4, 5, 6, 7, 8, and 9; Laureys *et al.*, 2016).

Recently, the interest in water kefir has increased, as this beverage may offer health benefits to its consumers (Marsh *et al.*, 2014a). For example, one of the key microorganisms during water kefir fermentation, *Lb. paracasei*, may possess probiotic properties (Zagato *et al.*, 2014; Zavala *et al.*, 2016). Despite this increased interest, the commercial exploitation of water kefir beverages remains limited, partially because the water kefir fermentation process is still difficult to control (Chapter 5). To aquire greater control over the water kefir fermentation process, the impact of the most relevant process conditions needs to be investigated.

The backslopping time may have a pronounced influence on the species diversity during water kefir fermentation, as is also the case during sourdough fermentation (Vrancken *et al.*, 2011; De Vuyst *et al.*, 2014b). Long backslopping times will increase the acidic stress, may select for acid-tolerant microorganisms, and may impact the water kefir grain growth during fermentation (Chapter 7). In contrast, short backslopping times will reduce the acidic stress and may allow the growth of less acid-tolerant microorganisms, but may flush out slow-growing ones. The latter may be even more pronounced when the grains are rinsed before each backslopping step, as is often the case during water kefir fermentation processes. Rinsing of the water kefir grains may remove residual substrates and metabolites or even detach microorganisms from the grains. The former may result in low substrate and metabolite concentrations, less acidic stress, and a high water kefir grain growth (Chapter 7), whereas the latter may result in a slow water kefir fermentation process (Chapter 4). From an industrial point of view, rinsing of the water kefir grains before each backslopping step is not desirable, as it may result in a loss of substrates and metabolites, extra waste, and a low fermentation rate. However, rinsing of the water kefir grains before each backslopping step may select for only those microorganisms that are strongly attached onto the grains, while removing contaminating ones. Rinsing of the grains before each backslopping step may thus be necessary to maintain a stable water kefir microbiota, but this has not been investigated yet.

The incubation temperature likely exerts a large influence on the water kefir fermentation rate, as is also the case during milk kefir fermentation (Zajšek & Goršek, 2010b). A high incubation temperature will increase the fermentation rate, which is desirable from an industrial point of view. However, the incubation temperature may also affect the microbial species diversity and community dynamics during water kefir fermentation, as is the case during sourdough fermentation (Vrancken *et al.*, 2011; Bessmeltseva *et al.*, 2014; De Vuyst *et al.*, 2014b). Such a shift in the microbial communities may be reflected in the metabolite production. Additionally, the incubation temperature may directly affect the metabolism of certain microorganisms, as is the case for the production of lactic acid and acetic acid by *Lactobacillus casei* (Qin *et al.*, 2012), and the production of ethanol and glycerol by *S.*

cerevisiae (Yalcin & Ozbas, 2008). However, the influence of the temperature during water kefir fermentation has not been investigated yet.

This chapter aimed to determine the impact of the backslopping time, a rinsing of the water kefir grains before each backslopping step, and the incubation temperature on the microbial species diversity, substrate consumption, and metabolite production during the water kefir fermentation process. A modelling approach was used to allow a quantitative analysis of the effects of rinsing and temperature on the process characteristics.

2 Materials and methods

2.1 Water kefir grain inoculum and prefermentations

Two water kefir grain inocula were obtained one month apart from the household water kefir fermentation process described in Chapter 3. To obtain the necessary amount of water kefir grains, each inoculum was cultivated through a series of consecutive prefermentations through backslopping until > 1,300 g of water kefir grain wet mass was produced. The prefermentations were performed in glass 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 recultivated in fresh medium under the same conditions as mentioned above.

2.2 Fermentations

The first grain inoculum, obtained through the series of prefermentations mentioned above, was used to start eight series of water kefir fermentations differing in backslopping time and rinsing of the water kefir grains before each backslopping step. The backlopping times were 1 (fermentation series 1D-R and 1D-NR), 2 (2D-R and 2D-NR), 3 (3D-R and 3D-NR), or 4 d (4D-R and 4D-NR). For each backslopping time, one fermentation series was started with a rinsed grain inoculum, whereafter the water kefir grains were rinsed before each backslopping step (1D-R, 2D-R, 3D-R, and 4D-R). Another fermentation series was started with a non-rinsed grain inoculum, and neither were the water kefir grains rinsed before each backslopping step (1D-NR, 2D-NR, 3D-NR, and 4D-NR). Rinsing of the grains was performed with 2 l of tap water per 50 g of water kefir grains.

The second grain inoculum, also obtained through a series of prefermentations as mentioned above, was used to start eight series of water kefir fermentations differing in incubation temperature and backslopping time. The incubation temperatures were 18 (fermentation series 18C-3D and 18C-4D), 21 (21C-2D and 21C-3D), 25 (25C-2D and 25C-3D), or 29 °C (29C-1D and 29C-2D). The backslopping times were 1 (29C-1D), 2 (21C-2D, 25C-2D, and 29C-2D), 3 (18C-3D, 21C-3D, and 25C-3D), or 4 d (18C-4D). All water kefir fermentation series were started with a rinsed grain inoculum, and the water kefir grains were always rinsed before each backslopping step.

Each fermentation series was performed in independent biological triplicates. The fermentations were carried out in 250-ml glass bottles equipped with a water lock (PTFE). They were started with 10 g of sugar (Candico Bio), 5 g of dried figs (King Brand), 160 ml of tap water (Brussels, Belgium), and 50 g of a rinsed (R) or non-rinsed (NR) grain inoculum

(depending on the fermentation series). The bottles were incubated in a water bath at 21 °C unless stated otherwise (depending on the fermentation series). The contents of the bottles were mixed by gently turning them at the start and at the end of each backslopping step. After the respective backslopping time for each fermentation series, the backslopping practice was applied, whereby the water kefir grains were separated from the water kefir liquors by sieving and rinsed or not rinsed (depending on the fermentation series), whereafter 50 g of water kefir grains were recultivated in fresh medium 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 rinsed and non-rinsed water kefir grains of the first grain inoculum, for the non-rinsed water kefir grains of the second grain inoculum, and for the non-rinsed water kefir grains of all fermentation series at the end of backslopping step 8. The culture-dependent microbial species diversity of the LAB and yeasts was determined for the rinsed and non-rinsed water kefir grains of the first grain inoculum, for the non-rinsed water kefir grains of the second grain inoculum, and for the non-rinsed water kefir grains of all fermentation series at the end of backslopping step 8. The culture-independent microbial species diversity was determined for the water kefir liquors and the rinsed and non-rinsed water kefir grains of the first grain inoculum; for the water kefir liquors and the non-rinsed water kefir grains of the second grain inoculum; and for the water kefir liquors and non-rinsed water kefir grains of all fermentation series at the end of backslopping step 8. The substrate and metabolite concentrations in the liquors of all fermentation series were determined at the end of backslopping steps 1 and 8. The results are presented as the mean ± standard deviation of the three independent biological replicates performed for each fermentation series.

2.4 pH and water kefir grain wet and dry mass 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 in Chapter 7.

2.5 Microbial enumerations

The viable counts of the presumptive LAB were determined on de Man-Rogosa-Sharpe (MRS) agar medium, those of the presumptive yeasts on yeast extract-peptone-dextrose (YPD) agar medium, and those of the presumptive AAB on modified deoxycholate-mannitol-sorbitol (mDMS) agar medium, as described in Chapter 7.

2.6 Culture-dependent microbial species diversity analyses

The culture-dependent microbial species diversity in the water kefir liquors and on the water kefir grains was determined by randomly picking up 10 to 20 % of the total number of colonies from the respective agar media with 30 to 300 colonies. The isolates were subcultivated on their respective agar media until the third generation, which was used for dereplication via matrix-assisted laser desorption/ionization time-of-flight mass spectrometetry (MALDI-TOF MS) fingerprinting, as described in Chapter 7. The fingerprint peptide patterns obtained were clustered numerically by means of the BioNumerics software version 5.10 (Applied Maths, Sint-Martens-Latem, Belgium), as described in Chapter 7.

Representative bacterial isolates within each cluster were identified by sequencing part of their 16S rRNA gene from genomic DNA, and representative yeast isolates within each cluster were identified by squencing their 26S large subunit (LSU) rRNA gene and internal transcribed spacer (ITS) region from genomic DNA, as described in Chapter 3.

2.7 Exopolysaccharide production

All bacterial isolates were grown on MRS agar medium supplemented with 10 g l^{-1} of sucrose at 30 °C for 7 days to visually assess their exopolysaccharide (EPS) production capacity.

2.8 Culture-independent microbial species diversity analyses

The culture-independent microbial species diversity of bacteria and yeasts in the water kefir liquors and on the water kefir grains was determined after preparing total DNA extracts from the cell pellets of the water kefir liquors and 0.2 g of crushed water kefir grains, respectively, as described in Chapter 7. The culture-independent microbial community profiles were obtained by amplifying selected genomic fragments in the total DNA with the universal prokaryotic primer pair (V3), the LAB-specific primer pair (LAC), the *Bifidobacterium*-specific primer pair (Bif), and the universal eukaryotic primer pair (Yeast); and separating the PCR amplicons through denaturing gradient gel electrophoresis (DGGE), as described in Chapter 3. Selected bands of the community profiles were cut from the gels and identities were assigned through sequencing, as described in Chapter 3.

2.9 Substrate and metabolite concentration determinations

Samples for substrate and metabolite concentration analyses were prepared as described in Chapter 2. The concentrations of sucrose, glucose, fructose, glycerol, and mannitol were determined through high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), those of D- and L-lactic acid and acetic acid through high-performance liquid chromatography with ultraviolet detection (HPLC-UV), those of ethanol through gas chromatography with flame ionization detection (GC-FID), and those of the aroma compounds through static headspace gas chromatography with mass spectrometry detection (SH-GC-MS), as described in Chapter 7.

2.10 Statistics

An ANOVA was performed to test for differences between the eight fermentation series, followed by a series of post-hoc pairwise comparisons with Fisher's least significant difference (LSD) test, as described in Chapter 7. All statistical tests were performed in R 3.2.0 with a significance level of 0.05.

3 Kinetic model development

3.1 Model equations

The concentrations of ethanol [Eth], lactic acid [LA], acetic acid [AA], glycerol [Gly], and mannitol [Mtl] (g l^{-1}) during water kefir fermentation were described as a function of time, based on their initial concentrations $[Eth]_0$, $[LA]_0$, $[AA]_0$, $[Gly]_0$, and $[Mtl]_0$ (g l^{-1}) and their volumetric production rates k_{Eth}, k_{LA}, k_{AA}, k_{Gly}, and k_{Mtl} (g l^{-1} h^{-1}), as described in

Chapter 9. A general expression for each metabolite (P), taking its initial concentration ($[P]_0$) into account, was used:

$$[P] = [P]_0 + k\,t \qquad (1)$$

3.2 Influence of rinsing of the grains on the volumetric production rates and the initial concentrations of the metabolites

To compare the volumetric production rates between the water kefir fermentation processes started with rinsed or non-rinsed grains, a linear model was developed, whereby the initial metabolite concentrations ($[P]_0$) depended on the rinsing of the grains (Rinsing), and the volumetric production rates (Time) depended on the rinsing of the grains (Time:Rinsing), as follows:

P ~ Rinsing + Time + Time:Rinsing (2)

For the metabolites of which the volumetric production rates were not significantly different between the water kefir fermentation processes started with rinsed or non-rinsed grains (see Results), the interaction term could be removed:

P ~ Rinsing + Time (3)

For the metabolites of which the estimated initial concentrations were not significantly different between the water kefir fermentation processes started with rinsed or non-rinsed grains (see Results), the linear model could be further simplified, as follows:

P ~ Time (4)

3.3 Influence of the incubation temperature on the volumetric production rates of the metabolites

The volumetric production rates were assumed to be dependent on the temperature as described by the Arrhenius equation, wherein A is a pre-exponential factor (g l^{-1} h^{-1}), E_a the activation energy for the reaction (J mol^{-1}), R the universal gas constant (J mol^{-1} K^{-1}), and T the incubation temperature (K):

$$k = A\,e^{-Ea/(RT)} \qquad (5)$$

The metabolite concentration [P] as a function of time, as described above (equation 1), was extended with the Arrhenius equation to account for the incubation temperature.

To estimate the A and E_a values, a non-linear model was developed:

$$P \sim [P]_0 + A\,e^{-Ea/(RT)} * Time \qquad (6)$$

The calculation of the Q_{10}-values was based on the E_a values.

3.4 Fitting the models to the experimental data

The estimations of the biokinetic parameters were performed in R 3.2.0 and the results are presented as the mean ± standard error.

The initial concentrations and volumetric production rates for the production kinetics of the metabolites during the water kefir fermentation processes started with rinsed (fermentation series 1D-R, 2D-R, and 3D-R) or non-rinsed grains (1D-NR, 2D-NR, and 3D-NR) were estimated by fitting linear models to the linear portions of the experimental data (which was from 0 to 72 h of fermentation) at the end of backslopping step 1.

The parameters of the Arrhenius equations used to describe the influence of the temperature on the volumetric production rates were estimated by fitting non-linear models to the experimental data, according to Klicka & Kubácek (1997). Hereto, the initial concentrations of the metabolites were assumed to be similar to the estimated initial concentrations of the water kefir fermentation process started with rinsed water kefir grains and the experimental data were those at the end of backslopping step 1 of the two water kefir fermentation processes with different backslopping times performed for each incubation temperature.

4 Results

4.1 Water kefir grain wet and dry mass and pH

For the eight fermentation series differing in backslopping time and rinsing of the grains before each backslopping step, the water kefir grain growth was similar for all backslopping times, though slightly higher when the grains were rinsed before each backslopping step (Figure 1; Tables 1 and 2). This indicated that most of the water kefir grain wet mass was produced during the first 24 h of fermentation. The pH at the end of each backslopping step was low when the backslopping time was long and when the water kefir grains were not rinsed compared to when they were rinsed before each backslopping step (Figure 1; Tables 1 and 2).

For the eight fermentation series differing in incubation temperature and backslopping time, the water kefir grain growth was always similar (Figure 1; Tables 3 and 4). Furthermore, the pH at the end of each backslopping step was low when the backslopping time was long.

The water kefir grain dry mass was always around 13-17 % (m m^{-1}) and was high when the residual total carbohydrate concentrations were high (Tables 2 and 4).

4.2 Microbial enumerations

The viable counts of the yeasts on the rinsed and non-rinsed water kefir grains of the first grain inoculum and on the non-rinsed water kefir grains of the second grain inoculum were 7.6 ± 0.1, 7.7 ± 0.1, and 7.4 ± 0.1 log cfu g^{-1} of grains, respectively. Those of the LAB were 8.8 ± 0.1, 8.9 ± 0.1, and 8.4 ± 0.4 log cfu g^{-1} of grains, respectively. Rinsing of the grains did not significantly decrease their viable counts of yeasts and LAB. The viable counts of the AAB were below the limit of quantification for all water kefir grain inocula.

The viable counts of the yeasts on the grains at the end of backslopping step 8 were approximately 7.5 log cfu g^{-1} of grains for all fermentation series (Tables 2 and 4). Those of the LAB were around 8.5 log cfu g^{-1} of grains for all fermentation series (Tables 2 and 4). This resulted in relatively similar ratios of the viable counts of the LAB to those of the yeasts of approximately 10. The viable counts of the AAB on the grains were approximately 4.5 log cfu g^{-1} of grains for most water kefir fermentation series, but were significantly lower for the fermentation series 1D-NR and even much lower for the fermentation series 1D-R.

