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An Update on Water Kefir: Microbiology, Composition and Production

Kieran M. Lynch[1], Stuart Wilkinson[2], Luk Daenen[2] and Elke K. Arendt[1,3*]

[1]*School of Food and Nutritional Sciences, University College Cork, Cork, Ireland*

[2]*Global Innovation & Technology Centre, Anheuser-Busch InBev nv/sa, Brouwerijplein 1, 3000 Leuven, Belgium*

[3]*APC Microbiome Ireland, University College Cork, Cork, Ireland*

* Corresponding author:

Mailing address: School of Food and Nutritional Sciences, University College Cork, Cork, Ireland.

Tel +353 21 490 2064; Fax +353 21 427 0213

E-mail address: e.arendt@ucc.ie

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ABSTRACT

Water kefir is a sparkling, slightly acidic fermented beverage produced by fermenting a solution of sucrose, to which dried fruits have been added, with water kefir grains. These gelatinous grains are a symbiotic culture of bacteria and yeast embedded in a polysaccharide matrix. Lactic acid bacteria, yeast and acetic acid bacteria are the primary microbial members of the sugary kefir grain. Amongst other contributions, species of lactic acid bacteria produce the exopolysaccharide matrix from which the kefir grain is formed, while yeast assist the bacteria by a nitrogen source that can be assimilated. Exactly which species predominate within the grain microbiota, however, appears to be dependent on the geographical origin of the grains and the fermentation substrate and conditions. These factors ultimately affect the characteristics of the beverage produced in terms of aroma, flavour, and acidity, for example, but can also be controlled and exploited in the production of a beverage of desired characteristics. The production of water kefir has traditionally occurred on a small scale and the use of defined starter cultures is not commonly practiced. However, as water kefir increases in popularity as a beverage – in part because of consumer lifestyle trends and in part due to water kefir being viewed as a health drink with its purported health benefits – the need for a thorough understanding of the biology and dynamics of water kefir, and for defined and controlled production processes, will ultimately increase. The aim of this review is to provide an update into the current knowledge of water kefir.

Introduction

Water kefir is a sparkling, slightly acidic fermented beverage, typically produced by fermenting a solution of sucrose to which fresh or dried fruits have been added (sometimes with a slice of lemon), with so-called water kefir 'grains'. This beverage is similar to but distinct from milk or dairy kefir which is produced typically with bovine milk using milk kefir grains (table 1). This review focuses specifically on water kefir. The reader is referred to Arslan (2015) and Prado et al. (2015) for reviews on milk kefir (Arslan, 2015; Prado et al., 2015). The gelatinous grains of water kefir are a symbiotic mixture of bacteria and yeast embedded in a primarily polysaccharide matrix (Martínez- Torres et al., 2017), and have, along with the resulting beverage, been variously known by different names: sugar(y) kefir, 'Tibicos', 'Tibi' (tibi grains or tibi complex), 'Tibetan mushroom', 'kefir d'aqua', 'Japanese beer seeds', 'Beer plant', 'Ginger-beer plant' 'bebées', 'Australian bees', 'African bees', 'California bees', 'Ale nuts', 'tepache de tibicos' and 'Balm of Gilead' (Corona et al., 2016; Diniz et al., 2003; Kebler, 1921; Laureys and De Vuyst, 2014; Martínez- Torres et al., 2017; Pidoux, 1989; Ward, 1892). As reported by Ward (1892) and Kebler (1921) such grains, first exhibited by Balfour in 1887 and called 'ginger-beer plant', may have been introduced to Britain around 1855 by soldiers returning from Crimea, and appeared similar to those grains used to produce 'kephir', a dairy beverage described by Kern in 1881 (Kebler, 1921; Ward, 1892). It has additionally been reported that ginger beer plants originated from the Caucasus region (Pidoux et al., 1988). However, it appears there could be more than once source of these kefir grains, or indeed distinct origins of similar grains, as it is said that tibi grains originate from a Mexican cactus (*Opunita*) where they were taken off the leaves (Horisberger, 1969; Pidoux, 1989). In fact, Pidoux et al. (1988) made the distinction between three different grains: Tibi grains (originating from Mexico), ginger-beer plant (originating from the Caucasus region) and sugary kefir grains, the latter found in France, but the origins

of which were stated as being unknown (Pidoux et al., 1988). As appears to be the practice today, and for the purposes of this review, all such grains used for the fermentation of a sugar water solution will be referred to simply as water kefir grains. Fermentations are typically of a spontaneous nature and unlike for milk kefir production, the use of defined starter cultures is not common. The grains can be reused for successive fermentations through back-slopping, or removal of the grains from the previous fermentation and placing them into fresh substrate for a new fermentation. Technically, this could be practiced *ad infinitum* (Pidoux et al., 1988) and traditionally the grains are passed on or shared from one generation to the next. Lactic acid bacteria, particularly species of *Lactobacillus*, are the primary bacterial members of the complex grain community, with acetic acid bacteria having a secondary role, depending on the presence of oxygen. Yeast, both *Saccharomyces* and non-*Saccharomyces* species are also dominant members. Aside from the use of different substrates for sugar water kefir and milk kefir, both products have a similar bacterial community (dominated by LAB), but the yeast community can be significantly different (milk kefir contains primarily non-*Saccharomyces* yeasts) (see table 1). Water kefir is traditionally produced at home and to date neither an industrial-scale process, nor defined strain starter cultures have been developed. Mirroring the origin of the grains, consumption of water kefir is traditionally high in South American, Eastern Europe and Russia. The benefits of kefir consumption have been tenuously linked to the presence of 'probiotic' lactic acid bacteria. Nevertheless, like with milk kefir, consumers are increasingly open to fermented and probiotic benefits in more types of foods than ever before, especially if the product has a 'traditional' origin or story associated with it (Mellentin, 2019), a trend that water kefir can capitalise on. In addition, compared to milk kefir, water kefir has an advantage in appealing to a wider diversity of consumers e.g. vegan (Buchet, 2019). The aim of this review is to provide an update into the current knowledge and science of water kefir.

Production of water kefir

Water kefir grains are gelatinous structures of 5 to 20 mm in diameter and with an irregular, cauliflower-like shape (Waldherr et al., 2010). Figure 3 shows scanning electron microscopy images of a typical water kefir grain. The approximate dry matter content of the grains can vary from 10 to 14% (w/w) (Laureys et al., 2017; Magalhães et al., 2011, 2010; Pidoux, 1989; Pidoux et al., 1988). The general process of water kefir beverage production is outlined in figure 4. Fermentations are typically spontaneous with water kefir grains being placed into a sucrose medium with or without dried fruits or fruit extracts. Fermentations are performed at a temperature between 21 to 30^{o}C for 4 to 8 days (Table 2). Water kefir grains can be recovered from the fermented liquid and essentially re-used *ad infinitum* by placing them in fresh sugar-water medium. The use of defined starter cultures in not common, as discussed below, and there are few studies in this area. Typically, water kefir has been produced on a home-scale under non- or minimally-aseptic conditions (Horisberger, 1969; Pidoux et al., 1988).

Table sugar or brown sugar (sucrose) is the most commonly used carbon source while fresh or dried fruits are added as the source of nitrogen. Fresh or dried figs, sometimes with added slices of lemon, are most commonly added for this purpose. Preparation of extracts of the fruit, rather than addition of the whole fruit, may be desirable; such extracts can be prepared in a standardised manner and pasteurisation is possible. Otherwise, depending on the type of fruit, how it has been processed (e.g. dried), how it has been packaged (e.g. with or without preservative measures) and the process of water kefir production (e.g. scalding of fruits during preparation), there may be a contribution of (potentially undesirable) microorganisms from the fruit to the water kefir e.g. *Enterobacteriaceae* and/or *Pseudomonas* (Randazzo et

al., 2016). Fig extract has been used in a number of scientific studies (Gulitz et al., 2013; Laureys and De Vuyst, 2014; Verce et al., 2019).

For water kefir produced at home fermentations are considered to be complete once the desired level of sourness (acidity) has been achieved. During experimental investigations or at semi-industrial scale, typical parameters measured include pH, TTA, sugar concentration (often by some form density measurement) and alcohol. In addition, particularly in the case of research studies, individual organic acids and sugars may be measured by high performance liquid chromatography or high-performance anion exchange chromatography. Flavour and aroma compounds may by measured by gas or liquid chromatography coupled with mass spectroscopy (GC-MS or LC-MS) (Corona et al., 2016; Laureys et al., 2018, 2017; Randazzo et al., 2016).

Substrates and factors influencing fermentation characteristics

Figure 5 shows other potential carbon and/nitrogen sources that could be used in water kefir production; however, fresh or dried figs appear to be most commonly used. The reason for their popularity is not fully known. Reiß (1990) found that figs produced the most optimum fermentation when compared to other fruits. It was observed that omission of figs significantly slowed the consumption of glucose and thus the fermentation rate, while substitution with other dried fruit, namely raisins, dates and plums, modified the fermentation and rate of production of lactic acid and acetic acid. In addition, the increase in grain mass over 18 days was significantly reduced (1 to 4% versus 70%) in all but the medium containing figs (Reiß, 1990). In addition, as reported by Reiß (1990), Porchet (1934) found that grain multiplication was significant in the presence of figs but lower with bananas, raisins, plums, apricots, potatoes and carrots, and non-existent with apples, pasteurized grape

juice or milk (Porchet, 1934). Reiß (1990) suggested, that figs contain an as yet unknown growth-promoting factor that is cold water-extractable and moderately heat stable (Reiß, 1990). A potential growth-promoting factor is calcium. Laureys et al. (2019) found that the buffering capacity and calcium content impacted the water kefir fermentation characteristics, particularly grain growth (mass increase). Higher buffering capacity and calcium concentrations of the water used for fermentation promoted increased growth. When the buffering capacity and/or calcium content were below a certain level, the pH values were significantly lower, even at the start of the fermentation (c. 3.2 versus 3.6), and grain growth was observed to decrease through the back-slopping process. Excessive acid stress due to a consistently low pH was suggested as the reason for the low grain growth. In addition, it was suggested that this low pH inhibited the production and, potentially, the activity of LAB glucansucrases, thus preventing glucan formation and associated grain growth (Laureys et al., 2019). It was suggested that the positive effect of calcium may be due to its effect on promoting glucansucrase activity. As can be seen in Table 3, dried figs have the highest content of calcium; however, interestingly, raw figs do not contain a significantly higher calcium content than some of the alternative fruits. Therefore, given the positive impact of buffering capacity and calcium content on the fermentation and grain growth, the characteristics of the water used for the fermentations needs considered; thus, hard water, containing a higher content calcium and magnesium ions, is expected to be more suitable for water kefir fermentations.