4.3 Culture-dependent microbial species diversity

The main yeasts and LAB found culture-dependently in the grain inocula were *S. cerevisiae*, *Dekkera bruxellensis*, *Lb. paracasei*, *Lb. hilgardii*, and *Lb. nagelii*. The communities of the yeasts and LAB were similar on the rinsed and non-rinsed water kefir grains of the first grain inoculum and on the non-rinsed grains of the second grain inoculum

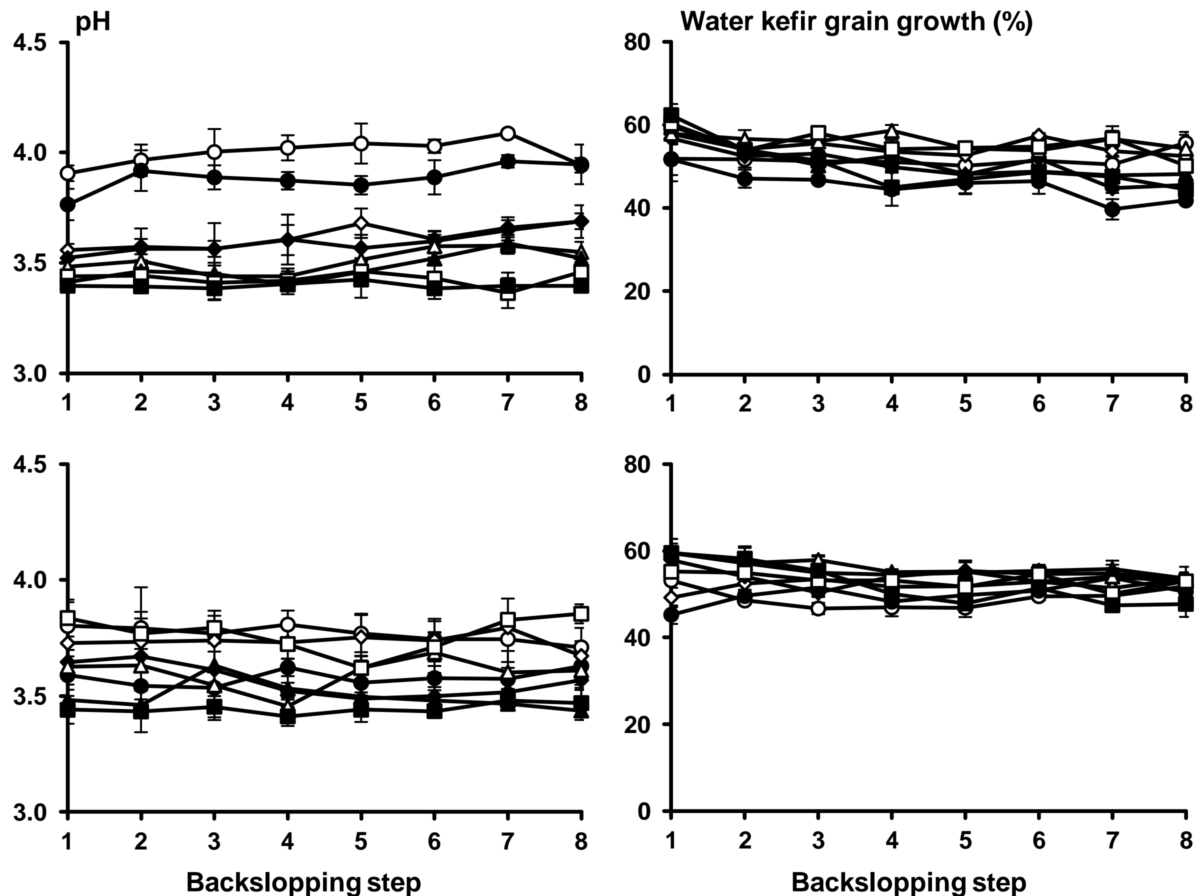

Figure 1. pH and water kefir grain growth at the end of each backslopping step for eight series of water kefir fermentations differing in backslopping time and rinsing of the grains before each backslopping step [backslopping time of 1 d with (1D-R, ○) or without rinsing (1D-NR, ●), backslopping time of 2 d with (2D-R, ◊) or without rinsing (2D-NR, ♦), backslopping time of 3 d with (3D-R, Δ) or without rinsing (3D-NR, ▲), and backslopping time of 4 d with (4D-R, □) or without rinsing (4D-NR, ■)] (top), and for eight series of water kefir fermentations differing in incubation temperature and backslopping time [incubation temperature of 17 °C with a backslopping time of 3 (17C-3D, ○) or 4 d (17C-4D, ●), incubation temperature of 21 °C with a backslopping time of 2 (21C-2D, ◊) or 3 d (21C-3D, ♦), incubation temperature of 25 °C with a backslopping time of 2 (25C-2D, Δ) or 3 d (25D-3D, ▲), and incubation temperature of 29 °C with a backslopping time of 1 (29C-1D, □) or 2 d (29C-2D, ■)] (bottom). C, temperature; D, days of backslopping; R, rinsed; NR, non-rinsed.

(Figure 2). These microorganisms were also found in all fermentation series at the end of backslopping step 8.

For the eight fermentation series differing in backslopping time and rinsing of the water kefir grains before each backslopping step, the relative abundances of *Lb. paracasei* and *D. bruxellensis* increased and those of *Lb. nagelii* and *S. cerevisiae* decreased with longer backslopping times (Figure 2). Furthermore, the relative abundances of *D. bruxellensis* were higher when the water kefir grains were not rinsed before each backslopping step. Additionally, *Leuconostoc pseudomesenteroides* was found in the series with a backslopping

Table 1. Characteristics of eight series of water kefir fermentations differing in backslopping time and rinsing of the grains between the backslopping steps [backslopping time of 1 d with (1D-R) or without rinsing (1D-NR), 2 d with (2D-R) or without rinsing (2D-NR), 3 d with (3D-R) or without rinsing (3D-NR), or 4 d with (4D-R) or without rinsing (4D-NR)] at the end of backslopping step 1. Significant differences ($p < 0.05$) between the series are indicated with different superscripts (a, b, c, d, e, and f). D, days of backslopping; R, rinsed; NR, non-rinsed.

Characteristic	1D-R	1D-NR	2D-R	2D-NR	3D-R	3D-NR	4D-R	4D-NR
Water kefir grain growth (%)	51.8 ± 3.9^{c}	51.7 ± 5.3^{c}	57.9 ± 2.1^{ab}	56.7 ± 1.3^{bc}	57.9 ± 2.6^{ab}	59.7 ± 2.2^{ab}	60.3 ± 1.7^{ab}	62.4 ± 2.7^{a}
pH	3.91 ± 0.04^{a}	3.77 ± 0.07^{b}	3.56 ± 0.03^{c}	3.52 ± 0.04^{cd}	3.48 ± 0.03^{de}	3.41 ± 0.03^{f}	3.44 ± 0.02^{ef}	3.40 ± 0.01^{f}
Sucrose (g l^{-1})	3.4 ± 0.9^{b}	4.3 ± 0.7^{a}	1.0 ± 0.2^{c}	1.0 ± 0.1^{c}	0.9 ± 0.3^{c}	1.1 ± 0.4^{c}	1.2 ± 0.2^{c}	0.9 ± 0.3^{c}
Glucose (g l^{-1})	4.4 ± 0.6^{a}	4.2 ± 0.7^{a}	1.8 ± 0.8^{b}	1.0 ± 0.4^{c}	0.4 ± 0.4^{cd}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}
Fructose (g l^{-1})	16.9 ± 2.2^{a}	16.4 ± 1.6^{a}	12.8 ± 4.2^{ab}	9.9 ± 1.1^{bc}	6.1 ± 4.1^{c}	1.8 ± 1.9^{d}	0.2 ± 0.1^{d}	0.1 ± 0.0^{d}
Total carbohydrates (g l^{-1})	24.7 ± 3.7^{a}	24.9 ± 2.6^{a}	15.6 ± 5.1^{b}	11.8 ± 1.4^{bc}	7.3 ± 4.7^{cd}	2.9 ± 2.3^{de}	1.4 ± 0.2^{e}	1.0 ± 0.3^{e}
Ethanol (g l^{-1})	6.0 ± 0.2^{f}	9.1 ± 0.9^{e}	11.7 ± 0.2^{d}	15.3 ± 0.8^{c}	15.5 ± 2.1^{c}	19.9 ± 0.9^{ab}	18.7 ± 0.2^{b}	21.5 ± 0.7^{a}
Lactic acid (g l^{-1})	1.04 ± 0.13^{e}	1.34 ± 0.11^{d}	1.89 ± 0.07^{c}	2.26 ± 0.12^{b}	2.35 ± 0.36^{b}	2.87 ± 0.13^{a}	2.91 ± 0.12^{a}	3.11 ± 0.05^{a}
Acetic acid (g l^{-1})	0.41 ± 0.01^{f}	0.56 ± 0.02^{e}	0.74 ± 0.03^{d}	0.92 ± 0.02^{c}	0.96 ± 0.10^{c}	1.15 ± 0.06^{b}	1.15 ± 0.04^{b}	1.30 ± 0.03^{a}
Glycerol (g l^{-1})	0.95 ± 0.09^{d}	1.16 ± 0.10^{cd}	1.60 ± 0.54^{bcd}	1.73 ± 0.22^{bc}	1.94 ± 0.49^{ab}	2.49 ± 0.82^{a}	1.88 ± 0.15^{abc}	2.60 ± 0.50^{a}
Mannitol (g l^{-1})	0.23 ± 0.11^{c}	0.19 ± 0.01^{c}	0.36 ± 0.11^{bc}	0.34 ± 0.07^{bc}	0.53 ± 0.18^{ab}	0.51 ± 0.14^{ab}	0.48 ± 0.09^{ab}	0.58 ± 0.17^{a}
2-Methyl-1-propanol (mg l^{-1})	4.4 ± 0.5^{f}	6.1 ± 0.3^{e}	7.5 ± 0.2^{d}	9.0 ± 1.0^{bc}	8.4 ± 1.4^{cd}	10.2 ± 0.3^{a}	9.7 ± 0.1^{ab}	10.7 ± 0.4^{a}
Isoamyl alcohol (mg l^{-1})	18.8 ± 1.5^{e}	25.9 ± 0.5^{d}	33.6 ± 0.7^{c}	38.8 ± 1.5^{b}	40.7 ± 3.2^{b}	43.8 ± 0.4^{a}	43.5 ± 0.8^{a}	45.9 ± 0.6^{a}
Ethyl acetate (mg l^{-1})	2.6 ± 0.3^{f}	5.8 ± 0.4^{e}	8.1 ± 0.9^{d}	11.0 ± 1.4^{c}	12.2 ± 0.9^{c}	15.9 ± 1.1^{b}	17.5 ± 2.2^{b}	20.8 ± 0.7^{a}
Isoamyl acetate (mg l^{-1})	0.073 ± 0.003^{d}	0.089 ± 0.006^{cd}	0.094 ± 0.004^{bc}	0.108 ± 0.015^{ab}	0.101 ± 0.018^{bc}	0.126 ± 0.012^{a}	0.111 ± 0.009^{ab}	0.123 ± 0.007^{a}
Ethyl hexanoate (mg l^{-1})	0.16 ± 0.01^{c}	0.17 ± 0.01^{bc}	0.18 ± 0.01^{ab}	0.20 ± 0.01^{a}	0.18 ± 0.01^{ab}	0.20 ± 0.01^{a}	0.19 ± 0.01^{a}	0.19 ± 0.01^{a}
Ethyl octanoate (mg l^{-1})	0.22 ± 0.04^{c}	0.26 ± 0.05^{c}	0.30 ± 0.09^{bc}	0.33 ± 0.09^{abc}	0.35 ± 0.06^{abc}	0.42 ± 0.10^{ab}	0.41 ± 0.09^{ab}	0.44 ± 0.08^{a}
Glycerol/ethanol (mmol/mol)	0.080 ± 0.009	0.064 ± 0.006	0.068 ± 0.022	0.056 ± 0.005	0.064 ± 0.020	0.062 ± 0.020	0.050 ± 0.004	0.060 ± 0.011
Lactic acid/ethanol (mmol/mol)	0.089 ± 0.009^{a}	0.076 ± 0.002^{c}	0.083 ± 0.001^{ab}	0.075 ± 0.001^{c}	0.078 ± 0.002^{bc}	0.074 ± 0.001^{c}	0.080 ± 0.003^{bc}	0.074 ± 0.003^{c}
Acetic acid/ethanol (mmol/mol)	0.052 ± 0.001^{a}	0.048 ± 0.003^{bc}	0.049 ± 0.002^{ab}	0.046 ± 0.002^{bc}	0.048 ± 0.003^{bc}	0.044 ± 0.001^{c}	0.047 ± 0.001^{bc}	0.047 ± 0.002^{bc}
Acetic acid/lactic acid (mmol/mol)	0.59 ± 0.06	0.63 ± 0.03	0.59 ± 0.03	0.61 ± 0.02	0.62 ± 0.04	0.60 ± 0.02	0.59 ± 0.04	0.63 ± 0.01
D-Lactic acid (% of total)	45.0 ± 3.6	43.9 ± 1.0	44.7 ± 0.6	45.0 ± 0.4	45.6 ± 0.3	45.8 ± 0.4	46.1 ± 0.2	45.9 ± 0.2

Table 2. Characteristics of eight series of water kefir fermentations differing in backslopping time and rinsing of the grains between the backslopping steps [backslopping time of 1 d with (1D-R) or without rinsing (1D-NR), 2 d with (2D-R) or without rinsing (2D-NR), 3 d with (3D-R) or without rinsing (3D-NR), and 4 d with (4D-R) or without rinsing (4D-NR)] at the end of backslopping step 8. Significant differences ($p < 0.05$) between the series are indicated with different superscripts (a, b, c, d, and e). D, days of backslopping; R, rinsed; NR, non-rinsed.

Characteristic	1D-R	1D-NR	2D-R	2D-NR	3D-R	3D-NR	4D-R	4D-NR
Yeasts (log cfu g^{-1})	7.6 ± 0.2^{ab}	7.5 ± 0.1^{bcd}	7.4 ± 0.1^{d}	7.5 ± 0.1^{bcd}	7.5 ± 0.1^{cd}	7.6 ± 0.1^{bc}	7.6 ± 0.1^{ab}	7.7 ± 0.1^{a}
Lactic acid bacteria (log cfu g^{-1})	8.6 ± 0.1	8.6 ± 0.1	8.4 ± 0.1	8.3 ± 0.1	8.5 ± 0.1	8.6 ± 0.1	8.5 ± 0.2	8.5 ± 0.2
Acetic acid bacteria (log cfu g^{-1})	BQL	2.8 ± 1.5^{b}	4.6 ± 0.2^{a}	4.6 ± 0.2^{a}	4.3 ± 0.2^{a}	4.6 ± 0.1^{a}	4.3 ± 0.9^{a}	4.0 ± 1.0^{ab}
Lactic acid bacteria/yeasts (cfu/cfu)	10.0 ± 4.9	13.3 ± 6.3	9.8 ± 1.9	7.3 ± 2.4	11.8 ± 1.1	11.8 ± 0.8	8.4 ± 3.8	6.4 ± 2.2
Water kefir grain growth (%)	55.7 ± 2.7^{a}	41.8 ± 1.0^{e}	52.5 ± 1.5^{ab}	45.6 ± 0.8^{de}	54.4 ± 3.3^{a}	48.2 ± 1.2^{cd}	50.2 ± 2.4^{bc}	44.5 ± 3.3^{de}
Water kefir grain dry mass (%)	15.6 ± 0.3^{c}	17.1 ± 0.1^{a}	15.5 ± 0.3^{c}	16.4 ± 0.1^{b}	14.9 ± 0.3^{d}	15.4 ± 0.2^{c}	14.4 ± 0.4^{e}	14.5 ± 0.3^{de}
pH	3.94 ± 0.03^{a}	3.95 ± 0.09^{a}	3.69 ± 0.04^{b}	3.69 ± 0.07^{b}	3.55 ± 0.05^{c}	3.52 ± 0.03^{c}	3.46 ± 0.06^{cd}	3.40 ± 0.02^{d}
Sucrose (g l^{-1})	2.4 ± 0.3^{c}	10.1 ± 0.6^{a}	1.6 ± 0.1^{d}	4.0 ± 0.4^{b}	1.5 ± 0.1^{d}	1.6 ± 0.1^{d}	1.4 ± 0.1^{d}	1.4 ± 0.1^{d}
Glucose (g l^{-1})	5.3 ± 0.2^{b}	6.4 ± 0.3^{a}	4.8 ± 0.8^{b}	5.5 ± 0.4^{b}	2.5 ± 0.2^{d}	3.4 ± 0.4^{c}	0.8 ± 0.9^{e}	0.6 ± 0.4^{e}
Fructose (g l^{-1})	23.8 ± 0.7^{a}	24.0 ± 1.6^{a}	23.3 ± 1.3^{ab}	23.7 ± 1.1^{a}	18.1 ± 1.2^{c}	19.5 ± 1.0^{bc}	10.2 ± 5.4^{d}	10.0 ± 2.5^{d}
Total carbohydrates (g l^{-1})	31.5 ± 0.6^{b}	40.5 ± 2.2^{a}	29.7 ± 2.0^{b}	33.2 ± 1.6^{b}	22.2 ± 1.5^{c}	24.4 ± 1.3^{c}	12.5 ± 6.4^{d}	12.0 ± 3.0^{d}
Ethanol (g l^{-1})	5.5 ± 0.1^{e}	5.2 ± 1.4^{e}	7.2 ± 1.0^{de}	7.9 ± 1.1^{de}	10.2 ± 0.9^{cd}	12.2 ± 0.5^{bc}	14.8 ± 3.1^{b}	20.1 ± 3.1^{a}
Lactic acid (g l^{-1})	0.87 ± 0.03^{e}	0.91 ± 0.21^{e}	1.25 ± 0.10^{d}	1.32 ± 0.08^{d}	1.68 ± 0.12^{c}	1.87 ± 0.04^{c}	2.20 ± 0.27^{b}	2.54 ± 0.31^{a}
Acetic acid (g l^{-1})	0.34 ± 0.01^{d}	0.37 ± 0.04^{d}	0.65 ± 0.08^{c}	0.65 ± 0.05^{c}	0.94 ± 0.06^{b}	0.93 ± 0.09^{b}	1.17 ± 0.11^{a}	1.17 ± 0.02^{a}
Glycerol (g l^{-1})	0.72 ± 0.04^{de}	0.61 ± 0.06^{e}	0.89 ± 0.11^{d}	0.84 ± 0.05^{de}	1.22 ± 0.07^{c}	1.27 ± 0.07^{c}	1.64 ± 0.27^{b}	2.02 ± 0.30^{a}
Mannitol (g l^{-1})	0.20 ± 0.08^{cd}	0.15 ± 0.10^{d}	0.31 ± 0.09^{cd}	0.16 ± 0.05^{d}	0.66 ± 0.21^{b}	0.35 ± 0.10^{c}	0.84 ± 0.02^{a}	0.57 ± 0.02^{b}
2-Methyl-1-propanol (mg l^{-1})	4.6 ± 0.6^{b}	3.9 ± 0.6^{b}	4.5 ± 0.7^{b}	4.3 ± 0.7^{b}	4.4 ± 0.7^{b}	4.8 ± 0.4^{b}	5.6 ± 2.1^{ab}	7.2 ± 1.2^{a}
Isoamyl alcohol (mg l^{-1})	14.2 ± 1.7^{e}	14.2 ± 0.1^{e}	20.8 ± 3.5^{cd}	20.0 ± 2.4^{de}	24.0 ± 3.6^{cd}	25.9 ± 1.1^{bc}	31.5 ± 6.6^{ab}	36.7 ± 3.6^{a}
Ethyl acetate (mg l^{-1})	2.6 ± 0.2^{d}	2.4 ± 0.8^{d}	6.4 ± 0.7^{c}	6.6 ± 0.6^{c}	13.4 ± 2.1^{a}	9.2 ± 2.0^{b}	13.6 ± 2.6^{a}	10.8 ± 0.6^{b}
Isoamyl acetate (mg l^{-1})	0.068 ± 0.001^{c}	0.066 ± 0.009^{c}	0.068 ± 0.003^{c}	0.069 ± 0.003^{c}	0.075 ± 0.006^{bc}	0.080 ± 0.007^{bc}	0.086 ± 0.015^{b}	0.110 ± 0.017^{a}
Ethyl hexanoate (mg l^{-1})	0.16 ± 0.01^{bc}	0.16 ± 0.02^{bc}	0.15 ± 0.01^{c}	0.16 ± 0.01^{bc}	0.17 ± 0.01^{bc}	0.18 ± 0.02^{bc}	0.18 ± 0.01^{b}	0.23 ± 0.03^{a}
Ethyl octanoate (mg l^{-1})	0.32 ± 0.13^{bc}	0.29 ± 0.06^{c}	0.34 ± 0.06^{bc}	0.38 ± 0.09^{bc}	0.47 ± 0.07^{abc}	0.51 ± 0.22^{abc}	0.54 ± 0.18^{ab}	0.65 ± 0.13^{a}
Glycerol/ethanol (mmol/mol)	0.066 ± 0.005^{a}	0.060 ± 0.009^{abc}	0.061 ± 0.001^{ab}	0.054 ± 0.004^{cd}	0.060 ± 0.002^{abc}	0.052 ± 0.001^{d}	0.056 ± 0.003^{bcd}	0.050 ± 0.004^{d}
Lactic acid/ethanol (mmol/mol)	0.082 ± 0.002^{abc}	0.090 ± 0.003^{a}	0.089 ± 0.008^{a}	0.086 ± 0.007^{ab}	0.084 ± 0.002^{abc}	0.078 ± 0.002^{bc}	0.077 ± 0.007^{c}	0.065 ± 0.004^{d}
Acetic acid/ethanol (mmol/mol)	0.047 ± 0.002^{bc}	0.056 ± 0.010^{abc}	0.071 ± 0.016^{a}	0.064 ± 0.013^{ab}	0.071 ± 0.012^{a}	0.059 ± 0.005^{abc}	0.062 ± 0.008^{abc}	0.045 ± 0.006^{c}
Acetic acid/lactic acid (mmol/mol)	0.58 ± 0.04^{c}	0.62 ± 0.09^{bc}	0.79 ± 0.12^{a}	0.74 ± 0.10^{ab}	0.84 ± 0.12^{a}	0.75 ± 0.06^{ab}	0.80 ± 0.04^{a}	0.70 ± 0.07^{abc}
D-lactic acid (% of total)	42.1 ± 0.3^{cd}	40.7 ± 2.0^{d}	42.7 ± 0.8^{bc}	44.2 ± 0.7^{ab}	44.7 ± 0.9^{a}	44.9 ± 0.6^{a}	45.7 ± 0.5^{a}	45.1 ± 0.3^{a}

BQL: below quantification limit.

Table 3. Characteristics of eight series of water kefir fermentations differing in incubation temperature and backslopping time [incubation temperature of 17 °C with a backslopping time of 3 d (17C-3D) or 4 d (17C-4D), 21 °C with a backslopping time of 2 d (21C-2D) or 3 d (21C-3D), 25 °C with a backslopping time of 2 d (25C-2D) or 3 d (25D-3D), and 29 °C with a backslopping time of 1 d (29C-1D) or 2 d (29C-2D)] at the end of backslopping step 1. Significant differences ($p < 0.05$) between the series are indicated with different superscripts (a, b, c, d, and e). C, temperature; D, days of backslopping.

Characteristic	17C-3D	17C-4D	21C-2D	21C-3D	25C-2D	25C-3D	29C-1D	29C-2D
Water kefir grain growth (%)	53.2 ± 0.8 ^{c}	45.3 ± 2.1 ^{e}	49.2 ± 2.2 ^{d}	57.9 ± 0.9 ^{ab}	59.7 ± 3.0 ^{a}	59.5 ± 3.3 ^{a}	55.2 ± 2.3 ^{bc}	59.5 ± 2.2 ^{a}
pH	3.80 ± 0.10 ^{a}	3.59 ± 0.04 ^{cd}	3.73 ± 0.14 ^{ab}	3.65 ± 0.05 ^{bc}	3.63 ± 0.05 ^{bc}	3.48 ± 0.05 ^{de}	3.84 ± 0.08 ^{a}	3.44 ± 0.06 ^{e}
Sucrose (g l^{-1})	1.7 ± 0.1	1.9 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.7 ± 0.1	1.9 ± 0.3	1.0 ± 0.8
Glucose (g l^{-1})	5.4 ± 1.0 ^{a}	2.7 ± 0.6 ^{c}	4.7 ± 1.0 ^{ab}	3.0 ± 0.5 ^{c}	3.5 ± 0.3 ^{bc}	0.8 ± 0.6 ^{d}	5.7 ± 0.5 ^{a}	0.5 ± 0.7 ^{d}
Fructose (g l^{-1})	24.5 ± 2.8 ^{ab}	18.2 ± 1.6 ^{c}	22.7 ± 2.2 ^{abc}	19.4 ± 0.7 ^{bc}	20.7 ± 0.8 ^{abc}	12.1 ± 3.9 ^{d}	24.9 ± 1.3 ^{a}	7.5 ± 6.9 ^{d}
Total carbohydrates (g l^{-1})	31.6 ± 3.9 ^{a}	22.8 ± 2.2 ^{b}	29.0 ± 3.3 ^{ab}	24.0 ± 1.1 ^{b}	25.7 ± 1.1 ^{ab}	14.6 ± 4.4 ^{c}	32.5 ± 2.0 ^{a}	9.0 ± 8.3 ^{c}
Ethanol (g l^{-1})	6.8 ± 0.8 ^{d}	10.8 ± 0.8 ^{b}	7.8 ± 1.4 ^{cd}	10.1 ± 0.5 ^{b}	9.3 ± 0.8 ^{bc}	14.0 ± 1.7 ^{a}	6.2 ± 0.8 ^{d}	14.6 ± 1.8 ^{a}
Lactic acid (g l^{-1})	1.11 ± 0.09 ^{d}	1.74 ± 0.13 ^{b}	1.33 ± 0.25 ^{cd}	1.67 ± 0.08 ^{b}	1.64 ± 0.06 ^{bc}	2.35 ± 0.30 ^{a}	1.13 ± 0.17 ^{d}	2.52 ± 0.19 ^{a}
Acetic acid (g l^{-1})	0.56 ± 0.03 ^{d}	0.81 ± 0.04 ^{c}	0.61 ± 0.05 ^{d}	0.81 ± 0.06 ^{c}	0.82 ± 0.03 ^{c}	1.16 ± 0.16 ^{a}	0.54 ± 0.04 ^{d}	1.02 ± 0.03 ^{b}
Glycerol (g l^{-1})	0.78 ± 0.05 ^{c}	1.07 ± 0.10 ^{b}	0.87 ± 0.12 ^{c}	1.08 ± 0.03 ^{b}	1.12 ± 0.06 ^{b}	1.49 ± 0.14 ^{a}	0.78 ± 0.03 ^{c}	1.46 ± 0.09 ^{a}
Mannitol (g l^{-1})	0.39 ± 0.01 ^{d}	0.60 ± 0.07 ^{b}	0.37 ± 0.03 ^{de}	0.52 ± 0.03 ^{bc}	0.51 ± 0.02 ^{c}	0.71 ± 0.07 ^{a}	0.31 ± 0.02 ^{e}	0.58 ± 0.04 ^{bc}
2-Methyl-1-propanol (mg l^{-1})	3.8 ± 0.2 ^{d}	4.8 ± 0.4 ^{cd}	4.8 ± 0.7 ^{cd}	5.3 ± 0.4 ^{bc}	4.9 ± 0.6 ^{cd}	6.4 ± 0.9 ^{b}	3.8 ± 0.7 ^{d}	8.0 ± 1.2 ^{a}
Isoamyl alcohol (mg l^{-1})	18.1 ± 2.0 ^{de}	25.7 ± 2.5 ^{bc}	21.2 ± 3.1 ^{cd}	27.0 ± 3.7 ^{b}	23.7 ± 2.5 ^{bc}	33.7 ± 3.8 ^{a}	16.1 ± 1.5 ^{e}	36.3 ± 3.1 ^{a}
Ethyl acetate (mg l^{-1})	4.1 ± 0.8 ^{c}	9.5 ± 2.3 ^{b}	4.9 ± 1.1 ^{c}	9.7 ± 0.4 ^{b}	8.5 ± 2.5 ^{b}	14.4 ± 1.4 ^{a}	3.2 ± 0.8 ^{c}	10.6 ± 0.6 ^{b}
Isoamyl acetate (mg l^{-1})	0.066 ± 0.005 ^{cd}	0.080 ± 0.006 ^{a}	0.072 ± 0.007 ^{abc}	0.073 ± 0.005 ^{abc}	0.068 ± 0.003 ^{bcd}	0.079 ± 0.005 ^{a}	0.061 ± 0.002 ^{d}	0.075 ± 0.002 ^{ab}
Ethyl hexanoate (mg l^{-1})	0.15 ± 0.01 ^{c}	0.17 ± 0.01 ^{a}	0.16 ± 0.01 ^{bc}	0.17 ± 0.01 ^{ab}	0.15 ± 0.01 ^{cd}	0.16 ± 0.01 ^{bc}	0.14 ± 0.01 ^{d}	0.15 ± 0.01 ^{c}
Ethyl octanoate (mg l^{-1})	0.38 ± 0.14	0.52 ± 0.13	0.33 ± 0.07	0.41 ± 0.11	0.30 ± 0.09	0.44 ± 0.07	0.25 ± 0.04	0.42 ± 0.01
Glycerol/ethanol (mmol/mol)	0.058 ± 0.003 ^{bc}	0.050 ± 0.003 ^{d}	0.056 ± 0.003 ^{bc}	0.054 ± 0.002 ^{cd}	0.060 ± 0.002 ^{ab}	0.053 ± 0.002 ^{cd}	0.063 ± 0.005 ^{a}	0.050 ± 0.003 ^{b}
Lactic acid/ethanol (mmol/mol)	0.084 ± 0.003	0.083 ± 0.008	0.087 ± 0.002	0.085 ± 0.002	0.090 ± 0.004	0.086 ± 0.003	0.093 ± 0.003	0.088 ± 0.005
Acetic acid/ethanol (mmol/mol)	0.064 ± 0.004 ^{ab}	0.057 ± 0.004 ^{bc}	0.061 ± 0.006 ^{abc}	0.062 ± 0.005 ^{abc}	0.068 ± 0.007 ^{a}	0.063 ± 0.001 ^{ab}	0.067 ± 0.004 ^{a}	0.054 ± 0.006 ^{c}
Acetic acid/lactic acid (mmol/mol)	0.76 ± 0.03 ^{a}	0.70 ± 0.02 ^{a}	0.70 ± 0.08 ^{a}	0.73 ± 0.04 ^{a}	0.76 ± 0.05 ^{a}	0.74 ± 0.03 ^{a}	0.72 ± 0.06 ^{a}	0.61 ± 0.03 ^{b}
D-lactic acid (% of total)	42.3 ± 0.9 ^{d}	42.0 ± 0.7 ^{d}	43.9 ± 1.0 ^{bc}	45.1 ± 0.6 ^{ab}	45.4 ± 0.4 ^{a}	45.8 ± 0.4 ^{a}	42.7 ± 1.3 ^{cd}	45.5 ± 0.6 ^{a}

Table 4. Characteristics of eight series of water kefir fermentations differing in incubation temperature and backslopping time [incubation temperature of 17 °C with a backslopping time of 3 d (17C-3D) or 4 d (17C-4D), 21 °C with a backslopping time of 2 d (21C-2D) or 3 d (21C-3D), 25 °C with a backslopping time of 2 d (25C-2D) or 3 d (25D-3D), and 29 °C with a backslopping time of 1 d (29C-1D) or 2 d (29C-2D)] at the end of backslopping step 8. Significant differences ($p < 0.05$) between the series are indicated with different superscripts (a, b, c, d, and e). C, temperature; D, days of backslopping.