While fruits such as figs and various alternatives are typically used as fermentation substrates, other than fruits and substrates have been tested, including vegetables, such as carrots, ginger, fennel, and onions (Fiorda et al., 2017), dairy substrates such as cows and goats milk (Hsieh et al., 2012) and dairy substitutes such as soy (Tu et al., 2019). For example, a beverage, known as ‘Tepache’, consisting brown sugar, pineapple and cinnamon

and fermented with kefir grains is popular in South American countries (de la Fuente-Salcido et al., 2015; Fiorda et al., 2017). Importantly, and as has already been discussed, the grain origin, and equally consequential, the substrate used (carbon and nitrogen sources), can significantly influence the diversity and dominant strains constituting a particular water kefir fermentation (Hsieh et al., 2012; Marsh et al., 2013; Miguel et al., 2011). Laureys et al. (2018) studied the effect of oxygen, nutrient concentration, and nutrient source on the water kefir fermentation. Acetic acid bacteria were found to be present under both anaerobic (use of an air-lock to exclude oxygen ingress) and aerobic (muslin cloth allowing air exchange) conditions; under aerobic conditions AAB were more abundant. The main species detected were *A. fabarum*, *G. roseus*/*oxydans*, and *A. indonesiensis*. *G. roseus*/*oxydans* and *A. indonesiensis* were more abundant under anaerobic fermentation conditions, while *A. fabarum* was more abundant under aerobic conditions. Proliferation of AAB resulted in high acetic acid concentrations and low pH values, which were accompanied by gradually decreasing grain growth over the back-slopping period, likely related to excessive acidic stress, as discussed earlier. In addition, the proliferation of AAB species in the aerobic fermentations correlated with higher ethyl acetate concentrations and lower concentrations of fruity esters (Laureys et al., 2018). Low nutrient concentrations (i.e. no dried figs added) caused a slow fermentation and high total residual carbohydrate concentrations, low metabolite concentrations, and high pH values. In addition, there was a slow and gradual decrease of the grain growth over the back-slopping cycles. Low nutrient concentrations were also accompanied by high viable counts of AAB species, probably caused by the limited expulsion of oxygen due to the low metabolic activity (and low CO_2 production) of fermentative microorganisms. In contrast, high nutrient concentrations (i.e. one or two dried figs added) caused a fast fermentation, and high metabolite concentrations without a decrease of the total residual carbohydrate concentrations or pH values. This was likely due to

sufficient carbohydrate and buffering compounds being present, enabling high metabolite production without a significant drop in total carbohydrate or pH, and was observed for dried figs. In addition, the nutrient concentration shifted the diversity and dominant species in the fermentation – high nutrient concentrations favoured the growth of yeasts over LAB species and increased the relative abundance of *Lb. nagelii* and *S. cerevisiae*; in contrast low nutrients promoted a dominance of *Lb. hilgardii* and *D. bruxellensis*. Furthermore, high nutrient concentrations resulted in low ratios of the concentrations of acetic acid to ethanol and acetic acid to lactic acid, reflecting the change in species diversity (Laureys et al., 2018). With respect to the specific nutrient source, dried figs, dried apricots and dried raisins resulted in stable water kefir fermentations, but fresh figs or a mixture of yeast extract and peptone did not. Low pH values were observed from the beginning of the yeast extract-peptone fermentations, not due to high acid concentrations, but due to low buffering capacity. Thus, grain growth gradually decreased throughout these back-slopping cycles, for reasons discussed already. In general, fermentations with dried raisins resulted in high total residual carbohydrate concentrations, low metabolite concentrations, thus resembling fermentations with low nutrient concentrations. In contrast, fermentations with fresh figs or yeast extract-peptone resulted in low total residual carbohydrate concentrations, but high metabolite concentrations, thus resembling the fermentations with high nutrient concentrations (Laureys et al., 2018). Therefore, the substrate used, and fermentation conditions should be considered carefully in light of the potential effects on which species predominate and consequential effects on the final beverage characteristics in terms of metabolites produced, their concentration and final beverage flavour and aroma (Table 4); nevertheless, such plasticity could be exploited to positive effect with respect to producing a diverse range of beverage products, with variety in flavour and aroma.

Scale-up of water kefir production

The scale up of water kefir production poses challenges which have limited the industrial production of water kefir. These include unstable fermentation processes resulting in beverage of varied quality and often low kefir grain growth, thus hindering scale up (Laureys et al., 2017). Laureys et al. (2017) performed one of the only reported investigations on a so-called industrial water kefir production process (batch fermentations of 6 L scale). This fermentation was suffering from the above issues of instability and negligible grain growth, with the grains being small and damaged compared to water kefir grains used in typical household water kefir. The company stored their stock grains at -20^{o}C, and because water kefir grains are 86-90% (w/w) water, it was suggested that freezing and thawing irreversibly damages the grain structure and/or the associated microorganisms, which do not recover, even after successive back-slopping (Gulitz et al., 2013; Laureys et al., 2017). In addition, demineralised water was used for the water kefir production. Demineralised water lacks buffering capacity due to the removal of ions. Thus, while the pH decreased, the level of acids produced was lower than expected. Low water kefir grain growth is associated with the use of water with low buffering capacity (and/or low calcium concentrations) as subsequently investigated further by the authors and discussed earlier (Laureys et al., 2019, 2017). High viable microbial counts have been associated with slow growing grains (Laureys et al., 2017) – it is hypothesised that the smaller size of the slow growing grains results in a larger surface area for microbial colonisation (figure 3), in addition to there being a higher amount of total residual glucose because it is not sequestered in glucan production (due to decreased glucansucrase activities) (Laureys et al., 2019). However, in the study of the industrial water kefir production, the amounts and ratio of LAB and yeast were found to be similar to those previously reported (Laureys et al., 2017; Laureys and De Vuyst, 2014). The slow progression of the fermentation in this case was suggested to be a consequence of the high

sucrose concentration used (approx. 25% (w/v) compared to typically 8 - 10% (w/v)) and associated effect of osmotic pressure on the microorganisms (Laureys et al., 2017). Furthermore, these industrial fermentations were performed essentially aerobically, with a muslin cloth covering the fermentation containers. This was reflected in a high prevalence of AAB. Finally, it was observed that a storage or rest period at low temperature (8 ^{o}C), which the company was employing as a buffer to account for beverage demand, resulted in variable, prolonged lag times in the subsequent production fermentation (Laureys et al., 2017).

Development of defined starter cultures

The use of defined starter cultures for the production of water kefir has not been extensively studied, and is not currently widely applied to the production of water kefir, to the authors knowledge; thus, the back-slopping of water kefir grains (i.e. undefined, complex cultures) from one fermentation to the next remains the primary method of production. To this end, past research has focused on recombining of isolated, defined strains with the aim of reconstituting the water kefir granule. However, these endeavours have mostly failed to reconstitute the granule (Ward, 1892; Xu et al., 2018). Notwithstanding, from their investigation into the microbiota and metabolic interactions of water kefir, Martínez-Torres et al. (2017) proposed a minimal and efficient consortium for water kefir fermentation consisting of *Lb. hilgardii*, *S. cerevisiae* and *A. tropicalis*. The following metabolic interactions were proposed: an initial production of alcohol by *S. cerevisiae*, followed by lactic acid and acetic acid production after 24 h by *Lb. hilgardii* and *A. tropicalis*, respectively; subsequently, acetic acid accumulated due to utilisation of ethanol by *A. tropicalis* (figure 6). In addition, colonies of an isolated *Lb. hilgardii* strain growing on solid sucrose–casein peptone medium were observed to be very similar to gelatinous water kefir

granules (figure 7) (Martínez- Torres et al., 2017). This appears to be the first report of such kefir-like granule formation by an isolated *Lactobacillus* strain. However, the authors did not experimentally test their minimal tri-strain defined starter in the production of water kefir beverage. Nevertheless, a few studies have used mixed culture starters in the production of so-called 'kefir-like beverages' with Mediterranean fruit (apple, quince, grape, kiwifruit, prickly pear and pomegranate) juices (Randazzo et al., 2016) and vegetable (carrot, fennel, melon, onion, tomato and strawberry) juices (Corona et al., 2016). The commercial freeze-dried water kefir starter culture used in these studies, "kefir d'acqua fai da te" (BioNova snc, Villanova sull'Arda, Italy), was reported by the manufacturer to contain approximately 10^9 CFU/g of *Lactobacillus, Lactococcus, Leuconostoc* and *Saccharomyces* (Corona et al., 2016; Randazzo et al., 2016). The strains were identified as *Lactobacillus fermentum*, *Lactobacillus kefiri*, *Lactococcus lactis*, *Leuconostoc mesenteroides* and *Saccharomyces cerevisiae* (Randazzo et al., 2016). Interestingly, despite being recommended for the production of water kefir (and their original isolation source being unreported), *Lactobacillus fermentum* has never been isolated from water kefir and both *Lactobacillus kefiri* and *Lactococcus lactis* are typically more associated with milk kefir, but have been isolated from Brazilian water kefir grains (discussed earlier). For the fruits juices, the yeast was mainly responsible for the fermentation changes, except for with prickly pear as a substrate, which showed an increase in lactic acid and acetic acid acids due to the action of LAB; the vegetable juices all underwent lactic fermentation. Apple-, grape- and carrot-based kefir-like beverages received the most positive evaluations by tastes (Corona et al., 2016; Randazzo et al., 2016).