Characteristic	17C-3D	17C-4D	21C-2D	21C-3D	25C-2D	25C-3D	29C-1D	29C-2D
Yeasts (log cfu g^{-1})	7.3 ± 0.1 ^{c}	7.6 ± 0.1 ^{a}	7.5 ± 0.1 ^{ab}	7.3 ± 0.1 ^{c}	7.4 ± 0.2 ^{bc}	7.3 ± 0.1 ^{bc}	7.5 ± 0.1 ^{ab}	7.4 ± 0.1 ^{bc}
Lactic acid bacteria (log cfu g^{-1})	8.5 ± 0.1	8.5 ± 0.2	8.5 ± 0.2	8.5 ± 0.1	8.5 ± 0.2	8.3 ± 0.2	8.4 ± 0.1	8.5 ± 0.1
Acetic acid bacteria (log cfu g^{-1})	5.0 ± 0.4	4.5 ± 0.4	5.1 ± 0.2	4.6 ± 0.1	5.0 ± 0.6	4.8 ± 0.5	4.6 ± 0.1	4.7 ± 0.2
Lactic acid bacteria/yeasts (cfu/cfu)	18.7 ± 2.4 ^{a}	8.6 ± 2.9 ^{b}	10.7 ± 4.2 ^{b}	18.8 ± 4.7 ^{a}	13.8 ± 6.5 ^{ab}	10.9 ± 3.1 ^{b}	8.0 ± 2.2 ^{b}	11.8 ± 2.1 ^{b}
Water kefir grain growth (%)	51.8 ± 3.1 ^{a}	50.6 ± 0.7 ^{ab}	53.6 ± 2.7 ^{a}	53.4 ± 1.6 ^{a}	52.9 ± 2.1 ^{a}	53.6 ± 1.2 ^{a}	53.0 ± 0.5 ^{a}	47.8 ± 3.0 ^{b}
Water kefir grain dry mass (%)	15.4 ± 0.5 ^{ab}	14.9 ± 0.3 ^{bc}	15.3 ± 0.3 ^{abc}	14.6 ± 0.4 ^{c}	14.8 ± 0.6 ^{bc}	13.8 ± 0.4 ^{d}	15.9 ± 0.4 ^{a}	15.0 ± 0.4 ^{bc}
pH	3.71 ± 0.08 ^{b}	3.63 ± 0.09 ^{bc}	3.67 ± 0.05 ^{bc}	3.57 ± 0.02 ^{cd}	3.61 ± 0.13 ^{bc}	3.44 ± 0.04 ^{e}	3.85 ± 0.04 ^{a}	3.47 ± 0.06 ^{de}
Sucrose (g l^{-1})	2.0 ± 0.2 ^{ab}	1.9 ± 0.1 ^{ab}	1.8 ± 0.1 ^{abc}	1.7 ± 0.1 ^{bc}	1.6 ± 0.1 ^{bc}	1.4 ± 0.1 ^{c}	2.2 ± 0.5 ^{a}	1.4 ± 0.5 ^{c}
Glucose (g l^{-1})	4.2 ± 1.6 ^{bc}	3.1 ± 1.1 ^{bcd}	4.5 ± 0.6 ^{b}	2.4 ± 0.5 ^{cde}	3.2 ± 2.0 ^{bcd}	1.0 ± 0.8 ^{e}	6.6 ± 0.7 ^{a}	1.6 ± 1.0 ^{de}
Fructose (g l^{-1})	22.5 ± 3.8 ^{ab}	20.3 ± 2.9 ^{ab}	23.9 ± 1.7 ^{ab}	18.0 ± 1.6 ^{bc}	19.9 ± 6.1 ^{b}	11.7 ± 4.3 ^{d}	26.3 ± 0.9 ^{a}	13.1 ± 3.9 ^{cd}
Total carbohydrates (g l^{-1})	28.6 ± 5.5 ^{ab}	25.3 ± 3.9 ^{b}	30.1 ± 2.3 ^{ab}	22.1 ± 2.1 ^{bc}	24.7 ± 8.2 ^{b}	14.0 ± 5.2 ^{c}	35.1 ± 2.0 ^{a}	16.1 ± 5.2 ^{c}
Ethanol (g l^{-1})	7.5 ± 2.4 ^{bcd}	9.6 ± 1.9 ^{bc}	6.5 ± 1.0 ^{cd}	10.5 ± 0.8 ^{b}	9.1 ± 3.0 ^{bc}	14.1 ± 2.3 ^{a}	4.9 ± 0.4 ^{d}	14.1 ± 1.5 ^{a}
Lactic acid (g l^{-1})	1.54 ± 0.35 ^{bcd}	1.59 ± 0.23 ^{bc}	1.42 ± 0.17 ^{cd}	1.92 ± 0.15 ^{b}	1.83 ± 0.36 ^{bc}	2.43 ± 0.34 ^{a}	1.08 ± 0.08 ^{d}	2.47 ± 0.26 ^{a}
Acetic acid (g l^{-1})	0.86 ± 0.15 ^{c}	0.86 ± 0.06 ^{c}	0.85 ± 0.09 ^{c}	1.07 ± 0.10 ^{b}	1.06 ± 0.10 ^{b}	1.36 ± 0.15 ^{a}	0.62 ± 0.03 ^{d}	1.13 ± 0.16 ^{b}
Glycerol (g l^{-1})	0.92 ± 0.21 ^{cd}	1.22 ± 0.29 ^{c}	0.91 ± 0.11 ^{cd}	1.26 ± 0.18 ^{bc}	1.26 ± 0.33 ^{bc}	1.61 ± 0.15 ^{ab}	0.80 ± 0.08 ^{d}	1.77 ± 0.19 ^{a}
Mannitol (g l^{-1})	0.86 ± 0.14 ^{bc}	0.64 ± 0.14 ^{c}	1.38 ± 0.28 ^{a}	1.18 ± 0.38 ^{ab}	1.40 ± 0.22 ^{a}	1.39 ± 0.31 ^{a}	0.66 ± 0.12 ^{c}	1.31 ± 0.28 ^{a}
2-Methyl-1-propanol (mg l^{-1})	3.6 ± 1.1 ^{c}	4.1 ± 0.6 ^{c}	3.6 ± 0.7 ^{c}	4.6 ± 0.4 ^{c}	4.6 ± 1.7 ^{c}	6.8 ± 1.6 ^{b}	3.8 ± 0.5 ^{c}	8.8 ± 1.6 ^{a}
Isoamyl alcohol (mg l^{-1})	19.4 ± 6.3 ^{cd}	22.4 ± 4.6 ^{cd}	17.8 ± 1.9 ^{cd}	24.1 ± 0.5 ^{bc}	22.4 ± 7.5 ^{cd}	32.5 ± 5.9 ^{a}	14.5 ± 1.9 ^{d}	31.4 ± 4.0 ^{ab}
Ethyl acetate (mg l^{-1})	8.4 ± 4.0 ^{b}	11.2 ± 2.7 ^{b}	8.4 ± 1.5 ^{b}	15.1 ± 1.3 ^{a}	9.9 ± 0.9 ^{b}	14.8 ± 0.5 ^{a}	3.5 ± 0.5 ^{c}	17.0 ± 2.0 ^{a}
Isoamyl acetate (mg l^{-1})	0.073 ± 0.007 ^{bcd}	0.073 ± 0.005 ^{bcd}	0.067 ± 0.003 ^{cd}	0.076 ± 0.004 ^{bc}	0.073 ± 0.008 ^{bcd}	0.091 ± 0.013 ^{a}	0.062 ± 0.002 ^{d}	0.082 ± 0.008 ^{ab}
Ethyl hexanoate (mg l^{-1})	0.17 ± 0.02	0.19 ± 0.02	0.15 ± 0.01	0.17 ± 0.01	0.15 ± 0.02	0.16 ± 0.02	0.13 ± 0.01	0.17 ± 0.02
Ethyl octanoate (mg l^{-1})	0.45 ± 0.17 ^{ab}	0.46 ± 0.17 ^{ab}	0.35 ± 0.10 ^{bc}	0.49 ± 0.11 ^{ab}	0.36 ± 0.05 ^{bc}	0.57 ± 0.03 ^{a}	0.24 ± 0.04 ^{c}	0.49 ± 0.09 ^{ab}
Glycerol/ethanol (mmol/mol)	0.063 ± 0.007	0.066 ± 0.026	0.071 ± 0.004	0.060 ± 0.009	0.070 ± 0.004	0.058 ± 0.005	0.081 ± 0.012	0.063 ± 0.004
Lactic acid/ethanol (mmol/mol)	0.107 ± 0.010 ^{a}	0.085 ± 0.005 ^{c}	0.113 ± 0.004 ^{a}	0.093 ± 0.001 ^{bc}	0.106 ± 0.017 ^{ab}	0.088 ± 0.002 ^{c}	0.113 ± 0.004 ^{a}	0.090 ± 0.003 ^{c}
Acetic acid/ethanol (mmol/mol)	0.090 ± 0.015 ^{abc}	0.070 ± 0.009 ^{d}	0.101 ± 0.005 ^{a}	0.078 ± 0.002 ^{bcd}	0.094 ± 0.021 ^{ab}	0.074 ± 0.004 ^{cd}	0.097 ± 0.008 ^{a}	0.062 ± 0.006 ^{d}
Acetic acid/lactic acid (mmol/mol)	0.84 ± 0.06 ^{a}	0.82 ± 0.07 ^{a}	0.90 ± 0.03 ^{a}	0.84 ± 0.02 ^{a}	0.88 ± 0.09 ^{a}	0.84 ± 0.03 ^{a}	0.86 ± 0.08 ^{a}	0.68 ± 0.04 ^{b}
D-lactic acid (% of total)	40.4 ± 1.7 ^{e}	42.2 ± 1.0 ^{de}	46.2 ± 1.4 ^{ab}	44.9 ± 1.0 ^{bc}	47.4 ± 0.5 ^{a}	45.9 ± 0.5 ^{ab}	43.9 ± 1.2 ^{cd}	46.0 ± 1.1 ^{ab}

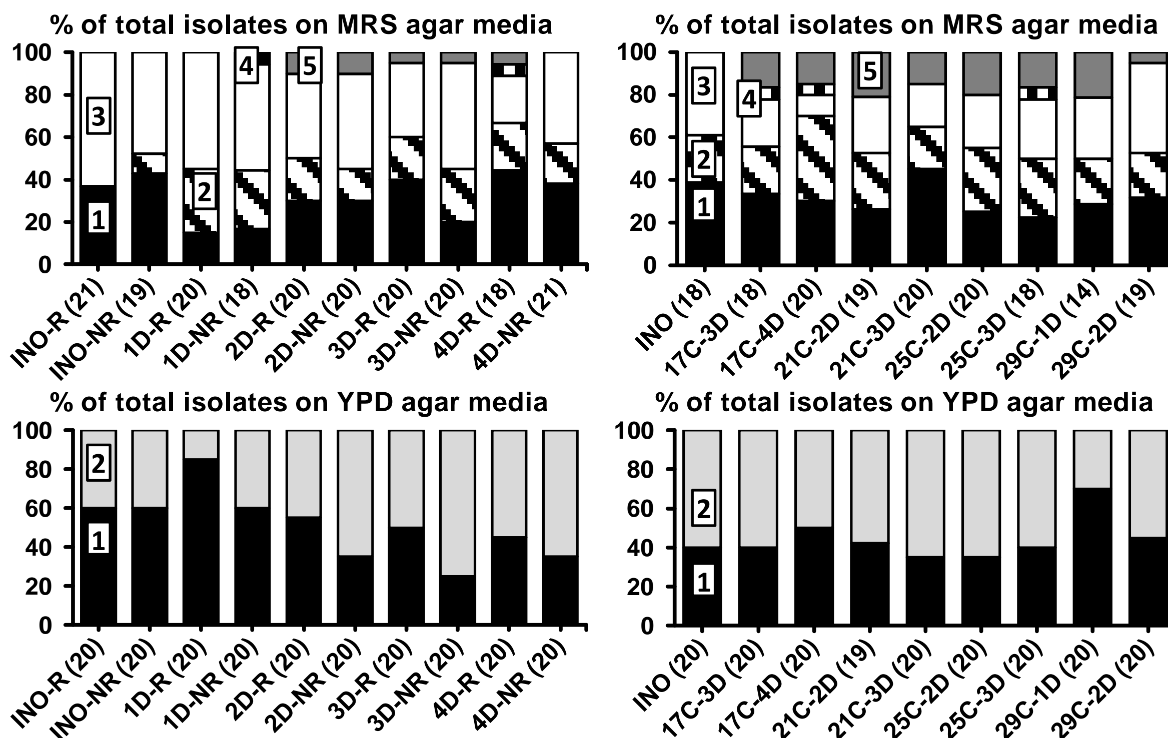

Figure 2. Culture-dependent species diversity of the rinsed (INO-R) and non-rinsed (INO-NR) grain inocula, of the non-rinsed grains of the eight series of water kefir fermentations differing in backslopping time and rinsing of the grains between each backslopping step, at the end of backslopping step 8 (left); and of the non-rinsed grain inoculum (INO) and the non-rinsed grains of the eight series of water kefir fermentations differing in incubation temperature and backslopping time, at the end of backslopping step 8 (right). The number of isolates are indicated between brackets. Isolates from MRS agar media: 1, *Lactobacillus paracasei* (100 % identity; GenBank accession no. AP012541); 2, *Lactobacillus hilgardii* (100 % identity; accession no. LC064898); 3, *Lactobacillus nagelii* (99 % identity; accession no. NR112754); 4, *Lactobacillus mali* (99 % identity; accession no. NR112691); and 5, *Leuconostoc pseudomesenteroides* (99 % identity; accession no. LC096220). Isolates from YPD agar media: 1, *Saccharomyces cerevisiae* [LSU (99 % identity; accession no. CP011558) and ITS (99 % identity; accession no. KC515374)]; and 2, *Dekkera bruxellensis* [LSU (99 % identity; accession no. GU291284) and ITS (99 % identity; accession no. FJ545249)]. LSU, large subunit rRNA gene; ITS, internal transcribed spacer. C, temperature; D, days of backslopping; R, rinsed; NR, non-rinsed.

time of 2 or 3 d and in the series with a backslopping time of 4 d, whereby the grains were rinsed before each backslopping step. *Lactobacillus mali* was found in the fermentation series with a backslopping time of 1 d without rinsing of the grains before each backslopping step and in the series with a backslopping time of 4 d with rinsing of the grains. All *Lb. mali* and *Leuc. pseudomesenteroides* strains and 40 % of the *Lb. hilgardii* strains produced EPS, whereby the proportion of EPS-producing *Lb. hilgardii* strains was similar for the eight fermentation series. Additionallly, 25 and 44 % of the *Lb. nagelii* strains from fermentation series 4D-R and 4D-NR, respectively, produced EPS.