Microbial diversity

Lactic acid bacteria of the genera *Lactobacillus*, acetic acid bacteria of the genera *Acetobacter* and *Saccharomyces* yeast appear to be the primary microbial members of the sugary kefir grain (Table 2). In addition, certain species have been observed to occur often in the grains, but which species dominate appears to be dependent on the geographical origin of the grains and the fermentation substrate.

Lactic acid bacteria

Of the seventeen studies detailed in Table 2 that have examined the bacterial composition of water kefir (not differentiating between grains and liquid) via culture-dependent and/or culture-independent methods, *Lactobacillus* species were isolated and/or detected in all; however, no one particular unifying or characteristic species has been identified (Galli et al., 1995; Gulitz et al., 2013, 2011; Laureys et al., 2018; Laureys and De Vuyst, 2017, 2014; Magalhães et al., 2010; Marsh et al., 2013; Miguel et al., 2011; Pidoux, 1989; Verce et al., 2019; Zanirati et al., 2015). The most commonly identified species include *Lb. hilgardii* (9/17 studies) and *Lb. nagelii* (8/17), followed by *Lb. casei* (7/17) and *Lb. paracasei* (6/17). *Lb. hilgardii* and *Lb. nagelii* have been reported to be key *Lactobacillus* species in water kefir grain communities, particularly due to their production of exopolysaccharides (EPS) (Fels et al., 2018). Indeed, while *Lb. hilgardii* was identified in all studies that examined Belgian water kefir, the species was present in only one of two studies performed in Germany and one of three studies which examined Brazilian water kefir (Laureys et al., 2018; Laureys and De Vuyst, 2017, 2014; Pidoux, 1989; Stadie et al., 2013; Verce et al., 2019). Similarly, *Lb. nagelii* was identified in each of the Belgian and German studies, but in only one of four studies examining grains from South America (Brazil and Mexico) (Gulitz et al., 2013, 2011;

Laureys et al., 2018; Laureys and De Vuyst, 2017, 2014; Magalhães et al., 2010; Verce et al., 2019). In addition, this species was identified in the single Brazilian study that found *Lb. hilgardii* (Zanirati et al., 2015). Furthermore, Zanirati et al. (2015) only identified *Lb. hilgardii* and *Lb. nagelii* via a culture-independent approach, but not via culture-dependent means. This finding highlights the importance of using culture-independent approaches in addition to culturing and isolating of strains. Thus, both *Lb. hilgardii* and *Lb. nagelii* appear to be primarily components of European kefir grains. These findings could be a consequence of the medium which the authors used for culturing (Zanirati et al., 2015). The medium, MRS reconstituted in acidic whey, may have selected for those *Lactobacillus* species (e.g. *Lb. casei*) that are more adapted to the dairy environment, while those species less adapted to this environment may have been supressed. Alternatively, it may have been that the more adapted *Lactobacillus* species were able to compete and outgrow *Lb. hilgardii* and *Lb. nagelii*. Therefore, the importance of the medium characteristics used for culture isolation are apparent. Second to *Lb. hilgardii* and *Lb. nagelii*, *Lb. casei* and *Lb. paracasei* have been commonly identified. Taking these species together as the *Lb. casei*/*paracasei* group, they represented the most common *Lactobacillus* species identified in water kefir grains, being found in nine of the seventeen previously mentioned studies. Another study, by Marsh et al. (2013), only identified the bacteria to genus level and therefore it is not possible to state which *Lactobacillus* species were present (Marsh et al., 2013). *Lb. casei* appears to be a prominent member of Brazilian water kefir grains, but is most notably absent from more recent studies on German and Belgian grains, with the exception of one study by Laureys and De Vuyst, (2014), but the authors did not distinguish between *Lb. casei* and *Lb. paracasei* in this instance (Laureys and De Vuyst, 2014). *Lb. paracasei* has been detected in a similar number of studies to *Lb. casei*, being primarily detected in Belgian and Brazilian water kefir grains (Laureys et al., 2018; Laureys and De Vuyst, 2017, 2014; Magalhães et al.,

2010; Miguel et al., 2011; Verce et al., 2019). Other lactobacilli which have been found less commonly, but still representing a geographical spread, include *Lb. hordei* (5/17) and *Lb. satsumensis* (4/17). *Lb. hordei* has been detected in European water kefir grains only (Gulitz et al., 2013, 2011; Laureys and De Vuyst, 2017, 2014; Verce et al., 2019), while *Lb. satsumensis* has been found in Brazilian, German and Belgian grains, albeit sporadically (Gulitz et al., 2013; Laureys and De Vuyst, 2017; Miguel et al., 2011; Zanirati et al., 2015). However, despite their low prevalence between various studies that have examined the microbial composition of water kefir, Stadie et al. (2013) stated that *Lb. hordei* was the most prominent bacteria in water kefir, at 31.3%, followed by *Lb. nagelii* at 22.7% (Stadie et al., 2013). Other species of lactobacilli have been detected, either in one or two studies alone, or, only in water kefir grains from a particular geographical location, as in the case of the detection of *Lb. brevis* and *Lb. plantarum* (Hollsberger, 1969; Pidoux, 1989; Pidoux et al., 1988). The *Lb. brevis* strain, however, was later re-identified as *Lb. hilgardii* (Pidoux et al., 1990). Another example is the detection of *Lb. harbinensis* exclusively and in all studies of Belgian grains (Laureys et al., 2018; Laureys and De Vuyst, 2017, 2014; Verce et al., 2019), while *Lb. buchneri* and *Lb. kefiri* have only been found in Brazilian grains (Magalhães et al., 2010; Miguel et al., 2011). The finding the *Lb. harbinensis* was always present in the Belgian water kefir grains could suggest that either this species is a specific member of water kefir grains from Belgium and/or the surrounding geographical area, or that the water kefir grains used in each study originated from the one source (and thus the water kefir grains used in each study were from the same 'lineage'). Considering this, and the geographical differences in species isolation as apparent above, and accounting for the small number of research groups that currently perform research on water kefir – which suggests that a potentially small pool of kefir grains have been studied to date – it is probable that the true diversity of, firstly, water kefir grains, and secondly, the microorganisms within, has not been realised.

Besides lactobacilli, another commonly detected member of the LAB group, which are present in water kefir grains, is *Leuconostoc* (7/17) (Galli et al., 1995; Gulitz et al., 2013, 2011; Magalhães et al., 2010; Pidoux, 1989). Only two species have been found consistently, namely *Leuc. mesenteroides* and *Leuc. citreum* in studies of French and German grains; notably, only a single species *Leuc. pseudomesenteroides* has been found in one Belgian study (Laureys et al., 2019). Indeed, in the two studies that examined water kefir grains from Germany, both *Leuc. mesenteroides* and *Leuc. citreum* were found on each occasion; however, this may reflect a single lineage of water kefir grains, as discussed earlier (Gulitz et al., 2013, 2011). Bifidobacteria (7/17), namely *B. psychraerophilum* and *B. aquikefiri*, have been found only in water kefir grains from Europe, the latter species being regularly detected in Belgian grains, from which it was first isolated (Gulitz et al., 2013; Laureys et al., 2018, 2016; Laureys and De Vuyst, 2017, 2014; Verce et al., 2019). Recently, a new species *B. tibiigranuli* has been described, isolated from German water kefir (Eckel et al., 2019). Members of the genus *Oenococcus* have also been found on occasion, including *O. oeni* and *O. kitaharae* (Laureys and De Vuyst, 2017; Zanirati et al., 2015), and a novel, newly proposed species, *Candidatus* Oenococcus aquikefiri by Verce et al. (2020) (Verce et al., 2020). This newly proposed *Oenococcus* species was recently confirmed by water kefir metagenome analysis to be *Oenococcus sicerae* (Verce et al., 2020). *Pediococcus* species were reportedly detected in a single study (Galli et al., 1995). Lactococci are typically rarely identified in water kefir grains; however, *Lactococcus lactis* has been found, mainly in recent studies of Brazilian water kefir grains (Magalhães et al., 2010; Zanirati et al., 2015). In addition, this species, reported using nomenclature as streptococci, was detected in one historical study on French grains by Pidoux (Pidoux, 1989). The finding of this primarily milk kefir-associated bacteria could be a consequence of the media used for microbial isolation and enumeration, for example M17 (Magalhães et al., 2010) and MRS reconstituted

in acidic whey (Zanirati et al., 2015). If these species are present in the water kefir community, even as minor components, their undetectable nature in most other studies could be due to the narrow selectivity of the media chosen for microbial isolation and growth. However, even if present at relatively low levels, it would be expected that culture-independent approaches would detect these species if they were present.

The presence of these dairy-associated bacteria in grains of a certain geographical origin could also relate to the possible divergent origin of grains which we today collectively refer to as water kefir grains. Another possibility is that those grains that contain typically dairy-associated species were, either historically or more recently, milk kefir grains that were applied in sugary kefir fermentation and adapted over time to that new substrate (Marsh et al., 2013; Miguel et al., 2011). In addition, other studies have observed that water kefir grains can ferment milk; however, the grain mass does not grow, probably because the dominant EPS producers (e.g. *L. hilgardii*) cannot utilise lactose or produce EPS from this disaccharide (Martínez- Torres et al., 2017). Further still, Hsieh et al. (2012) fermented water kefir grains with brown sugar in comparison to cows and goats' milk. Interestingly, changes in the microbial profiles and species present in the resulting fermented grains and beverage were apparent between the substrates. *Leuc. mesenteroides*, *Lb. mali* and *Lb. hordei* were found in the grains fermented using brown sugar whereas *Leu. mesenteroides*, *Lactococcus lactis*, *B. psychraerophilum* and *Enterococcus faecalis* were identified in the grains fermented using either cow's or goat's milk. This suggests that kefir grains may (initially) contain a broad diversity of microorganisms, with a relatively few and specific number of species subsequently being selected for based on the fermentation substrate (Hsieh et al., 2012). An interesting question is whether species which become less dominant due to the use of an unsuitable substrate (e.g. *Lactococcus lactis*, a primarily dairy-associated bacterium, in brown sugar) remain at a certain viable level within the grains, even after successive fermentations,

and could regain dominance again after being provided a suitable substrate (e.g. milk, in the case of *Lactococcus lactis*).

Acetic acid bacteria

Bacteria other than LAB which have been detected in water kefir grains from different geographical origins include members of the acetic acid bacteria (AAB), primarily *Acetobacter* (7/17); however, they are not always detectable, even between studies from the same research group (Laureys and De Vuyst, 2017, 2014). This could be due to differences in study design, as oxygen is a requirement for the growth of AAB, and its levels in the water kefir and the grain environment could dictate weather members of this group grow and are detectable (Laureys et al., 2018). *A. lovaniensis* and *A. fabarum* are the most commonly found species, being detected in three studies, *A. orientalis* has been found in two studies, while *A. tropicalis*, *A. indonesiensis* and *A. okenawensis* each have been found in a single (Gulitz et al., 2011; Laureys et al., 2018; Laureys and De Vuyst, 2014; Magalhães et al., 2010; Martínez- Torres et al., 2017). Other AAB have been detected rarely. Species of *Gluconobacter*, namely *G. liquefaciens* and *G. roseus/oxydans* have been found in grains from Brazil and Belgium, respectively (Laureys et al., 2018; Miguel et al., 2011); *Gluconacteobacter* was detected at low level via culture-independent approach in a single study (Marsh et al., 2013).

Other bacteria

Interestingly, Marsh et al. (2013) found, via a culture-independent approach, that the dominant bacteria in a number of water kefirs from different geographical locations (UK, Canada and USA), and the water kefir produced with them, was *Zymomonas*. The genus is

represented by a single species, *Zymomonas mobilis*, a Gram-negative, rod-shaped, aerotolerant anaerobe (Weir, 2016). This bacterium produces high levels of ethanol, rivalling *S. cerevisiae* in terms of yield, and is associated with traditional fermented beverages in tropical regions of America, Africa and Asia (e.g. *Agave* juice, or pulque, palm juice or palm wines, sugar cane juice) (Marsh et al., 2013; Weir, 2016). Interesting, *Zymomonas mobilis* has been detected in only two studies of water kefir (Hsieh et al., 2012; Marsh et al., 2013), despite the use of culture-independent methods also in other studies (Laureys et al., 2018; Verce et al., 2019); it is even more curious that it has not been found more often, given the abundant levels detected (>60% and >40% relative abundance in all grains and fermentates, respectively) in, and wide geographic spread of, the grains examined by Marsh et al. (2013). Notably, *Zymomonas* also produces levan extracellularly from sucrose in a similar manner to LAB (Doelle et al., 1993).

Yeast

In general, the diversity and breadth of yeast species in water kefir grains appears to be lower than that of the bacteria. Of the fifteen studies to examine the yeast composition of water kefir (Table 2), *Saccharomyces cerevisiae* was found in all but two, thus this species appears to be a key member of the grain community (Galli et al., 1995; Gulitz et al., 2013, 2011; Laureys et al., 2018; Laureys and De Vuyst, 2017, 2014; Magalhães et al., 2010; Marsh et al., 2013; Miguel et al., 2011; Verce et al., 2019; Zanirati et al., 2015). Other species of *Saccharomyces* have been identified on rare occasions, including *S. florentinus*, *S. pretoriensis*; however, the former species is now classified as *Zygotorulaspora florentina* (Galli et al., 1995). *S. bayanus* has also been found, as reported by Waldherr et al. (2010) (Waldherr et al., 2010). Based on the number of studies in which the genus was detected,

Dekkera (anamorph, *Brettanomyces*) appears to be the second most commonly found yeast in water kefir grains (7/15 studies); however, this yeast, apart from one study (Marsh et al., 2013), has been found almost exclusively in Belgian water kefir grains (Laureys et al., 2018; Laureys and De Vuyst, 2017, 2014; Verce et al., 2019). Thus, as discussed earlier, given the potential lack of diversity within kefir grain samples due to the possibility of research groups continually using the same lineage of grains for subsequent studies, while *Dekkera* appears as a common yeast in water kefir grains, the finding may be biased due to the geographically constrained distribution. In addition, *D. bruxellensis* was the sole species detected in Belgian grains, whereas Marsh et al. (2013) found both *D. bruxellensis* and *D. anomala* in their examination of grains from the USA, Canada and the UK, indicating a more broad geographical distribution of the genus than in Belgium alone (Marsh et al., 2013). As discussed by these authors, the low detection of *Dekkera* in most studies may be a consequence of the yeasts' slow doubling time when cultured on commonly used microbiological growth media (Marsh et al., 2013).

Species of *Zygotorulaspora* (5/15) and *Hanseniaspora* (5/15) are commonly ascribed as members of the water kefir grain community, with a broader distribution amongst grains from different locations; however, neither genus has been described in grains from South America. *Zygotorulaspora* is represented by a single species *Z. florentina*, in older studies being identified as *Saccharomyces florentinus* and/or *Zygosaccharomyces florentinus* (Galli et al., 1995; Gulitz et al., 2011; Laureys and De Vuyst, 2017; Pidoux, 1989). In addition, Marsh et al. (2013) did not detect this yeast in grains from the USA, Canada or the UK (Marsh et al., 2013). Species of *Hanseniaspora* have not been found in Belgian or South American studies. While the yeast diversity in Brazilian grains is high (Magalhães et al., 2010; Miguel et al., 2011), water kefir grains from Belgium are typically dominated by only two species, *S. cerevisiae* and *D. bruxellensis* (Laureys et al., 2018; Laureys and De Vuyst, 2014; Verce et

al., 2019). Again, this could be a consequence of a narrow lineage of grains. Two species of *Hanseniaspora* have been found, namely *H. valbyensis* and *H. vinae* (Galli et al., 1995; Gulitz et al., 2011; Marsh et al., 2013). Species of *Lachancea* (4/15), primarily *Lac. fermentati*, have also been identified in grains from different locations, including Brazil, Germany and the UK (Gulitz et al., 2011; Magalhães et al., 2010; Marsh et al., 2013; Miguel et al., 2011). *Candida* (4/15) species have been identified mainly in grains from South America, but also in a single study from France and Thailand; species detected include *Candida ethanolica* (Sarikkha et al., 2015), *C. californica* (Martínez- Torres et al., 2017), *C. lambica*, *C. valida* (Pidoux, 1989) and *C. valdiviana* (Miguel et al., 2011).

Other less commonly found yeast include *Torulaspora* (2/15), *Kazachstania* (2/15), *Pichia* (1/15), *Kluyveromyces* (1/15) and *Yarrowia* (1/15). *Torulaspora pretoriensis* is the sole species of this genus identified in water kefir grains, being found in grains from France, Italy, UK and USA (Galli et al., 1995; Marsh et al., 2013; Pidoux, 1989). *Kazachstania* (*Ka. aerobia*), Pichia (*P. cecembensis*, *P. membranifaciens*, *P. caribbica*, *P. fermentans*), *Kluyveromyces* (*Kl. lactis*) and *Yarrowia* (*Y. lipolytica*) have been detected primarily in Brazilian water kefir grains, these grains having the most diverse yeast microbiota compared to grains from other geographical areas. The finding of yeast such as *Candida* and *Kluyveromyces*, which are more typically associated with milk kefir, may reflect an origin as milk kefir grains, that were subsequently adapted for the production of sugary kefir (Fiorda et al., 2017).

With respect to microbial levels in water kefir grains, Gulitz et al. (2011) showed that the consortium consisted of 10^8 lactobacilli, 10^6 to 10^8 AAB and 10^6 to 10^7 yeasts per gram of grains (Gulitz et al., 2011). Thus, lactobacilli dominate and typically outnumber yeast by a factor of 10 to 100. The number of AAB can be similar or considerably lower than the lactobacilli, possibly dependant on oxygen availability in the system, as discussed above. In

addition, combined with the intermittent introduction of oxygen (often only during back-slopping), ethanol, a key energy source for AAB, is typically only present at significant levels at the end of the fermentation process (Laureys et al., 2018).

Metabolic interactions and metabolites

The main metabolic interactions that occur between the primary water kefir microbiota and the fermentation medium are detailed in figure 1. While the interactions and pathways shown are based on current knowledge, there may be as yet unknown interactions occurring – including between undiscovered, potentially uncultivable, species – or important compounds being produced which remain to be identified. This point is strengthened when considering that all attempts to reconstruct water kefir grains (e.g. individual granules) based on recombining individual isolated strains of bacteria and yeast have been unsuccessful (Gulitz et al., 2013; Waldherr et al., 2010; Xu et al., 2012).

Both the carbon source (typically sucrose) and nitrogen source (fresh or dried fruits typically) are central to the metabolism and growth of the water kefir grain microorganisms and the fermentative capacity of the grains as a whole, key to which are the trophic and cooperative interactions and metabolite exchange between the grain microorganisms. Water kefir is a challenging environment, high in sugar (can be up to 100 g/L at beginning) and low in nitrogen (amino acids); therefore, mutualistic cooperation between the microbial community is important (Stadie, 2013).