For the eight fermentation series differing in incubation temperature and backslopping time, the relative abundances of *Lb. nagelii* increased as the temperature increased. The relative abundances of *D. bruxellensis* were low in the fermentation series with an incubation temperature of 29 °C and a backslopping time of 1 d (Figure 2). Additionally, *Leuc.*

pseudomesenteroides was found in all fermentation series. *Lactobacillus mali* was found in the fermentation series with an incubation temperature of 17 °C and a backslopping time of 3 or 4 d and in the fermentation series with an incubation temperature of 25 °C and a backslopping time of 3 d. All *Lb. mali* and *Leuc. pseudomesenteroides* strains and 51 % of the *Lb. hilgardii* strains produced EPS, whereby the proportion of EPS-producing *Lb. hilgardii* strains was similar for the eight fermentation series. Additionally, 20 % of the *Lb. nagelii* strains from the fermentation series 25C-3D produced EPS.

Strains of *Lb. mali* and *Lb. nagelii* produced EPS that remained localized around the colonies, whereas isolates of *Lb. hilgardii* and *Leuc. pseudomesenteroides* produced EPS that spread over the whole plate.

4.4 Culture-independent microbial species diversity

At the end of backslopping step 8, the rRNA-PCR-DGGE community profiles obtained with the four different primer pairs (V3, LAC, Bif, and Yeast) were similar for the three independent biological replicates performed for each fermentation series.

The main bands in the community profiles obtained with the four primer pairs for the grains and liquors of the inocula were attributed to *S. cerevisiae*, *D. bruxellensis*, *Lb. paracasei*, *Lb. hilgardii*, *Lb. nagelii*, and *Bifidobacterium aquikefiri* (Figure 3). The relative intensities of the bands attributed to *Lb. hilgardii* were higher and those attributed to *D. bruxellensis* and *Lb. paracasei* were lower when the grain inoculum was rinsed (INO-R) than when it was not rinsed (INO-NR). Further, the microorganisms found in the grains and liquors of the inocula were also found in all fermentation series at the end of backslopping step 8.

For the eight series of fermentations differing in backslopping time and rinsing before each backslopping step, the relative intensities of the bands attributed to *S. cerevisiae*, *Lb. nagelii*, and *Lb. hilgardii* decreased and those of the bands attributed to *D. bruxellensis*, *Lb. paracasei*, and a non-identified *Oenococcus* species increased when the backslopping time increased (Figure 3). Additionally, high relative intensities of the bands attributed to *Leuc. pseudomesenteroides* were found in the fermentation series with backslopping times of 2 or 3 d. When the water kefir grains were rinsed before each backslopping step, the relative intensities of the bands attributed to *D. bruxellensis* and *Lb. nagelii* were lower and those of the bands attributed to *Lb. hilgardii* and *Leuc. pseudomesenteroides* were higher than when the grains were not rinsed before each backslopping step. The partial 16S rRNA gene sequence of the non-identified *Oenococcus* species was 100 % identical to the sequence of an *Oenococcus* species (accession no. LT220205) found in water kefirs before (Chapters 4, 7, and 9).

For the eight series of fermentations differing in incubation temperature and backslopping time, the relative intensities of the bands attributed to *Lb. mali* decreased and those of the bands attributed to *Lb. nagelii* increased when the incubation temperature increased (Figure 3). The relative intensities of the bands attributed to *Leuc. pseudomesenteroides* were highest when the incubation temperature was 21 or 25 °C. For each incubation temperature, the relative intensities of the bands attributed to *Leuc. pseudomesenteroides* and *Lb. hilgardii* were lowest and those of the bands attributed to *D. bruxellensis* and *Lb. paracasei* were highest in the series with the longest backslopping time.

In general, the relative intensities of the bands attributed to *D. bruxellensis*, *Leuc. pseudomesenteroides*, *Lb. mali*, and the non-identified *Oenococcus* species were higher for the liquors, whereas those of the bands attributed to *Lb. hilgardii* were higher for the grains (Figure 3).

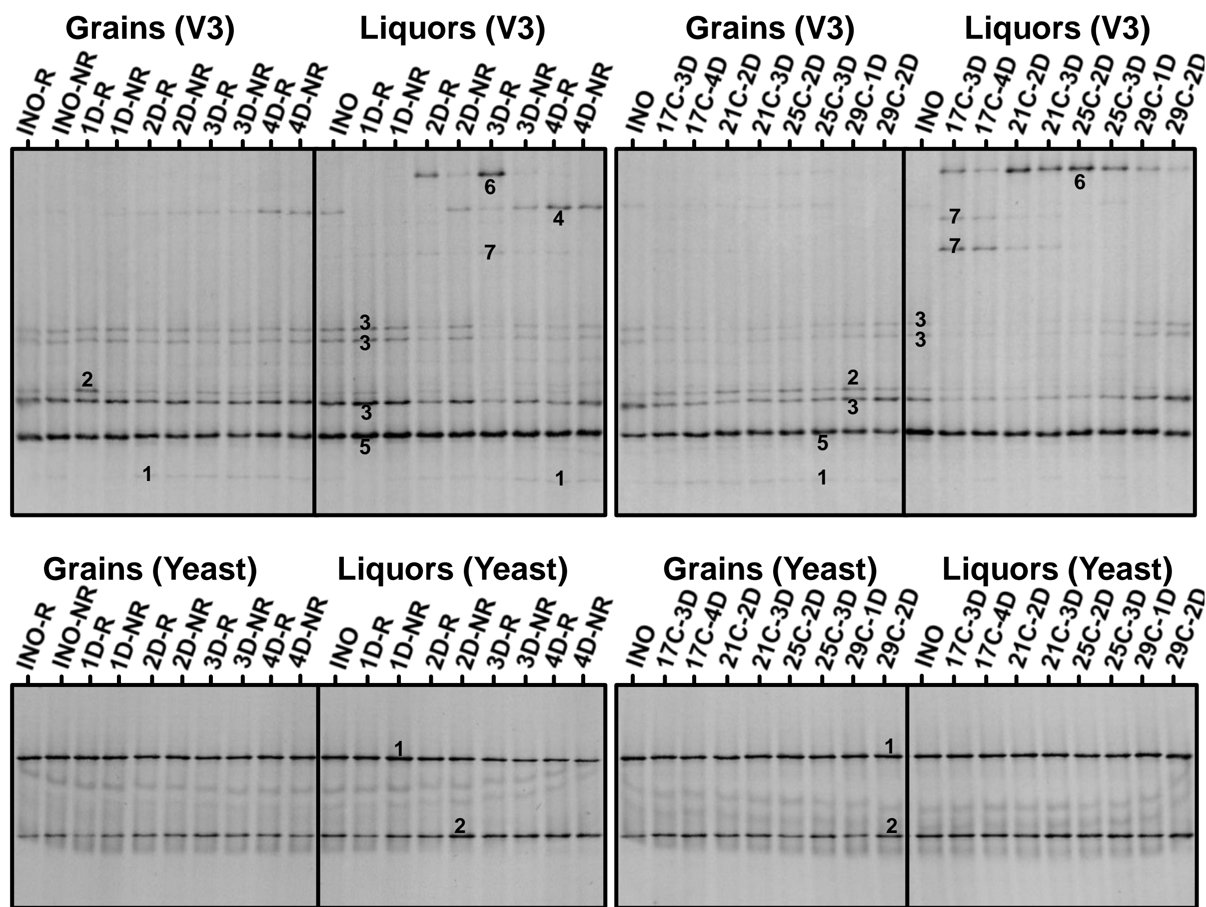

Figure 3. Culture-independent species diversity for the rinsed (INO-R) and non-rinsed (INO-NR) grains and the liquor of the grain inoculum (INO), and the non-rinsed grains and liquors of the eight series of water kefir fermentations differing in backslopping time and rinsing of the grains before each backslopping step at the end of backslopping step 8; and the non-rinsed grains (INO) and liquor of the grain inoculum (INO), and the non-rinsed grains and liquors of the eight series of water kefir fermentations differing in incubation temperature and backslopping time at the end of backslopping step 8. With the V3 primer pair: 1, *Lactobacillus casei*/*paracasei*/*zeae*/*rhamnosus* (99 % identity for all species; GenBank accession no. LC064894/AB289229/AB289313/JQ580982); 2, *Lactobacillus hilgardii/diolivorans* (100 % identity; accession no. LC064898/NR037004); 3, *Lactobacillus nagelii*/*ghanensis* (99 % identity; accession no. NR119275/NR043896); 4, *Oenococcus kitaharae* (97 % identity; accession no. NR041312); 5, *Bifidobacterium aquikefiri* (100 % identity; accession no. LN849254); 6, *Leuconostoc pseudomesenteroides* (99 % identity; accession no. LC096220); and 7, *Lactobacillus mali*/*hordei* (100 % identity; accession no. LC064888/NR044394). With the yeast primer pair: 1, *Saccharomyces cerevisiae* (100 % identity; accession no. NG042623); and 2, *Dekkera bruxellensis* (100 % identity; accession no. AY969049). C, temperature; D, days of backslopping; R, rinsed; NR, non-rinsed.

4.5 Substrate and metabolite concentrations

The concentrations of ethanol, glycerol, lactic acid, acetic acid, and aroma compounds were higher when the backslopping time was longer and when the water kefir grains were not rinsed before each backslopping step. In contrast, the concentrations of mannitol were higher when the grains were rinsed before each backslopping step. Overall, the ratios of the different

metabolites were not substantially impacted by the backslopping time, rinsing of the water kefir grains before each backslopping step, or the incubation temperature (Tables 1, 2, 3, and 4).

4.6 Kinetic models for the production of metabolites

4.6.1 Influence of rinsing of the water kefir grains on the volumetric production rates and the initial concentrations of the metabolites

The estimated volumetric production rates for each metabolite were not significantly different between the water kefir fermentation processes started with rinsed or non-rinsed grains (Table 5). This allowed to remove the interaction term from equation (2) of the linear model for all metabolites (equation 3). The estimated initial concentrations of ethanol, lactic acid, and acetic acid were significantly different between the water kefir fermentation processes started with rinsed or non-rinsed grains. The estimated initial concentrations of glycerol and mannitol were not significantly different between the water kefir fermentation processes started with rinsed or non-rinsed grains, and for these metabolites, the linear model was further simplified (equation 4). The initial concentrations and volumetric production rates obtained for the water kefir fermentation processes inoculated with rinsed or non-rinsed grains are illustrated in Figure 4. Overall, rinsing of the water kefir grains reduced the initial concentrations of the metabolites, but not the volumetric production rates for the production of these metabolites.

4.6.2 Influence of the incubation temperature on the volumetric production rates of the metabolites

For each metabolite, the values of A and E_a were estimated, and the estimated E_a values were used to calculate the Q_{10} values (Table 6). Furthermore, the estimated values of E_a and A for the production of ethanol were used to illustrate the applicability of the Arrhenius equation for ethanol production, and to illustrate the models for the concentrations of ethanol as a function of time at 17, 21, 25, and 29 °C (Figure 5). However, the effect of the inoculum

Table 5. The p-values for differences between the estimated values of the biokinetic parameters during the water kefir fermentation processes started with rinsed and non-rinsed grains; and the estimated initial concentrations and volumetric production rates for the production kinetics of ethanol ($[Eth]_0$ and k_{Eth}), lactic acid ($[LA]_0$ and k_{LA}), acetic acid ($[AA]_0$ and k_{AA}), glycerol ($[Gly]_0$ and k_{Gly}), and mannitol ($[Mtl]_0$ and k_{Mtl}) during the water kefir fermentation processes started with rinsed and non-rinsed grains. The results are presented as the mean ± standard error.

Parameter	p	Rinsed grain inoculum	Non-rinsed grain inoculum
$[Eth]_0$ (g l^{-1})	< 0.001	0.92 ± 0.52	4.61 ± 0.73
$[LA]_0$ (g l^{-1})	< 0.001	0.34 ± 0.09	0.74 ± 0.13
$[AA]_0$ (g l^{-1})	< 0.001	0.13 ± 0.03	0.31 ± 0.04
$[Gly]_0$ (g l^{-1})	0.164	0.48 ± 0.27	0.48 ± 0.27
$[Mtl]_0$ (g l^{-1})	0.609	0.05 ± 0.06	0.05 ± 0.06
k_{Eth} (mg l^{-1} h^{-1})	0.309	211 ± 13	211 ± 13
k_{LA} (mg l^{-1} h^{-1})	0.315	29.6 ± 2.3	29.6 ± 2.3
k_{AA} (mg l^{-1} h^{-1})	0.609	11.9 ± 0.7	11.9 ± 0.7
k_{Gly} (mg l^{-1} h^{-1})	0.501	24.2 ± 5.1	24.2 ± 5.1
k_{Mtl} (mg l^{-1} h^{-1})	0.892	6.5 ± 1.2	6.5 ± 1.2

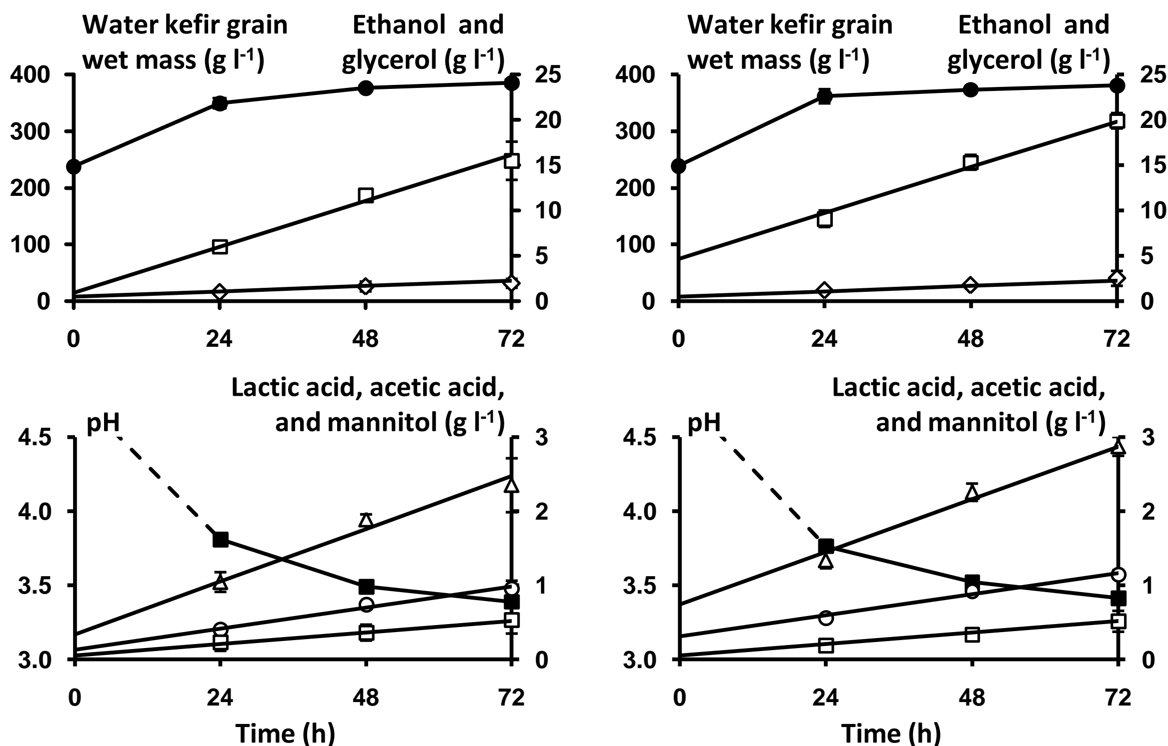

Figure 4. pH (■) and concentrations of water kefir grain wet mass (●), ethanol (□), glycerol (◊), lactic acid (Δ), acetic acid (○), and mannitol (□) as a function of time; as well as the model lines (solid lines) describing the concentrations of ethanol, glycerol, lactic acid, acetic acid, and mannitol during the first 72 h of fermentation of the water kefir fermentation series started with rinsed (left) or non-rinsed (right) grains.

could not be neglected. Indeed, the volumetric production rates of all metabolites at 21 °C calculated from the A and E_a values (131 mg l^{-1} h^{-1} for ethanol, 19.3 mg l^{-1} h^{-1} for lactic acid, 9.9 mg l^{-1} h^{-1} for acetic acid, 8.6 mg l^{-1} h^{-1} for glycerol, and 6.8 mg l^{-1} h^{-1} for mannitol) were lower than those reported for a similar fermentation performed at the same temperature but inoculated with a different inoculum (Table 5).

5 Discussion

Water kefir fermentation is usually performed at room temperature with a backslopping time of two to four days, whereby the water kefir grains are rinsed before each backslopping step (Pidoux, 1989; Waldherr *et al.*, 2010; Gulitz *et al.*, 2011, 2013; Laureys & De Vuyst, 2014; Chapters 3, 4, 5, 6, 7, 8, and 9). This chapter determined the short- and long-term influences of the backslopping time, rinsing of the water kefir grains before each backslopping step, and incubation temperature on the water kefir fermentation process.

Rinsing of the water kefir grains removed part of the metabolites from the grains, resulting in lower substrate and metabolite concentrations and higher pH values than when the grains were not rinsed. Rinsing of the grains did not remove substantial amounts of LAB or yeasts and did not decrease the volumetric metabolite production rates significantly. The volumetric metabolite production rates were strongly influenced by the viable counts of the LAB and yeasts in the grain inoculum, as they were higher during the water kefir

Table 6. Estimated values for the pre-exponential factors (A), the activation energies (E_a), and the Q_{10} values for the production kinetics of ethanol, lactic acid, acetic acid, glycerol, and mannitol during the water kefir fermentation processes started with rinsed grains. The results for A and E_a are presented as the mean ± standard error, and the results for the Q_{10} values are presented as the mean and the 95 % confidence interval.

Metabolite	A (mg l^{-1} h^{-1})	E_a (kJ mol^{-1})	Q_{10}
Ethanol	$(25.5 \pm 49.7) \cdot 10^{12}$	63.6 ± 4.8	2.37 [2.08; 2.69]
Lactic acid	$(113 \pm 242) \cdot 10^{12}$	71.9 ± 5.3	2.64 [2.30; 3.04]
Acetic acid	$(1.08 \pm 1.54) \cdot 10^{12}$	62.2 ± 3.5	2.32 [2.11; 2.55]
Glycerol	$(305 \pm 776) \cdot 10^{12}$	76.3 ± 6.3	2.81 [2.38; 3.32]
Mannitol	$(9.19 \pm 12.92) \cdot 10^{8}$	45.8 ± 3.4	1.86 [1.70; 2.04]

fermentation processes inoculated with non-rinsed grains of a grain inoculum with high viable counts of LAB and yeasts than during a similar fermentation process inoculated with non-rinsed grains of a grain inoculum with low viable counts of LAB and yeasts. This underlines the importance of the grain inoculum on the water kefir fermentation rate, confirming previous results (Chapters 4 and 9).

Short backslopping times resulted in low viable counts of AAB on the water kefir grains, which were even lower when the grains were rinsed before each backslopping step. Furthermore, rinsing of the grains before each backslopping step increased the relative abundances of *Lb. hilgardii* and *S. cerevisiae* (both associated with the water kefir grains), and decreased the relative abundances of *D. bruxellensis* and *Lb. nagelii* (both associated with the water kefir liquors) (Chapters 3 and 4).

Short backslopping times and rinsing of the grains before each backslopping step reduced the acidic stress, which impacted the microbial species diversity during the water kefir fermentation processes studied. Indeed, *Leuc. pseudomesenteroides* is sensitive to acidic stress (Ludwig *et al.*, 2009) and was less abundant when the backslopping times were long or when the water kefir grains were not rinsed before each backslopping step. In contrast, *Oenococcus* species are generally not sensitive to acidic stress (Alegría *et al.*, 2004) and the non-identified *Oenococcus* species was indeed present in higher relative abundances when the backslopping times were long and when the water kefir grains were not rinsed before each backslopping step. Furthermore, short backslopping times decreased the relative abundances of slow-growing microorganisms, as *D. bruxellensis* grows slower than *S. cerevisiae* (Schifferdecker *et al.*, 2014) and was present in low relative abundances when the backslopping times were short. The same mechanism may have caused the low relative abundances of *Leuc. pseudomesenteroides* at short backslopping times. The influence of the backslopping time is well-known for backslopped sourdough fermentation processes (De Vuyst *et al.*, 2014b).

When the incubation temperature increased, the relative abundances of *Lb. mali* decreased and those of *Lb. nagelii* increased. It is indeed known that the incubation temperature may influence the microbial species diversity during food fermentations, as encountered for example in backslopped sourdough fermentation processes (Meroth *et al.*, 2003; Vrancken *et al.*, 2011; De Vuyst *et al.*, 2014b). The relative abundances of *Leuc. pseudomesenteroides* were highest at intermediate incubation temperatures (21-25 °C), which is in agreement with the optimal growth temperature of *Leuconostoc* species of approximately 20-30 °C (Ludwig *et al.*, 2009) and the high relative abundance of particular *Leuconostoc* species at 23 °C during backslopped wheat sourdough fermentations (Vrancken *et al.*, 2011). The incubation

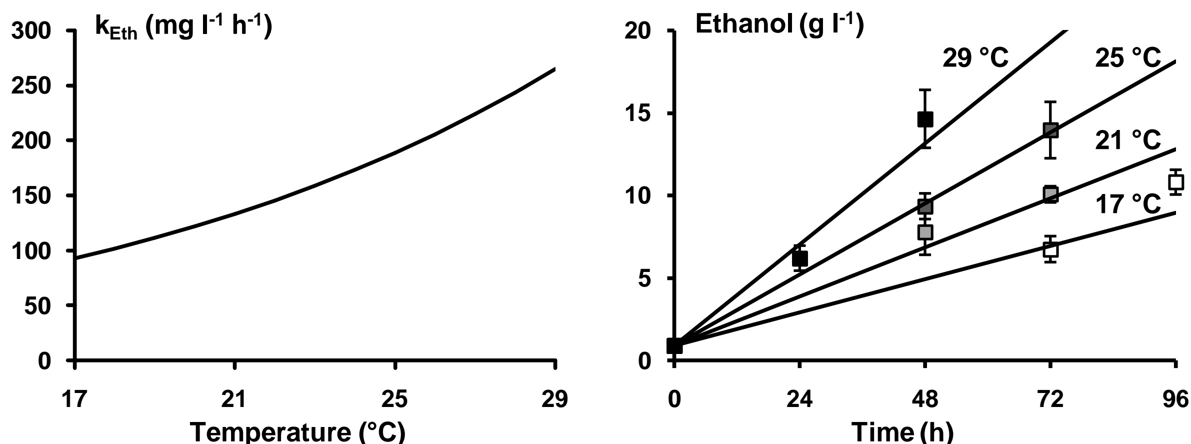

Figure 5. Arrhenius equation describing the volumetric production rates for the production of ethanol (k_{Eth}) as a function of the incubation temperature (left); the concentrations of ethanol after 72 and 96 h of incubation at 17 °C (□), after 48 and 72 h of incubation at 21 °C (■), after 48 and 72 h of incubation at 25 °C (■), and after 24 and 48 h of incubation at 29 °C (■) (right); and the model lines (solid lines) describing the concentrations of ethanol at incubation temperatures of 17 °C, 21 °C, 25 °C, and 29 °C (right).

temperature did not influence the yeast communities. This is in agreement with the optimal temperature of yeast growth (Kurtzman *et al.*, 2011).

Overall, a shift in the microbial communities did not substantially influence the concentrations of the different metabolites produced, except for mannitol. High concentrations of mannitol coincided with high relative abundances of *Lb. hilgardii*, an obligately heterofermentative LAB species (Ludwig *et al.*, 2009) that is able to reduce fructose into mannitol (Zaunmüller *et al.*, 2006).

Values of the pH higher than 3.4 ensured that the water kefir grain growth remained stable and high, as low pH values could decrease the water kefir grain growth (Chapter 7). The water kefir grain growth was slightly higher when the grains were rinsed before each backslopping step. This may be caused by the high pH values during these fermentation series, as the activity of glucansucrases is lower at low pH values (Waldherr *et al.*, 2010); or by the high relative abundances of *Lb. hilgardii* in these fermentation series, as this LAB species is thought to be responsible for the water kefir grain growth during fermentation (Pidoux, 1989; Waldherr *et al.*, 2010). Indeed, the main EPS-producing LAB species in the water kefir fermentation processes studied was *Lb. hilgardii*, but its abundance did not always correspond with the water kefir grain growth (Chapters 4, 7, and 8). Additionally, *Lb. mali* and *Leuc. pseudomesenteroides* produced EPS from sucrose, but these LAB species were probably not responsible for the water kefir grain growth, as they were more strongly associated with the water kefir liquors and their presence did not influence the water kefir grain growth. Furthermore, only a few *Lb. nagelii* strains from the fermentation series with the lowest pH values produced EPS from sucrose. This was in line with a previous report, where EPS-producing *Lb. nagelii* strains were found only in the water kefir fermentations with the lowest pH values (Chapter 4). This LAB species was not strongly associated with the grains and did not always produce EPS, indicating that it was probably not responsible for the water kefir grain growth.

The influence of the temperature on the volumetric production rates of ethanol, lactic acid, acetic acid, and glycerol was quantified by determining the parameters of the Arrhenius equation for each metabolite. The activation energy (E_a) for the production of ethanol during water kefir fermentation (Table 6) was similar to the E_a of 65 kJ mol^{-1} for the production of ethanol by *S. cerevisiae* (Ortiz-Muñiz *et al.*, 2010), the E_a of 69.5 kJ mol^{-1} for the production of ethanol by *D. bruxellensis* (Brandam *et al.*, 2007), and the E_a of 64.3 kJ mol^{-1} for the production of ethanol during milk kefir fermentation (Zajšek & Goršek, 2010b). The E_a for the production of lactic acid during water kefir fermentation (Table 6) was similar to the E_a of 71.5 kJ mol^{-1} for the production of lactic acid by *Lactobacillus delbrueckii* (a homofermentative LAB species) at pH 5.5 (Kempe *et al.*, 1956), the E_a of 77-79 kJ mol^{-1} for the production of lactic acid by *Lb. paracasei* at pH 6.0 (Adamberg *et al.*, 2003), and the E_a of 84.7 kJ mol^{-1} for the production of lactic acid by *Lactobacillus amylovorus* at pH 5.4 (Messens *et al.*, 2002).

In conclusion, rinsing of the water kefir grains before each backslopping step decreased the concentrations of metabolites and the relative abundances of liquor-associated microorganisms, and increased the water kefir grain growth and the relative abundances of grain-associated microorganisms. Short backslopping times decreased the relative abundances of slow-growing microorganisms, whereas long backslopping times decreased the relative abundances of acid-sensitive microorganisms. The microbial communities were also impacted by the incubation temperature. However, a shift in the microbial communities had only minor effects on the production of the different metabolites. The water kefir fermentation rate was mainly determined by the viable counts of the LAB and yeasts on the water kefir grain inoculum and by the incubation temperature, but not by rinsing of the water kefir grains.

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GENERAL DISCUSSION

When a water kefir fermentation process was started, the viable counts of the lactic acid bacteria (LAB) and yeasts in the water kefir liquors plateaued at approximately 7.0 and 6.5 log colony forming units (cfu) ml^{-1}, respectively, as soon as the water kefir grain inoculum was added, indicating no further growth. Also, the viable counts of the LAB and yeasts on the grains remained always at approximately 8.0 and 7.5 log cfu g^{-1}, respectively, indicating no further growth. The exact viable counts of the LAB and yeasts on the grains depended on the particular grain inoculum, whereas those in the liquors were less affected by the viable counts on the grain inoculum. The ratios of the viable counts of the LAB and yeasts on the grains to those in the liquors remained stable at around 10-100 during the entire course of a fermentation process, whereby the actual ratios depended on the particular grain inoculum. The absence of growth of the LAB and yeasts was explained by the much higher density of the microorganisms on the grains than in the liquors. When the grains were added to the fermentation medium, only a small part of the microorganisms detached from the grains into the liquor, whereby the overall density of the LAB and yeasts on the grains was not affected. The stable viable counts of the LAB and yeasts resulted in stable ratios of the viable counts of the LAB to those of the yeasts, both in the liquors and on the grains, during the entire course of a water kefir fermentation process. Overall, there were 2-10 LAB cells for each yeast cell, both in the liquors and on the grains. Although the ratios of the LAB to the yeasts remained stable, they increased when the buffer capacity of the water used for fermentation (and thus also the pH) increased or when the nutrient concentration (provided as dried figs) decreased. These characteristics of water kefir resemble the characteristics of milk kefir, a similar dairy-based fermented beverage (Kim *et al.*, 2015).

The LAB and yeasts were relatively strongly attached onto the water kefir grains, as rinsing of the grains did not decrease their viable counts. Consequently, the water kefir fermentation rate did not decrease upon rinsing of the grains, although the concentrations of the residual substrates and metabolites were lower, as a part of the residual substrates and metabolites were removed by the rinsing practice. When the amounts of grains and liquors were taken into account during a water kefir fermentation process, most of the microorganisms were always associated with the grains. Visualization of the microorganisms on the grains by scanning electron microscopy (SEM) revealed that the microorganisms were found onto the surface of the grains, but not inside. Furthermore, the LAB and yeasts were not structurally organized around each other. Some places were dominated by LAB cells and other places by yeast cells. These observations were in line with previous results from water kefir (Moinas *et al.*, 1980) and were also in line with results about the microbial colonization of milk kefir grains (Lu *et al.*, 2014).

The dry mass of the water kefir grains always increased at the start of the fermentation process due to the diffusion of carbohydrate substrates into the matrix of the water kefir grains. When the total residual carbohydrate concentrations were < 1 g l^{-1}, their dry mass was approximately 13 % (m m^{-1}). Their density was approximately 1.05 g ml^{-1}, which explained why they sank to the bottom of the fermentation bottles. Water kefir grains were brittle and broke easily, which explained why the the size of the water kefir grains decreased when the grain growth was low. When the water kefir grains were small, their viable counts were high. Small water kefir grains possess indeed a large specific surface and could thus harbor high counts of microorganisms, as the latter were mainly attached onto the surface of the grains. This explained why low grain growth was associated with a high fermentation rate. Similarly,

milk kefir grains desintegrate under unfavorable conditions, even though milk kefir grains are elastic and do not break easily (Nielsen *et al.*, 2014).

The composition and the production mechanism of water kefir grains differs from that of milk kefir grains. The water kefir grains of the present investigations were composed of glucose as the only monomer, which was conform with previous reports that water kefir grains are composed of dextran (Horisberger, 1969). The dextran of the water kefir grains is a homopolysaccharide assumed to be produced by *Lactobacillus hilgardii* (Waldherr *et al.*, 2010). In contrast, milk kefir grains are composed of kefiran, a heteropolysaccharide composed of glucose and galactose produced by *Lactobacillus kefiranofaciens* (Prado *et al.*, 2015). Nevertheless, the exact mechanism behind the production of kefir grain mass remains unclear and, at this moment, it is still impossible to recreate functional water or milk kefir grains by combining microorganisms isolated from them.