Sucrose metabolism and the action of yeast

Yeast species (e.g. *Saccharomyces*, *Zygotorulaspora*, *Dekkera*) hydrolyse sucrose via an extracellular ß-D-fructofuranosidase (invertase), producing glucose and fructose, which can be taken up by the cell via facilitated diffusion (Reed and Nagodawithana, 1991; Watson,

1993). The yeast use these monosaccharides for its metabolism and the production of ethanol, while also making these simple sugars available for the bacteria in the consortium. Ethanol and carbon dioxide production occur via the Embden-Meyerhof-Parnas Pathway (Glycolytic Pathway) (Stewart and Russell, 2009). The primary carbon source that is present can influence which yeast dominate in the community. For example, *Saccharomyces* species lack ß-galactosidase required for lactose utilisation, and are therefore cannot dominant in milk kefir (Dickinson and Kruckeberg, 2006). All LAB (lactobacilli and leuconostocs) species which have been commonly found in water kefir, with the exception of *L. hilgardii*, are able to produce acids from sucrose; this is understandable, given the nature of water kefir and the ecological advantage associated with the ability to utilise sucrose (Table 5) (Bechtner et al., 2019b). According to Bergey's Manual of Systematic Bacteriology, 11 to 89% of *L. hilgardii* strains are positive for acid production from sucrose (Miyamoto et al., 2005; Rouse et al., 2008; Vos et al., 2011). Importantly, sucrose is the substrate for dextran production, an α-glucan exopolysaccharide (EPS) and the main structure-forming component of the kefir grain.

Role and diversity of exopolysaccharides produced by lactic acid bacteria

The main α-glucan in the water kefir grain is dextran, a polysaccharide which contains predominantly α-(1→6) linked glucosyl units (Lynch et al., 2018; Monsan et al., 2001). Dextran is produced by a number of LAB species commonly associated with water kefir grains (Table 2), with the majority (approx. 81%) of members of the species *Lb. hilgardii*, *Lb. nagelii*, *Lb. hordei*, *Leuc. mesenteroides* and *Leuc. citreum* producing EPS (Fels et al., 2018; Gulitz et al., 2011; Stadie, 2013; Waldherr et al., 2010; Xu et al., 2018). This EPS is produced extracellularly from sucrose by the action of a secreted enzyme known as a

glycansucrase (Lynch et al., 2018). These glycansucrases may be released freely into the surrounding environment (e.g. soluble glucansucrase) or may remain bound to the cell (e.g. bound glucansucrase) (Côté et al., 2013).

Dextran production is likely a niche adaption to an environment high in sucrose, a characteristic of water kefir. Xu, et al. (2019) showed that an *L. hordei* isolate from water kefir encodes a sucrose specific phosphotransferase system (PTS) and extracellular glycosyltransferase (dextranase), two systems which enable sucrose utilisation, and both of which were found to be absent in a *L. hordei* strain isolated from barley (Bechtner et al., 2019a; Sun et al., 2015). LAB are known to produce different types of glucan, varying with respect to characteristics such as. the conformation of the glycosidic linkage, the percentage of the different linkages present (e.g. α-(1→6), α-(1→3)) and the presence or absence and location of branches (van Hijum et al., 2006). Indeed, different LAB water kefir isolates are known to produce structurally different dextrans, and it is likely that even within a single grain, that dextrans of different characteristics are produced by different members (Xu et al., 2018). In the context of water kefir, the solubility of dextran in water is important and it is self-evident that insoluble dextran is the principal structure forming component on which the biofilm of yeast and bacteria reside. Other α-glucans, including dextran, have been shown to be important in biofilm formation, such as mutan produced by *Strepotococcus mutans* in dental plaque (Russell, 2009). Mutan has been termed the water insoluble analogue of dextran; this insolubility has been attributed it a relative higher proportion of α-(1→3) linkages present compared to soluble dextran. However, insoluble α-glucans described as dextran are also reported in the case of water kefir and thus, the distinction between dextan and mutan on the basis of being soluble or not is not so clear. Aside from the proportion of α-(1→3) linkages, it is likely that the presence of mono- or di-substituted glucose resides (branching) are a determinant of dextran solubility (Côté et al., 2013). *Lb. hilgardii* is

commonly ascribed as the main producer of granule (insoluble) polysaccharide in the kefir grain (Fels et al., 2018; Pidoux et al., 1988; Waldherr et al., 2010). However, as seen is Table 2, studies exist in which *Lb. hilgarii* strains have not been detected in the grains under investigation (Gulitz et al., 2011), and thus other dextran-producing LAB must assume this role. Indeed, the dextran-producing diversity of water kefir LAB isolates is interesting, with some isolates producing more than one glucansucrase enzyme (Côté et al., 2013) and others producing more than one type of dextran (Pidoux et al., 1988). Pidoux et al. (1988) isolated both a non-gelling polysaccharide (soluble) and gelling polysaccharide (insoluble) from a strain of *Lb. hilgardii* (initially classified as *Lb. brevis*) that displayed different structural characteristics. It was found that the non-gelling polysaccharide had a higher proportion of α-(1→6) linkages; the gelling polysaccharide was also shown to be more similar to the polysaccharide in the kefir grains from which the strain was isolated. It was suggested that the culture conditions and medium components, or the micro-environment of the kefir grain could influence the dextransucrase activity and thus the characteristics of the produced glucan(s) (Pidoux et al., 1988). Such niche effects would not be present during pure culture of the strain, and could differentially influence the activity of the dextransucrase and the characteristics or proportions (ratio of soluble to insoluble) of the glucans formed. Cote et al. (2013) described a strain of *L. satsumensis* producing two α-glucans from sucrose. One was a water-soluble dextran, consisting of predominantly α-(1→6)-linked glucose units, and the other a water-insoluble glucan containing both α-(1→6)-linked and α-(1→3)-linked units. The culture medium contained at least two different glucansucrase enzymes varying in molecular weight, as was the case for another *L. satsumensis* strain also. Both cell-free and cell-associated glucansucrase activity was detected and analysis of the produced glucans showed that the insoluble glucan produced by the cell-free enzyme differed markedly from the glucan produced by the cell-associated enzyme; the cell-free glucansucrase produced an

insoluble polysaccharide with a high proportion of α-(1→3) linkages (Côté et al., 2013). Given that a single strain can produce glucans of different characteristics, and given that a large number glucan-producing strains of different species may be found in water kefir, then the actual number and diversity of different dextrans in a single water kefir grain is potentially large.

Such diverse dextrans likely have different functions. Somewhat counterintuitively, it has been shown that it is soluble dextran, rather than the insoluble polymer which mediates yeast aggregation and, as such, is equally important in biofilm formation. Xu et al. (2018) found that yeast (*S. cerevisiae*) aggregation is affected by soluble dextran produced by *L. hordei*, but not by insoluble dextran produced by *Lb. hilgardii* (Xu et al., 2018); this is despite the fact that the latter species is understood to be a primary producer of granule (insoluble) polysaccharide in the kefir grain (Fels et al., 2018; Pidoux et al., 1988; Waldherr et al., 2010). In co-cultivation studies, only *L. hordei* strains and/or their purified soluble EPS were able to cause the aggregation and network formation of *S. cerevisiae*, which was not caused *Lb. nagelii* or *Leuc. citreum* or their associated glucans. Furthermore, *Leuc. citreum* produced significantly different dextran structures to either *Lb. hilgardii* or *Lb. hordei*, which were comparably more similar to each other; however, despite the similarities of the dextrans produced by the latter two species, only *Lb. hordei* and associated glucan promoted yeast aggregation. Differences in molecular mass or side chain patterns between *Lb. nagelii* and *Lb. hordei* dextrans, potentially resulting in secondary structure differences (effecting particle shape, polydispersity in solution), was postulated as a reason for the different functional behaviours (Xu et al., 2018). However, in spite of the importance of *L. hordei* or its EPS in affecting yeast aggregation, co-cultivation with yeast did not influence dextransucrase expression (Xu et al., 2019b). Thus, evidently, different dextrans produced within the water kefir grain have different functionalities. Despite the insights into networking function of

certain dextrans within the grain, the higher order mechanism of physical formation, consolidation and growth of individual granules is not fully understood; however, some hypotheses have been put forward, as in the case of milk kefir (Dong et al., 2018).

The fructose liberated from sucrose, either via the action of LAB dextransucrase or yeast invertase may be assimilated by the LAB and used in glycolysis, as has been shown for *Lb. hordei* (Xu et al., 2019b), or alternatively, may be used as an electron acceptor by LAB and converted to mannitol by those species harbouring a mannitol dehydrogenase (Ortiz et al., 2013). Indeed, heterofermentative LAB species which have the potential to produce mannitol (e.g. *Leuc. citreum*) have been found in water kefir (Gulitz et al., 2011; Laureys and De Vuyst, 2014; Magalhães et al., 2010; Sahin et al., 2019). Mannitol has a sweet taste, which could be desirable in water kefir, however its formation is accompanied by acetate production, which may mask any sweetness benefit (Vrancken et al., 2010). Mannitol can also be produced by *S. cerevisiae* and may act as an additional carbon source within the kefir grain – *Lb. hordei* has been shown to have the ability to utilise mannitol and gluconate as sole carbon sources (Xu et al., 2019a). Levan, a polymer of fructose, have also been detected in water kefir beverages, albeit at low levels (Fels et al., 2018). While some EPS-producing LAB have the ability to produce fructans from sucrose via a similar mechanism to dextran production, there has been no previous description of fructan-producing LAB in the context of water kefir. Nevertheless, fructan production by minor members of the consortium (including potentially other LAB species) cannot be excluded; indeed, some acetic acid bacteria have been demonstrated to produce high amounts of fructans, including an isolate of *Gluconobacter frateurii* from water kefir (Jakob et al., 2013). A possible origin in the added fruit(s) cannot be excluded either.

Nitrogen flow within the water kefir grain ecosystem

Apart from hydrolysing sucrose and making the liberated monosaccharides available to other members of the consortium, yeast have the important role of providing the LAB and other community members with peptides and amino acids. While early studies suggested a possible parasitic relationship between *Zygotorulaspora florentina* and *Lb. hilgardii*, to the detriment of *Z. florentina* (Leroi and Pidoux, 1993), recent studies have demonstrated that a mutualistic relationship exists the between LAB and yeast (Stadie et al., 2013; Xu et al., 2019a). Stadie et al. (2013) showed in co-cultivation experiments between yeast (*Saccharomyces cerevisiae* or *Zygotorulaspora florentina*) and LAB (*Lb. hordei* or *Lb. nagelii*) that the growth of both microorganisms improved compared to single culture of either microorganisms. The yeast were found to provide the lactobacilli with the amino acids for the latter were auxotrophic - that is, unable to synthesis the required amino acid(s) themselves. For example, *Z. florentina* supplied *Lb. nagelii* with arginine in the form of arginine-containing compounds; in fact, *Z. florentina* only excreted amino acids essential for *Lb. nagelii* in co- or in mixed-culture, but not when they are cultivated alone. It was suggested that cocultivation of the two microorganisms partially affects autolysis of the yeast or triggers other mechanisms of (selective) nutrient release e.g. production of signalling molecules by the LAB which could modify yeast membrane permeability leading to autolysis. This is supported by the finding that a medium pre-fermented with *Z. florentina* could not support the growth of *Lb. nagelii*. The study also revealed that both *Z. florentina* and *S. cerevisiae* supply vitamin B6 to *Lb. hordei*. The trophic influence of the yeast on the lactobacilli was greater with *Z. florentina* compared *S. cerevisiae*, with higher levels of growth observed with the former. While yeast supply essential amino acids and vitamins, they benefit from the growth of LAB through the production of organic acids by the latter, which optimises the medium for the growth of the yeast by decreasing the pH (figure 2) (Stadie et al., 2013). This reliance of the lactobacilli on

the provision of essential nutrients by the yeast was confirmed in follow-up studies. Xu et al. (2019a, b) combined physiological, genomic and proteomic analysis of *Lb. hordei* to further delineate and illustrate the relationship between LAB and yeast (Xu et al., 2019b, 2019a). No gene encoding a cell wall-bound proteinase was found in the genome of *Lb. hordei* (strain TMW 1.1822); however it expressed on a proteome level the complete uptake system for peptides (oligopeptide transport system, Opp) and numerous peptidases; such peptides likely originate either from the fruits in the water kefir directly or via the proteolytic activity of plant or microbial enzymes or as excreted products of yeast or microbes of the consortium. Analysis of the genome also revealed biosynthesis pathways for 12 amino acids and incomplete pathways for 8, in addition to transporters for those amino acids for which the strain is auxotrophic. This suggests that such essential amino acids, originating from fruit material or yeast, as discussed, are assimilated (Xu et al., 2019a). In the presence of *S. cerevisiae*, and correlating with enhanced growth, the proteome of *Lb. hordei* was observed to be significantly affected, with upregulation of proteins involved in amino acid, carbohydrate and nucleotide metabolism and cell wall biosynthesis. Competition for the limited nitrogen resources was suggested as a reason for the differentially expressed genes in the presence of yeast (Xu et al., 2019b).

Other interactions occurring between microbial community members

Apart from nitrogen exchange, other mutualistic cross-feeding interactions may occur between members of the water kefir consortium. *Lb. hordei* has been shown to have the ability to utilise mannitol (produced by *S. cerevisiae* and other LAB species as discussed earlier) and gluconate as sole carbon sources (Xu et al., 2019a). Gluconate utilisation (and production) by *Lb. hordei* is possible via the phosphoketolase pathway (Xu et al., 2019a,

2019b) and is potentially a niche-specific adaption to a life in water kefir. As such, *Gluconobacter* species, if present could oxidise glucose (itself a potential by-product of fructosyltransferase activity, as mentioned earlier) to gluconate, with the latter serving as an additional energy source for *Lb. hordei* (Xu et al., 2019b). Proteomic analysis has also suggested that citrate present in the water kefir (originating from fruit such as lemon, or yeast) can be readily consumed by *Lb. hordei*, preferentially in the presence of *S. cerevisiae* and could be used as a carbon source and electron acceptor (Xu et al., 2019b).

A number of other compounds and potential modulators of water kefir flavour and aroma are also produced. *Lb. hordei* has the capacity to produce diacetyl, acetoin and 2,3-butanediol from pyruvate, compounds which could also modulate the sensory characteristics of the water kefir (Xu et al., 2019a). Indeed, redirection of pyruvate from the production of lactic acid and acetic acid to 2,3-butanediol has been demonstrated for *Lb. hordei* in the presence of *S. cerevisiae* and may represent a mechanism for optimising redox balance and reducing acid and ethanol stress (Xu et al., 2019b). In addition, aside from ethanol and carbon dioxide production by yeast, a number of volatile esters and higher alcohols have also been detected in water kefir, including isoamyl acetate, isoamyl alcohol, ethyl acetate, 2-methyl-1-propanol, ethyl octanoate, ethyl decanoate and ethyl hexanoate (Laureys and De Vuyst, 2014).

Aside from some of the primary mutualistic metabolic interactions described here, it is likely that other, as yet undiscovered interactions exist, some of which may be more parasitic than mutualistic. In addition, whilst here certain metabolic processes or roles have been ascribed to particular species, it is likely that other yeast and bacteria could play similar roles and that there is redundancy in the roles or processes they perform. This is particularly likely given the geographical diversity and origin of these grains, and the associated diversity in the microorganisms and species present. For example, *Lb. hilgardii*, described as the primary producer of granule forming EPS, has not always been detected in analysed water kefirs

(Gulitz et al., 2011), as is likewise for *Z. florentina*, a key stimulator of *Lactobacillus* growth (Marsh et al., 2013). Thus, other bacteria and yeast species must be important and fill the niche gap in these cases.

Safety of water kefir and potential beneficial effects

Safety of water kefir

Due to the pH of water kefir being typically below pH 4.5, even at the start of fermentation, and generally decreasing further thereafter, accompanied lactic acid and acetic acid production (Laureys et al., 2019, 2018; Laureys and De Vuyst, 2014), the risk of the growth of undesirable microorganism is low (FSAI, 2019). However, this is the case when using dried figs; use of other fruits may not provide such a low pH and therefore protection (Randazzo et al., 2016). Nevertheless, the tap water is typically boiled during the water kefir preparation. The substrates added, for example, fruits, may contribute undesirable microorganisms (e.g. *Enterobacteriaceae* and/or *Pseudomonas*), however the risk may be reduced if the fruits are peeled, washed and/or added the water for kefir preparation while it is still near boiling. Alternatively, the preparation of fruit extracts allows for easier processing of the substrate material, making it amenable to pasteurisation, which has been shown the reduce the microbial contaminant levels (Randazzo et al., 2016). In addition, with the exception of some *Enterobacter* species (Waldherr et al., 2010; Zanirati et al., 2015), pathogenic microorganisms have not been isolated or reported in water kefir grains (Table 2).

While the ethanol content which can range between 0.02–2.0 % (Laureys and De Vuyst, 2014; Martínez- Torres et al., 2017) could be another hurdle against undesirable microorganisms, it is generally produced late in the fermentation; in addition, in beverage products which are typically marketed for their health benefits, significant alcohol levels are undesirable, especially if labelled as non-alcoholic. In many countries the threshold below which the ethanol content must be to be labelled as non-alcoholic is 0.5% ABV, but this can vary by country (Bellut and Arendt, 2019). However, as with other types of fermented

beverages e.g. kombucha, if the product is unpasteurised and contains live microorganisms, there is a risk that continued fermentation in the bottle could result in continued ethanol formation during storage. This is a real potential issue when selling live, unpasteurised products, especially as many producers and secondary wholesalers do not employ cold shipping, which the authors have observed. In addition, many kefir producers opt to sell products with remaining live (unpasteurised) cultures in the bottle, as this can be a positive selling point with consumers because of the purported health benefits.

Potential health benefits

Many studies have reported nutritional and health benefits associated with regular consumption of milk kefir; however, few studies have examined if such benefits can be translated to water kefir. The myriad purported health benefits associated with milk kefir consumption include anti-inflammatory, anti-hypertensive, antioxidant, anti-allergenic and anti-carcinogenic effects, a hypocholesterolaemic effect, modulation of plasma glucose, and antibacterial and healing effects. In addition, there is improved digestion and tolerance of lactose (Rosa et al., 2017). However, many of the health benefits associated with milk kefir are linked, as such, to the properties of, and effects of fermentation on its substrate, milk, and therefore any translation of such benefits to water kefir are likely moot. Furthermore, as with other fermented beverages, most studies supporting beneficial effects of milk kefir have been performed in animal models and there is a great need for rigorous systematic clinical trials to truly support any claimed health benefits (Lynch et al., 2019; Rosa et al., 2017). Nonetheless, in the same way that milk kefir is a source of potentially 'probiotic', beneficial microorganisms, so too this applies to water kefir (Zanirati et al., 2015); however, it is important to recognise that true, so-called probiotic effects are strain-specific and that no

bacterial strain has received approval to be claimed as probiotic to date in the EU (Von Wright, 2019). Notwithstanding the difference in substrate, considering that in milk kefir, many of the beneficial effects are associated with the fermentative action of LAB and yeast, which produce bioactive components such as polysaccharides and peptides, the fact that water kefir is composed of a similar type of microbiota suggests that there is potential for such effects and activities in the latter also.