AAB were always present in water kefir, but their viable counts remained low as long as the fermentation was performed anaerobically. The persistent presence of these obligate aerobic microorganisms at low viable counts was explained by the periodic availability of either oxygen (at the start of the fermentation process) or ethanol (at the end of the fermentation process). The viable counts of the AAB increased indeed fast when the incubation was performed under aerobic conditions. In contrast with the LAB and yeasts (which were always predominantly associated with the grains), the AAB were predominantly associated with the liquors. Earlier studies report a great variability in the viable counts of the AAB between different water kefirs or different milk kefirs (Franzetti *et al.*, 1998; Gulitz *et al.*, 2011; Marsh *et al.*, 2013a). This high variability may be related with the presence or absence of oxygen during the fermentation process (Pothakos *et al.*, 2016). In both water kefir and milk kefir, successful fermentation was possible when the numbers of AAB were low, indicating that they are not essential for their fermentation processes. This is in contrast with the kombucha fermentation process, where AAB are always present in high numbers (Jayabalan *et al.*, 2014; Marsh *et al.*, 2014b). The presence of AAB in water kefir or milk kefir is probably undesirable, as they can produce high concentrations of acetic acid. Concentrations of acetic acid higher than 0.7 g l^{-1} are indeed not desired in wine due to its sharp acidic taste and aroma (Lambrechts & Pretorius, 2000). Additionally, growth of AAB in wine can decrease its fruity aroma (Bartowsky & Henschke, 2008). The presence of AAB is also undesired in most beers, with the exception of Belgian-style acidic ales, where AAB are present during the entire fermentation process (Spitaels *et al.*, 2014; Pothakos *et al.*, 2016). The proliferation of AAB in water kefir could be avoided by ensuring anaerobic conditions and by applying short backslopping times, whereby the grains were rinsed during backslopping.

No evidence was found for the presence of *Enterobacteriaceae*, *Enterococcus*, or *Streptococcus* during the fermentation processes started with four different inocula. This is in contrast with Belgian-style acidic ales and vegetable fermentations, where species of *Enterobacteriaceae* occur at the start of the fermentation process (Wouters *et al.*, 2013b; Spitaels *et al.*, 2014). Their absence during water and milk kefir fermentation can be explained by the inoculation of their fermentation process with a high amount of grain inoculum, which contains high numbers of microorganisms and substantial amounts of acids from the previous fermentation process, thereby rapidly acidifying the fermentation medium until < 4.0. In contrast, species of the genus *Streptococcus* and *Enterococcus* are often present in milk kefir (Marsh *et al.*, 2013a).

The key microorganisms of water kefir fermentation were *Lactobacillus paracasei*, *Lactobacillus hilgardii*, *Lactobacillus nagelii*, and *Saccharomyces cerevisiae*, as these microorganisms were always found in well-performing water kefir fermentation processes

and are also reported in the literature regularly (Waldherr *et al.*, 2010; Gulitz *et al.*, 2011, 2013; Marsh *et al.*, 2013b). Depending on the grain inoculum and the process conditions applied, other microorganisms could be present, such as the LAB species *Lactobacillus harbinensis*, *Lactobacillus mali*, *Lactobacillus satsumensis*, *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, and a not yet identified and thus probably novel *Oenococcus* species; the yeast species *Dekkera bruxellensis*, *Zygotorulaspora florentina*, *Dekkera anomala*, *Candida boidinii*, *Pichia membranifaciens*, *Wickerhamomyces anomalus*, and *Candida smithsonii*; the AAB species *Acetobacter fabarum*, *Acetobacter indonesiensis*, *Acetobacter orientalis*, *Gluconobacter cerinus*, *Gluconobacter japonicus*/*frateurii*, and *Gluconobacter roseus*/*oxydans*; the bifidobacterial species *Bifidobacterium aquikefiri*; and the *β*-proteobacterial species *Comamonas testosteroni*/*thiooxydans*. *Bifidobacterium aquikefiri* was originally detected only culture-independently, but was subsequently isolated from a water kefir fermentation process, characterized genotypically and phenotypically, and finally described as a novel species during the present study. A *Bifidobacterium* species with an identical 16S rRNA gene sequence was already found before in a water kefir from Germany (Gulitz *et al.*, 2013). As far as we know, *B. aquikefiri* has only been found in water kefir up to now and might be a water kefir-specific microorganism. A closely related *Bifidobacterium crudilactis* has been found in raw milk and raw milk products (Delcenserie *et al.*, 2007), and *Bifidobacteriaceae* have been found in several milk kefirs as well (Marsh *et al.*, 2013a). This substantiated the hypothesis that water kefir grains may originate from milk kefir grains instead of from the leaves of the *Opuntia* cactus, as has been stated before (Lutz, 1899).

Most of the LAB and yeasts found in water kefir are commonly associated with aquatic environments, as is the case for *Lb. nagelii* (wine), *Lb. hilgardii* (wine), *Lb. satsumensis* (rice wine), *Lb. mali* (cider), *Oenococcus* sp. (wine), *S. cerevisiae* (wine and beer), and *D. bruxellensis* (wine and beer). Although many aspects of water and milk kefirs are similar, their microbial species diversitiy differs substantially. For example, lactococci occur in high numbers in milk kefir (Dobson *et al.*, 2011), but were not found in the water kefirs of the present study. Only *S. cerevisiae* and *Leuc. mesenteroides* are found frequently in both water and milk kefirs. When water kefir grains are used to start a milk kefir fermentation process, the microbial species diversity indeed changes subtantially (Hsieh *et al.*, 2012).

The techniques used in the present study for investigating the microbial species diversity were at best semi-quantitative, due to the different biases of the different techniques. Certain of these results may be further investigated with more in-depth techniques such as quantitative PCR. Nevertheless, the different techniques used during this study complemented each other, and delivered valuable information about the water kefir microbial ecosystem. Overall, the microbial species diversity in the water kefir liquors was always more or less similar to that on the grains, although the relative abundances of the different species could differ between the grains and the liquors. Similar differences have also been found between the microbial species diversity analyses of milk kefir liquors and grains (Marsh *et al.*, 2013a). In the present study, the relative abundances of *Lb. hilgardii* and *S. cerevisiae* were consistently higher on the grains than in the liquors, indicating that these microorganisms were most strongly attached onto the grains. This was further confirmed by the increase of their relative abundances when the water kefir grains were rinsed during backslopping.

Certain process conditions substantially altered the microbial species diversity during backslopped water kefir fermentation processes. A low buffer capacity of the water used for fermentation (and thus low pH values) increased the relative abundances of *Lb. nagelii* and the non-identified *Oenococcus* species, whereas a high buffer capacity of the water (and thus high pH values) increased the relative abundances of *Leuc. pseudomesenteroides*. These results were consistent with the low acid tolerance of *Leuconostoc* species and the overall

higher acid tolerance of *Oenococcus* and *Lactobacillus* species (Ludwig *et al.*, 2009). Short backslopping times (and thus high pH values) increased the relative abundances of *Lb. nagelii* and *S. cerevisiae*, whereas long backslopping times increased the relative abundances of *Lb. paracasei*, the non-identified *Oenococcus* species, and *D. bruxellensis*. This could be explained by slow growth and high stress resistance of the latter two species compared to the former two ones (Dicks *et al.*, 1995; Schifferdecker *et al.*, 2014). When the water kefir grains were rinsed during backslopping, not only the relative abundances of *Lb. hilgardii* and *S. cerevisiae* increased, but also those of *Leuc. pseudomesenteroides*. The increase of the former two species could be explained by their strong attachment onto the water kefir grains, whereas that of the latter species could be explained by its short lag phase and low acid tolerance, as *Leuconostoc* species were not strongly associated with the grains. Similarly, *Leuconostoc* species dominate the first stage of vegetable fermentations due to their short lag phase, but disappear afterwards due to their acid sensitivity (Wouters *et al.*, 2013b). Rinsing of the grains indeed removed residual substrates and metabolites (such as organic acids) from the grains, reducing the acidic stress. On the long term, the relative abundances of *Lb. mali* increased at an incubation temperature of 17 °C, those of *Leuc. pseudomesenteroides* at 21-25 °C, and those of *Lb. nagelii* at 29 °C.

Low nutrient concentrations during fermentation increased the relative abundances of *Lb. hilgardii* and *D. bruxellensis*, whereas high nutrient concentrations increased those of *Lb. nagelii* and *S. cerevisiae*. Indeed, *D. bruxellensis* has lower nutrient requirements than *S. cerevisiae* (Uscanga *et al.*, 2000). Similarly, *Lb. hilgardii* probably has lower nutrient requirements than *Lb. nagelii*. Furthermore, stable water kefir fermentation processes were possible with dried figs, dried apricots, and raisins as a source of nutrients, but not with fresh figs or a solution of yeast extract and peptone (YP solution). This was in contrast with an earlier study, which reported that dried figs are necessary for water kefir fermentation, as they possess a water-soluble growth-promoting factor (Reiß, 1990). The microbial species diversity of water kefir fermentations carried out with dried apricots resembled that of fermentations carried out with dried figs, those carried out with raisins resembled that of fermentations with low nutrient concentrations, and those carried out with fresh figs or YP solution resembled that of fermentations with high nutrient concentrations. Low nutrient concentrations during water kefir fermentation resulted in a slow metabolism and pH decrease, which allowed the growth of *C. testosteroni/thiooxydans*. This obligate aerobic and acid-sensitive environmental microorganism did not occur in normal water kefir fermentation processes, as carbon dioxide produced by yeasts flushed out oxygen and created anaerobic conditions, while the pH decreased fast until below 4.0. The ingredients of water kefir fermentation may thus vary considerably, as long as sufficient but not excessive nutrients are available.

The most common AAB species were *Gl. roseus/oxydans*, *Ac. fabarum*, and *Ac. indonesiensis*, whereby *Ac. fabarum* was the most dominant species under aerobic conditions. *Gluconobacter japonicus/frateurii* was only found in water kefir fermentations with low nutrient concentrations. Aerobic fermentation conditions caused a decrease of the relative abundances of *B. aquikefiri*. This was probably caused by the high concentrations of acetic acid (produced by the AAB species) rather than the presence of oxygen, as *B. aquikefiri* was not sensitive to oxygen. Furthermore, the presence of *B. aquikefiri* in water kefir coincided with high concentrations of acetic acid. At this moment, it is not known if the presence of *B. aquikefiri* is desirable in water kefir, as acetic acid can contribute a harsh acidic flavor (Lambrechts & Pretorius, 2000).

Several LAB species, including *Lb. hilgardii*, *Leuc. pseudomesenteroides*, *Lb. nagelii*, *Lb. satsumensis*, and *Lb. mali* were able to produce EPS from sucrose. Nevertheless, *Lb. hilgardii*

was always the main EPS-producing LAB species. This LAB species is indeed assumed to be responsible for the water kefir grain growth during water kefir fermentation (Pidoux, 1989; Pidoux *et al.*, 1990; Waldherr *et al.*, 2010). The water kefir grain growth during fermentation varied widely, but was around 50 % under normal conditions. The grain inoculum used to start a water kefir fermentation process had a large influence on the grain growth during fermentation, but grain growth was not fixed and could change gradually over multiple backslopping steps. The mere presence of EPS-producing *Lb. hilgardii* strains was not sufficient for good water kefir grain growth and also the relative abundance of *Lb. hilgardii* did not directly influence the water kefir grain growth. However, excessive acidic stress during fermentation, whereby the pH decreased below 3.4, caused a decreasing water kefir grain growth, probably by inhibiting the production of glucansucrases by *Lb. hilgardii*. The optimal pH for the dextransucrase produced by *Lb. hilgardii* is indeed around 4.5 (Waldherr *et al.*, 2010). Excessive acidic stress during water kefir fermentation may result from an insufficient buffer capacity of the water used for fermentation, as was shown during this study. The calcium concentration of the water used for fermentation also impacted the water kefir grain growth, although less pronounced than the acidic stress, probably by activating and/or stabilizing the glucansucrases. Similarly, the production of kefiran by milk kefir microorganisms is high when the calcium concentrations are high (Yokoi & Watanabe, 1992). These findings about the water kefir grain growth during fermentation may be investigated into more detail with metatranscriptomic analyses.

Excessive nutrient concentrations could decrease the water kefir grain growth without increasing the acidic stress. This might be caused by a change of the microbial species diversity, as *Lb. nagelii* was more prevalent in water kefir with high nutrient concentrations than *Lb. hilgardii*. Alternatively, high nutrient concentrations may directly decrease the amount and size of the exopolysaccharides produced by LAB, as has already been found for *Streptococcus thermophilus* (Degeest & De Vuyst, 1999). Furthermore, the cultivation medium is indeed known to influence the ratio of the different glycosidic bonds in the exopolysaccharides produced by *Lb. hilgardii* (Pidoux *et al.*, 1988).

The water kefir grain growth suffered from substrate inhibition by sucrose at commonly used sucrose concentrations of around 60 g l^{-1}. This was in accordance with results obtained with a pure dextransucrase enzyme (Hehre, 1946). When the water kefir grain growth was low, more glucose remained available for the production of metabolites (such as organic acids), resulting in more acidic stress, which could cause a vicious circle of continuously decreasing water kefir grain growth. Maintaining optimal water kefir grain growth will therefore require a constant evaluation of the process parameters.

The main metabolites in the end-products of a water kefir fermentation process were ethanol (15-25 g l^{-1}), glycerol (1.5-2.5 g l^{-1}), lactic acid (1.5-3.5 g l^{-1}), acetic acid (0.5-1.5 g l^{-1}), and mannitol (0.5-1.0 g l^{-1}). In contrast to the production of water kefir grain wet mass, the metabolite production was not inhibited by sucrose concentrations up to 100 g l^{-1}. The majority of the metabolic activity during water kefir fermentation was associated with the grains, which was expected, as the majority of the microorganisms was also associated with the grains. This allowed adjusting the water kefir fermentation rate by changing the concentration of the grain inoculum. However, the influence of the concentration of the grain inoculum on the fermentation rate was less than expected, as substantial metabolic activity occured in the liquor. Therefore, the liquor could also be used as an alternative inoculum to start a water kefir fermentation process, which eliminated the need for and the production of water kefir grain mass. However, the production of ethanol, glycerol, lactic acid, and acetic acid proceeded at only half the rate of a similar process inoculated with water kefir grains. Moreover, the production of mannitol was almost absent in a fermentation process started

with a liquor inoculum. Nevertheless, further experimentation is needed to confirm if the use of liquor as an alternative inoculum is a viable strategy for the long-term production of water kefir, as only one backslopping step was performed during the present study. These results also indicated that mannitol was probably produced by *Lb. hilgardii*, as this microorganism was always more prevalent on the grains than in the liquors. This obligately heterofermentative LAB species can indeed increase its energy efficiency by reducing fructose into mannitol for redox balancing (Zaunmüller *et al.*, 2006). However, despite the high initial concentrations of free fructose, resulting from the production of glucan EPS and the higher preference of the water kefir microorganisms for glucose than fructose, only relatively low concentrations of mannitol were produced during water kefir fermentation.

The main aroma compounds were the higher alcohols 2-methyl-1-propanol, isoamyl alcohol, and 2-phenylethanol, and the esters ethyl acetate, isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate. The higher alcohols were always present in concentrations around their threshold values and are therefore not expected to greatly influence the aroma of the water kefir beverage. In contrast, the esters (except for ethyl acetate) were usually present in concentrations that exceeded their threshold values and are thus expected to substantially impact the aroma of water kefir beverages. These higher esters might be desirable in water kefir, as they can contribute fruity and flowery aromas to the beverage (Lambrechts & Pretorius, 2000). When the AAB proliferated under aerobic conditions, the concentrations of the higher esters decreased and those of ethyl acetate increased. This was in accordance with the decrease of the fruitiness of wine due to the growth of AAB (Bartowsky & Henschke, 2008).