The antibacterial activity of milk kefir has been attributed to the presence of organic acids, hydrogen peroxide, acetaldehyde, carbon dioxide and bacteriocins produced through fermentation (Rosa et al., 2017). Silva et al, (2009) demonstrated antimicrobial activity during kefir fermentation, using kefir grains to ferment different sugar sources, namely, molasses, demerara sugar, and brown sugar. Brown sugar promoted the greatest antimicrobial activities, against the microorganisms *Candida albicans*, *Salmonella typhi*, *Shigella sonnei*, *Staphylococcus aureus* and *Escherichia coli* (Silva et al., 2009). The inhibition of the fungus *Aspergillus flavus* by water kefir has also been demonstrated (Gonda et al., 2019). The immunomodulatory and anti-inflammatory capacity of milk kefir has been associated with various bioactive compounds produced during the fermentation process (Rosa et al., 2017). With regard to water kefir, few studies have investigated its immunomodulatory and anti-inflammatory activity. Diniz et al. (2003) observed a significant inhibition of granuloma tissue formation and paw edema in rats fed Tibetan mushroom (water kefir) solution and associated grains (Diniz et al., 2003). A later study by the same group investigated the anti-inflammatory properties of an isolated carbohydrate fraction from sugary kefir (fermented on molasses). While an anti-inflammatory capacity was not demonstrated *in-vitro* in cellular respirometry and macrophage cell culture, the carbohydrate fraction significantly inhibited paw edema in rats fed with same (Moreira et al., 2008). The authors did not characterise the carbohydrate fraction. The antioxidant capacity of water kefir and microorganism-derived

components (EPS) has also been tested. Alsayadi et al. (2013) evaluated the antioxidant activity of water kefir and its extract using the 2,2,-diphenyl-1-pricrylhydrozyl (DPPH) method, and inhibition of ascorbate autoxidation and the reducing power of the water kefir were also determined. Strong DPPH radical scavenging and ascorbate oxidation inhibition activity were found (Alsayadi et al., 2013). Similarly, high DPPH scavenging activity was observed with culture supernatants from water kefir, in particular from *Acetobacter pasteurianus* (Luang-In et al., 2018). In addition, isolated EPS from *A. pasteurianus* displayed high ferric reducing antioxidant power (Luang-In et al., 2018). Bioactive peptides, produced through the action of fermentative microorganisms have been implicated as effectors of certain health effects of milk kefir, for example, its anti-hypertensive effects (Rosa et al., 2017). In a water kefir medium, Mechmeche et al. (2019) showed the formation of bioactive peptides (amides and aromatic compounds) from a substrate of tomato seed protein isolate and the fermented medium also displayed high radical scavenging activity (Mechmeche et al., 2019). Finally, milk kefir has been reported to have a hypocholersterolemic effect, potentially mediated through the action of the kefir grain LAB (e.g. via binding and sequestering cholesterol or through the action of bile salt hydrolase activity) (Rosa et al., 2017). This has similarly been shown for water kefir by Rocha-Gomes et al. (2018). Wistar rats that received a diet containing water kefir prepared with brown sugar over 42 days, showed an improved plasma and hepatic lipid profile in comparison to the control group, with water kefir being more effective than milk kefir (Rocha-Gomes et al., 2018).

Other compounds within and specific to water kefir could also have beneficial health effects, although their level within water kefir can be low and thus the effect negligible. For example EPSs produced by LAB from sucrose, such as dextran and levan, could be potentially prebiotic (Fels et al., 2018; Lynch et al., 2018). Mannitol, which can be produced by some

water kefir microorganisms (e.g. *Leuconostoc* species) has a sweet taste and has been shown to have antioxidant activity (Laureys and De Vuyst, 2014).

Ultimately, differences in the method of production, and factors such as grain origin, substrate, fermentation temperature and time will influence the potential health promoting components within, and benefits associated with consumption of, water kefir. For example, extended fermentation time, up to two weeks could be used to produce a water kefir vinegar-type beverage, the benefits of which may be similar to those purported for traditional vinegar consumption (Lynch et al., 2019; Martínez- Torres et al., 2017). Importantly, as water kefir is primarily a sugar (sucrose)-based beverage, producers need to be mindful of the residual sugar levels in the final product, which, given the detrimental effects of sugar consumption on health and particular concern related to the over-consumption of sugar-sweetened beverages, should be low, especially as these products are often marketed as health drinks (Lustig, 2013; WHO, 2015).

Water kefir market

Due to consumer lifestyle trends towards increased awareness of health and wellness, many are opting to choose, more often, non-dairy products. Alternatively, consumers may avoid dairy consumption for health (lactose intolerance, allergies) or ethical (animal welfare) reasons. Thus, today, these is increasing research into alternatives to dairy products and such non-dairy products are becoming increasing popular worldwide and constituting an ever large part of companies product portfolios (Corona et al., 2016). Therefore, water kefir is the ideal non-dairy alternative to milk kefir, and not just for vegetarian and vegan consumers.

One great advantage that water kefir has over milk kefir is the diverse range of substrates from which it can be produced, ranging from different forms of sugar, to various fruits and vegetables, to non-dairy alternatives, from fresh or dried (fruit) substrates, and from raw, minimally processed substrates or extracts (Corona et al., 2016; Fiorda et al., 2017; Randazzo et al., 2016; Tu et al., 2019) (figure 6). Furthermore, the ability to modulate the fermentation process and parameters (nutrient level, oxygen, fermentation temperature and time) introduces, still, further scope for creating unique products and flavours. Thus, the potential for flavour and aroma modulation, and product development and differentiation is huge.

A report from *Mintel* (08/2019) suggests water kefirs are seeking to capitalise on consumers' preference for category blurring, multiple functionalities and adventurous flavour combinations. Furthermore, the report suggests that water kefirs satisfy these preferences because, they are considered a vegan version of dairy fermented drinks and also compete with water for hydration, juices for fruit content and nutrition, ready-to-drink products for convenience, they are linked with probiotic content and digestion, and because they have a clear advantage on taste compared to kombucha (Buchet, 2019). Similarly, a report by NewNutrition Business highlighted that while in the past the probiotic market was dominated

by dairy, today, amid an increasing awareness of the benefits of fermentation, consumers are getting their probiotics from a much wider variety of foods. Consumers are open to fermented and probiotic benefits in more types of foods than in the past, and this is reinforced if the food is in some way 'traditional' with a back-story of historic usage (Mellentin, 2019). The traditional 'home-made' view of water kefir ticks these boxes. As evidence of this trend, in the USA, consumption of fermented foods in restaurants was up 149% in 2018. However, in the period between 2017 and 2019, sales of milk kefir fell 25%, with the space being eroded by kombucha, and consumers moving with the latest and newest ideas and trends (Mellentin, 2019). Water kefir, with its broad appeal, could capitalise on this trend.

Conclusions and Future Prospects

The granules that we today identify as water kefir grains most likely arose in different geographical locations, possibly at different time points. While all contain a mixture of LAB, yeast and AAB, the diversity, levels, and those species that dominate can vary, between grains from different geographical locations, but even within the same region. EPS-producing LAB are key members in the water kefir granule, however, the main EPS producers and the characteristics of the EPS produced can vary between water kefir grains. Nevertheless, at least one LAB producing a water insoluble dextran which forms the main structural component of the water kefir grain, is always present. Yeast are key microorganism within the grain community, with *Saccharomyces cerevisiae* being identified in virtually all water kefir grains. Yeast have a key role in making peptides and amino acids available to the LAB through a mutualistic relationship, while the LAB positively modify the environment for yeast via a reduction in pH. Acetic acid bacteria may or may not be present at significant levels in the water kefir, this being primarily dependent on the presence of oxygen. Aside from sucrose, the primary carbon source in water kefir, the nitrogen source (e.g. dried fruits) used can vary; however, different substrates can have positive or negative effects – effecting the fermentation stability, metabolite production and grain growth. Important factors aside from the substrate include the characteristics of the water and the availability of calcium. Many attempts to re-create a stable water kefir community by recombining isolated strains have failed; however, proposals for the minimum number of strains necessary for an efficient fermentation have been put forward. In general, the microbial safety of water kefir is assured as a consequence of its low pH; nonetheless, producers must be aware of the potential for secondary fermentation and continued ethanol production in the bottle. Various health benefits have been associated with water kefir, which are primarily associated with the activity of its fermentative microorganisms, but like other fermented beverages, these claims

are primarily based on *in-vitro* tests in animal models; thus, rigorous human clinical trials are warranted. Thus, future research should focus on areas such as further understanding the interactions and cross-talk between microbial community members with a view to modulation beverage flavour and aroma or indeed functional and health benefits of consumption. In addition, more research in the area of defined starter cultures for water kefir production is required, particularly to enable a shift from a product that is currently mainly produced on a small scale to one that is amenable to production on a semi-industrial or industrial scale.

In conclusion, water kefir beverage represents a promising alternative to dairy kefir at a time of change in consumer habits and a move towards increasing consumption of non-dairy products. While production is relatively straight forward, a thorough understanding of the composition, community dynamics and production processes are needed in order to fully exploit the scientific and commercial potential of this product.