Many aspects of the water kefir fermentation process were investigated in detail during the present study and resulted in technical knowledge that allows greater control over the fermentation process. For example, the results indicated that the low water kefir grain growth during the industrial water kefir production process studied could be attributed to the absence of viable EPS-producing *Lb. hilgardii* strains, low pH values during fermentation, low calcium concentrations of the water used for fermentation, and excessive nutrient concentrations and/or excessive sucrose concentrations during fermentation. The instability of this production process could be explained by the low water kefir grain growth during fermentation, which decreased the size of the water kefir grains. Nevertheless, no concrete information was obtained about what should constitute an optimal water kefir fermentation or water kefir beverage. To be able to produce tasty and healthy water kefir products that satisfy the contemporary consumers, the technical knowledge obtained during this study can be used to produce a variety of water kefir beverages, which will allow uncovering the preferences of the consumers regarding water kefir beverages.

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SUMMARY

During the present study, water kefir fermentation was investigated in detail. Lactic acid bacteria (LAB) and yeasts were always the main microorganisms during the fermentation process and their viable counts in the water kefir liquors and on the water kefir grains remained stable during the entire course of the water kefir fermentation process. The majority of the LAB and yeasts was always associated with the water kefir grains and only a small part of these microorganisms detached from the grains into the liquor when the fermentation process was started. The main LAB species that were found in all water kefir fermentations performed were *Lactobacillus hilgardii*, *Lactobacillus nagelii*, and *Lactobacillus paracasei*, and the main yeast species was *Saccharomyces cerevisiae*. Additionally, many other LAB and yeast species could be present, depending on the particular inoculum, the ingredients, and the process conditions. Acetic acid bacteria (AAB) were usually present in low numbers. The main microbial metabolites produced were always ethanol, glycerol, lactic acid, acetic acid, and mannitol. Additionally, many aroma compounds such as higher alcohols and esters were produced during water kefir fermentation. The main aroma compounds were ethyl hexanoate and ethyl octanoate, as the concentrations of these compounds in the water kefir liquors were much higher than their threshold concentrations.

During the fermentation processes, the water kefir grain wet mass usually increased. The microorganism that was assumed to be responsible for water kefir grain growth was *Lb. hilgardii*. This microorganism was indeed the most prevalent exopolysaccharide (EPS)-producing LAB species during the present investigations. However, the mere presence of EPS-producing *Lb. hilgardii* strains was not sufficient for good water kefir grain growth. Instead, the water kefir grain growth was determined by the inoculum, and could change gradually over multiple backslopping steps. The evolution of the water kefir grain growth could not be explained by that of the microbial species diversity. Excessive acidic stress (pH < 3.4) and insufficient calcium concentrations in the water resulted in a decreasing water kefir grain growth. The influence of the calcium concentrations on the water kefir grain growth could not be explained by acidic stress, as the pH decreased when the calcium concentrations increased. Furthermore, both insufficient and excessive nutrient concentrations could result in a decreased water kefir grain growth. Additionally, high sucrose concentrations could decrease the water kefir grain growth through substrate inhibition.

The production of water kefir grain wet mass might be considered as waste, as water kefir fermentation is usually performed to produce liquor for its use as a beverage. To control the water kefir grain growth, sucrose could be substituted partly with fructose and/or glucose. Glucose was the preferred substrate for the water kefir microorganisms, as it was consumed faster than fructose.

The water kefir grain dry mass was around 13 % (m m^{-1}) and the grain density was around 1.05 g cm^{-3}, which explained why the grains sank to the bottom of the fermentation bottles. The water kefir microorganisms were found predominantly on the surface of the grains, but the bacteria and yeasts were not structurally arranged around each other. Some places were predominated by bacterial cells and other places by yeast cells. Furthermore, water kefir grains were brittle and broke easily. So, when the water kefir grain growth was low, the water kefir grains became smaller continuously. Small water kefir grains possessed a large specific surface, which explained why small grains harbored more microorganisms than large grains. This in turn explained why the fermentation rate increased when the water kefir grain growth decreased.

The water kefir fermentation rate could be controlled by adding more or less water kefir grains, as the majority of the microorganisms was present on the grains. Nevertheless,

substantial metabolic activity occurred in the liquor too. Moreover, it was possible to start a water kefir fermentation with water kefir liquor instead of water kefir grains, which would eliminate the need for and the production of grains. However, when a water kefir fermentation process was started with liquor, the fermentation rate was much lower than when it was started with grains. The fermentation rate could also be controlled by the incubation temperature, as increasing incubation temperatures resulted in increasing fermentation rates. On the long term, the incubation temperature also impacted the microbial species diversity, whereby the relative abundances of *Lactobacillus mali* increased at low temperatures, those of *Leuconostoc* increased at intermediate temperatures, and those of *Lb. nagelii* increased at high temperatures. Rinsing of the water kefir grains during backslopping did not remove the microbial cells, and therefore did not decrease the fermentation rate. However, rinsing of the grains removed part of the substrates and metabolites from the grains, resulting in overall lower total residual carbohydrate and metabolite concentrations. Furthermore, rinsing of the grains resulted in increased relative abundances of *Lb. hilgardii* and *S. cerevisiae*, probably because these microorganisms were attached most strongly onto the water kefir grains.

The relative abundances of *Lb. hilgardii* and *Dekkera bruxellensis* increased at low nutrient concentrations, whereas those of *Lb. nagelii* and *S. cerevisiae* increased at high nutrient concentrations. Stable water kefir fermentation was not only possible with dried figs, but also with raisins and dried apricots. In contrast, water kefir fermentation with fresh figs or a solution of yeast extract and peptone was not stable, as this resulted in decreasing water kefir grain growth. Low nutrient concentrations resulted in slow fermentations and high nutrient concentrations resulted in fast fermentations. The very low fermentation rate when insufficient nutrients were present allowed a *Comamonas* species to grow. This microorganism is obligately aerobic and acid-sensitive, and is not expected to be a problem during common water kefir fermentation processes. When oxygen was present during water kefir fermentation, AAB proliferated. This resulted in high acetic acid concentrations, which might be undesirable.

The findings mentioned above indicated that the low water kefir grain growth during the industrial water kefir production process studied could be attributed to the abscense of EPS-producing *Lb. hilgardii* strains, low pH values, low calcium concentrations, high nutrient concentrations, and/or excessive sucrose concentrations during fermentation. The instability of this industrial production process could be explained by the low water kefir grain growth during fermentation, which decreased the size of the water kefir grains.

SAMENVATTING

Gedurende dit onderzoek werd waterkefirfermentatie in detail onderzocht. Melkzuurbacteriën (MZB) en gisten waren altijd de meest prevalente micro-organismen tijdens het fermentatieproces en hun celaantallen in de waterkefirvloeistof en op de waterkefirkorrels bleven stabiel gedurende het volledige verloop van een waterkefirfermentatieproces. Het merendeel van de MZB en gisten was altijd geassocieerd met de waterkefirkorrels en slechts een klein deel van deze micro-organismen kwam los van de korrels en in de vloeistof terecht bij de start van het fermentatieproces. De meest prevalente MZB-soorten in alle uitgevoerde waterkefirfermentaties waren *Lactobacillus hilgardii*, *Lactobacillu nagelii* en *Lactobacillus paracasei* en de meest prevalente gistsoort was *Saccharomyces cerevisiae*. Daarbovenop konden vele andere MZB- en gistsoorten aanwezig zijn, afhankelijk van het specifieke inoculum, de ingrediënten en de procescondities. Azijnzuurbacteriën (AZB) waren gewoonlijk aanwezig in lage celaantallen. De meest prevalente metabolieten die geproduceerd werden tijdens waterkefirfermentatie waren ethanol, glycerol, melkzuur, azijnzuur en mannitol. Daarnaast werden ook vele aromacomponenten geproduceerd, zoals hogere alcoholen en esters. De voornaamste aromacomponenten waren ethylhexanoaat en ethyloctanoaat omdat hun concentraties in de vloeistof veel hoger waren dan hun drempelwaarden.

De natte massa waterkefirkorrels nam gewoonlijk toe tijdens het fermentatieproces. Er werd verondersteld dat *Lb. hilgardii* hiervoor verantwoordelijk was. Dit micro-organisme was inderdaad de meest voorkomende exopolysacharide (EPS)-producerende MZB-soort tijdens deze studie. Echter, de loutere aanwezigheid van EPS-producerende stammen van *Lb. hilgardii* was niet voldoende voor goede waterkefirkorrelaangroei. De waterkefirkorrelaangroei werd wel bepaald door het specifieke inoculum en kon gradueel veranderen over meerdere terugfermentatiestappen. De evolutie van de waterkefirkorrelaangroei kon niet verklaard worden door een verandering van de microbiële soortdiversiteit. Excessieve zuurtestress ($pH < 3.4$) en onvoldoende calcium in het water resulteerde in een dalende waterkeforkorrelaangroei. De invloed van de calciumconcentratie op de waterkefirkorrelaangroei kon niet verklaard worden door zuurtestress, omdat de pH daalde wanneer de calciumconcentratie steeg. Daarnaast konden onvoldoende en excessieve nutriëntenconcentraties ook resulteren in een verlaagde waterkefirkorrelaangroei. Daarbovenop konden hoge sucroseconcentraties de waterkefirkorrelaangroei doen dalen door substraatinhibitie.

De productie van natte massa waterkefirkorrels kan beschouwd worden als een afvalstroom, want waterkefirfermentatie wordt gewoonlijk uitgevoerd om vloeistof te produceren die gebruikt kan worden als drank. Om de waterkefirkorrelaangroei te controleren kon sucrose gedeeltelijk gesubstitueerd worden door fructose en/of glucose. Glucose was het geprefereerde substraat voor de waterkefirmicro-organismen omdat het sneller werd geconsumeerd dan fructose.

De droge massa waterkefirkorrels bedroeg ongeveer 13 % ($m\ m^{-1}$) en de densiteit van de korrels was ongeveer 1.05 $g\ cm^{-3}$, hetgeen verklaarde waarom de korrels naar de bodem van de fermentatie zakten. De waterkefirmicro-organismen waren vooral aanwezig op het oppervlak van de waterkefirkorrels, maar de bacteriën en gisten waren niet structureel rond elkaar georganiseerd. Sommige plaatsen op de korrels werden gedomineerd door bacteriële cellen en andere door gistcellen. Verder waren de waterkefirkorrels ook broos en braken ze gemakkelijk. Dus wanneer de waterkefirkorrelaangroei laag was, werden de korrels geleidelijk aan kleiner. Kleine waterkefirkorrels bezaten een groter specifiek oppervlak, hetgeen verklaarde waarom kleine korrels meer micro-organismen bevatten dan grote korrels.

Dit verklaarde ook waarom de fermentatiesnelheid steeg wanneer de waterkefirkorrelaangroei daalde.

De waterkefirfermentatiesnelheid kon gecontroleerd worden door meer of minder waterkefirkorrels toe te voegen, want de meerderheid van de micro-organismen was geassocieerd met de korrels. Desalniettemin vond er ook substantiële metabolische activiteit plaats in de vloeistof. Het was bovendien mogelijk om een waterkefirfermentatie te starten met waterkefirvloeistof in plaats van waterkefirkorrels, waardoor de nood voor en de productie van korrels geëlimineerd zou worden. Echter, de fermentatiesnelheid was lager wanneer een waterkefirfermentatieproces gestart werd met vloeistof dan wanneer het gestart werd met korrels. De fermentatiesnelheid kon ook gecontroleerd worden via de incubatietemperatuur, want stijgende incubatietemperaturen resulteerden in stijgende fermentatiesnelheden. Op lange termijn had de incubatietemperatuur ook een impact op de microbiële soortdiversiteit, waarbij de relatieve hoeveelheid *Lactobacillus mali* steeg bij lage temperaturen, deze van *Leuconostoc* steeg bij gemiddelde temperaturen en deze van *Lb. nagelii* steeg bij hoge temperaturen. Spoelen van de waterkefirkorrels tijdens terugfermentatie verwijderde geen significante aantallen microbiële cellen en deed de fermentatiesnelheid dus ook niet dalen. Echter, spoelen van de korrels verwijderde wel een deel van de substraten en metabolieten van de korrels, waardoor de uiteindelijke concentraties van de totale residuele koolhydraten en metabolieten verlaagde. Spoelen van de korrels resulteerde verder in een hogere relatieve hoeveelheid *Lb. hilgardii* en *S. cerevisiae*, waarschijnlijk omdat deze micro-organismen het sterkst met de waterkefirkorrels geassocieerd waren.

De relatieve hoeveelheden *Lb. hilgardii* en *Dekkera bruxellensis* stegen bij lage nutriëntenconcentraties, terwijl deze van *Lb. nagelii* en *S. cerevisiae* stegen bij hoge nutriëntenconcentraties. Stabiele waterkefirfermentatie was niet alleen mogelijk met gedroogde vijgen, maar ook met rozijnen en gedroogde abrikozen. Daarentegen was waterkefirfermentatie met verse vijgen of met een oplossing van gistextract en pepton niet stabiel, want dit resulteerde in een dalende waterkefirkorrelaangroei. Lage nutriëntenconcentraties resulteerden in trage fermentaties en hoge nutriëntenconcentraties in snelle fermentaties. De lage fermentatiesnelheid bij lage nutriëntenconcentraties liet de groei van een *Comamonas*-soort toe. Dit micro-organisme is obligaat aeroob en zuurgevoelig, waardoor geen problemen verwacht worden in normale waterkefirfermentaties. Wanneer zuurtstof aanwezig was tijdens de waterkefirfermentaties, konden AZB uitgroeien. Dit resulteerde in hoge azijnzuurconcentraties, die ongewenst kunnen zijn.

Deze bevindingen gaven aan dat de lage waterkefirkorrelaangroei tijdens het bestudeerde industriële waterkefirproductieproces teruggeleid kon worden tot de afwezigheid van EPS-producerende stammen van *Lb. hilgardii*, lage pH-waarden, lage calciumconcentraties, hoge nutriëntenconcentraties en/of excessieve sucroseconcentraties tijdens fermentatie. De onstabiliteit van dit industrieel productieproces kon verlaard worden door de lage waterkefirkorrelaangroei tijdens de fermentatie, waardoor de grootte van de waterkefirkorrels daalde.

CURRICULUM VITAE

David Laureys was born on December 28, 1984, in Lokeren (Belgium). He graduated from the Sint-Lodewijkscollege, Lokeren (Belgium), in 2003. He obtained his Bachelor of Science in Bioscience Engineering, specialization Chemistry and Food Technology, at Ghent University (Ghent, Belgium) in 2009, and his Master of Bioscience in Bioscience Engineering, specialization Food Science and Nutrition at Ghent University in 2011. In October 2011, he started his PhD in the Research Group of Industrial Microbiology and Food Biotechnology (IMDO) of the Faculty of Sciences and Bioengineering Sciences of the Vrije Universiteit Brussel, under the supervision of Prof. Dr. ir. Luc De Vuyst as promoter. In the framework of a Joint PhD, he carried out part of his experimental work in the Laboratory of Microbiology of the Faculty of Sciences of Ghent University under the supervision of Prof. Dr. Peter Vandamme as co-promotor. David Laureys' research focused on the microbial species diversity, substrate consumption, and metabolite production during water kefir fermentations. He is author of three scientific papers published in peer-reviewed international journals and co-author of one chapter in a peer-reviewed book. He participated at the International Food Microbiology conference held in Nantes (France), where he was selected for an oral presentation (as well as finalist for the best oral paper presentation) and a poster presentation. Further, he participated at three national conferences, where he was selected for a poster presentation. He also contributed with three oral presentations at other research meetings and is co-author of one scientific report. He was granted an award for the best poster presentation at the 3rd Annual Congress of the Belgian Nutrition Society conference.

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