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Tables and figures

Table 1: Comparison of water kefir and milk kefir

Water kefir	Milk kefir
Produced using water kefir grains	Produced using milk kefir
Main substrate is a sucrose solution to which dried fruits or fruits extracts is added	Main substrate is milk from a bovine mammal e.g. cow or goat milk
A greater diversity of substrates can be fermented	The diversity in terms of different substrates that can be fermented is lower
Grains are transparent, mucilaginous, and less resilient	Grains are white or cream colour and more resilient
The grain exopolysaccharide is primarily composed of α-glucans	The grain exopolysaccharide is primarily composed of kefiran
Acetic acid bacteria species more prevalent	Acetic acid bacteria species less prevalent
Saccharomyces yeast species are dominant	*Saccharomyces* yeast species are a minor component
Lactococcus bacterial species rarely present	*Lactococcus* bacterial species more dominant
Candida yeast species rarely found	*Candida* yeast species more likely to be present
Suitable for consumers who are vegan or lactose intolerant	Not suitable for consumers who are vegan or lactose intolerant

Table 2: Studies examining the microbial composition of water kefir

Lactic acid bacteria species	Acetic acid bacteria species	Other bacterial species	Yeast species	Fermentation substrate	Culture -	Fermentation time (single)	Fermentation temperature	Country (source of grains)	Reference
Lactobacillus (Lb.) brevis, Streptococcus (St.) lactis	n.d.	n.d.	*Saccharomyces (S.) cerevisiae*	-	-	-	-	Not stated, said to be Tibi grains	(Horisberger, 1969)
Lb. casei, Lb. hilgardii, Leuconostoc (Leuc.) mesenteroides ssp. *dextranicum, Lb. casei* ssp. *rhamnosus, Lb. plantarum, St. lactis, St. cremoris*	n.d.	n.d.	*Zygosaccharomyces (Zy.) florentinus, Torulaspora pretoriensis, Kloeckera (Hanseniaspora) apiculate, Candida (C.) lambica, C. valida*	6% sugar-water solution	Dependant	Not stated	Room temp.	France	(Pidoux, 1989)
Lb. casei subsp. casei, Lb. casei subsp. *pseudoplantarum Leuc. mesenteroides* subsp. *mesenteroides, Pediococcus spp., Lb. buchneri, Lb. fructiovorans, Lb. collinoides,*	n.d.	n.d.	*S. cerevisiae, Hanseniaspora (H.) valbyensis, H. vinae, S. florentinus, S. pretoriensis*	Not stated	Dependant	N/A	N/A	Italy	(Galli et al., 1995)
Short and long rod-shaped lactobacilli, dominance of bacterial cocci	n.d.	n.d.	*Zy. florentinus, H. valbyensis*	2 tablespoons grains in 500 ml tap water plus 30 g sucrose and 1/2 a dried fig	N/A	3 d	Approx. 22 °C	Germany	(Neve and Heller, 2002)
Lb. paracasei, Lb. parabuchneri, Lb. kefiri, Lactococcus lactis, Lb. casei, Lb. paracasei subsp. *paracasei, Leuc. citreum, Lb. paracasei* subsp. *tolerans, Lb. buchneri*	*Acetobacter (A.) lovaniensis*	n.d.	*S. cerevisiae, Kluyveromyces lactis, Lachancea meyersii, Kazachstania (Kz.) aerobia*	250 g grains in 2.25L distilled water with sugar (5% of brown sugar)	Dependant and independent	24 h	25 °C	Brazil	(Magalhaes et al., 2010)
Lb. hordei, Lb. nagelii, Leuc.. mesenteroides, Leuc.. citreum	*A. fabarum, A. orientalis*	n.d.	*S. cerevisiae, Lachancea (La.) fermentati, H. valbyensis, Zygotorulaspora (Z.) florentina*	10% sucrose water kefir, containing two dry figs and a slice of organic lemon	Dependent	3 d	21 °C	Germany	(Gulitz et al., 2011)
Lb. casei, Lb. sunkii, Lb. kefiri, Lb. satsumensis, Lb. paracasei, Lb. helveticus, Lb. buchneri	*Gluconobacter (G.) liquefaciens, A. lovaniensis*	*Bacillus cereus*	*S. cerevisiae, Pichia (P.) cecembensis, Yarrowia lipolytica, P. membranifaciens, P. caribbica, P. fermentans, C. valdiviana, Zy. (Lachancea) fermentati, Kz. aerobia*	Not stated	Dependant and independent	N/A	N/A	Brazil	(Miguel et al., 2011)
Lactobacillus, Leuconostoc, Bifidobacterium	*Gluconacetobacter, Acetobacter*	*Zymomonas*	*Dekkera anomala, Dekkera (D.) bruxcellensis, S. cerevisea, H. valbyensis, H. vineae, La. fermentati, Torulaspora, Zy. lentus, Meyerozyma caribbica*	10% sucrose, one dried, organic fig	Independent	24 h	25 °C	Various (UK, Canada, United States)	(Marsh et al., 2013)
Lb hordei, Lb. nagelii, Lb. hilgardii, Lb satsumensis, Leuc. citreum,	n.d.	n.d.	Not studied	100 ml/L of fig extract + 80 g/L	Independent	3 d	21 °C	Germany	(Gulitz et al., 2013)

Leuc. mesenteroides, Bifidobacterium (B.) psychraerophilum				sucrose.					
Lb. casei/paracasei, Lb. hilgardii, Lb. harbinensis, Lb. nagelii, Lb. hordei/mali, B. psychraerophilum/crudilactis,	*A. lovaniensis/fabarum*	n.d.	*S. cerevisiae, and D. bruxellensis*	6 g sucrose in 85 mL medium (medium = 65 mL tap water + 20 mL fig extract)	Dependant and independent (PCR-DGGE)	Up to 8 d	21 °C	Belgium	(Laureys and De Vuyst, 2014)
Lb. casei, Lactococcus lactis, Lb. perolens, Lb. parafarraginis, Lb. diolivorans, Oenococcus (O.) oeni, Lb. kefiranofaciens, Lb. hilgardii, Lb. satsumensis, Lb. nagelii, O. kitaharae	n.d.	*Klebsiella pneumoniae, Enterobacter ludwigii*	Not studied	Brown sugar solution (5% w/v)	Dependant and independent	24 h	Room temp.	Brazil	(Zanirati et al., 2015)
Lb. perolens, Lb. rhamnosus	*Gluconobacter japonicus*	*Bacillus cereus*	*S. cerevisiae, C. ethanolica, D. bruxellensis*	3% (w/v) brown sugar plus 2.6% (w/v) di-ammonium hydrogen orthophosphate	Independant	3 d	25 °C – 28 °C	Thailand	(Sarikkha et al., 2015)
Lb. hilgardii, Lb. nagelii, Lb. satsumensis, Lb. harbinensis, L. paracasei, Lb. mali/hordei, Lb. harbinensis, Oenococcus spp., B. aquikefiri	n.d.	n.d.	*S. cerevisiae, S. florentina, D. bruxellensis*	Various sugar concentrations, one to two dried figs	Dependant and independent	8 d	21 °C	Belgium	(Laureys and De Vuyst, 2017)
Lb. ghanensis, Lb. casei/paracasei, Lb. hilgardii	*A. orientalis, A. tropicalis and A. okinawensis*	*Pseudarthrobacter chlorophenolicus*	*S. cerevisiae, C. californica and P. membranifaciens*	100 mL of 5% panela* solution, 1 g grains	Dependant	8 d	26 °C	Mexico	(Martínez-Torres et al., 2017)
Lb. paracasei, Lb. hilgardii, Lb. nagelii, Lb. harbinensis, B. aquikefiri	*G. roseus/oxydans, A. indonesiensis, A. fabarum*	n.d.	*S. cerevisiae, D. bruxellensis*	10 g of sugar, 160ml of tap water, 5g of dried figs	Dependant and independent	3 d	21 °C	Belgium	(Laureys et al., 2018)
Lb. harbinensis, Lb. hilgardii, Lb. nagelii, Lb. paracasei, Lb. hordei/mali, B. aquikefiri, Candidatus O. aquikefiri	n.d.	n.d.	*S. cerevisiae, D. bruxellensis*	Unrefined cane sugar (7.1%, m/v) and fig extract (17.6%, v/v)	Independent	8 d	21 °C	Belgium	(Verce et al., 2019)
Lb. hilgardii, Lb. nagelii, Lb. paracasei, Leuc. pseudomesenteroides, Lb. harbinensis, Lb. mali/hordei, B. aquikefiri	n.d.	n.d.	*S. cerevisiae, D. bruxellensis*	50 g water kefir grains plus 10 g of sugar, 5 g of dried figs, to 160 ml of water	Dependant and independent	3 d	21 °C	Belgium	(Laureys et al., 2019)

Note: Original genus and species designations, as published, are shown

* Panela: a sugar cane product mainly composed of 80 - 89% sucrose, 10% reducing sugars and about 0.4% protein (Martínez-Torres et al., 2017).

n.d.: not detected

Table 3: Calcium content of various potential water kefir substrates

Product	FDC ID	Calcium (mg/100g)
Fig, dried, uncooked	326905	162
Figs, raw	173021	35
Raisins	341504	50
Apricot, dried, uncooked	341472	55
Banana, raw	341529	5
Apple, raw	341508	6
Plum, raw	341614	6
Potatoes, raw, skin	170032	30
Carrots, raw	342354	33
Grape juice, 100%	341731	11

Data source: USDA FoodData Central (FDC); fdc.nal.usda.gov

Table 4: General consequences of various factors on the water kefir fermentation

	Factor	Level	
		Low	High
Extrinsic	**Nutrient**	• Slow fermentation • High total residual carbohydrates • Low metabolite production • High pH values • Low or no grain growth • High abundance of AAB	• Fast fermentation • High metabolite production • High total residual carbohydrate • High (relative) pH • Lower LAB : yeast ratio • Lower ratios AcOH : EtOH, AcOH : lactic acid
	Buffering capacity	• Low pH values • Low grain growth	• High metabolite production • High total residual carbohydrate • High (relative) pH
	Oxygen	• Low abundance of AAB	• High abundance of AAB
tr in	**pH**	• Low grain growth	• High grain growth

	Grain growth	• Small grains • High viable counts • High metabolite production	• Large grains • Lower viable counts

Table 5: Sucrose utilisation by LAB species which have been associated with water kefir grains

Species	**Acid production from sucrose**	**EPS production from sucrose**	**Reference**
L. nagelii	+	+	(Bechtner et al., 2019a; Vos et al., 2011)
L. hilgardii	11 – 89% of strains	+	(Vos et al., 2011; Waldherr et al., 2010)
L. hordei/mali	+	+	(Rouse et al., 2008; Xu et al., 2018)
L. casei	+	-	(Vos et al., 2011)
L. paracasei	+	-	(Vos et al., 2011)
L. satumensis	+	+	(Côté et al., 2013; Vos et al., 2011)
L. harbinensis	+	-	(Miyamoto et al., 2005)
L. buchneri	11 – 89% of strains	-	(Vos et al., 2011)
Leuc. mesenteroides	+	+	(Jeanes et al., 1956; Vos et al., 2011)
Leuc. citreum	+	+	(Maina et al., 2008; Vos et al., 2011)

+, all strains positive for acid production from sucrose; EPS production from sucrose described in at least a single study

Figures

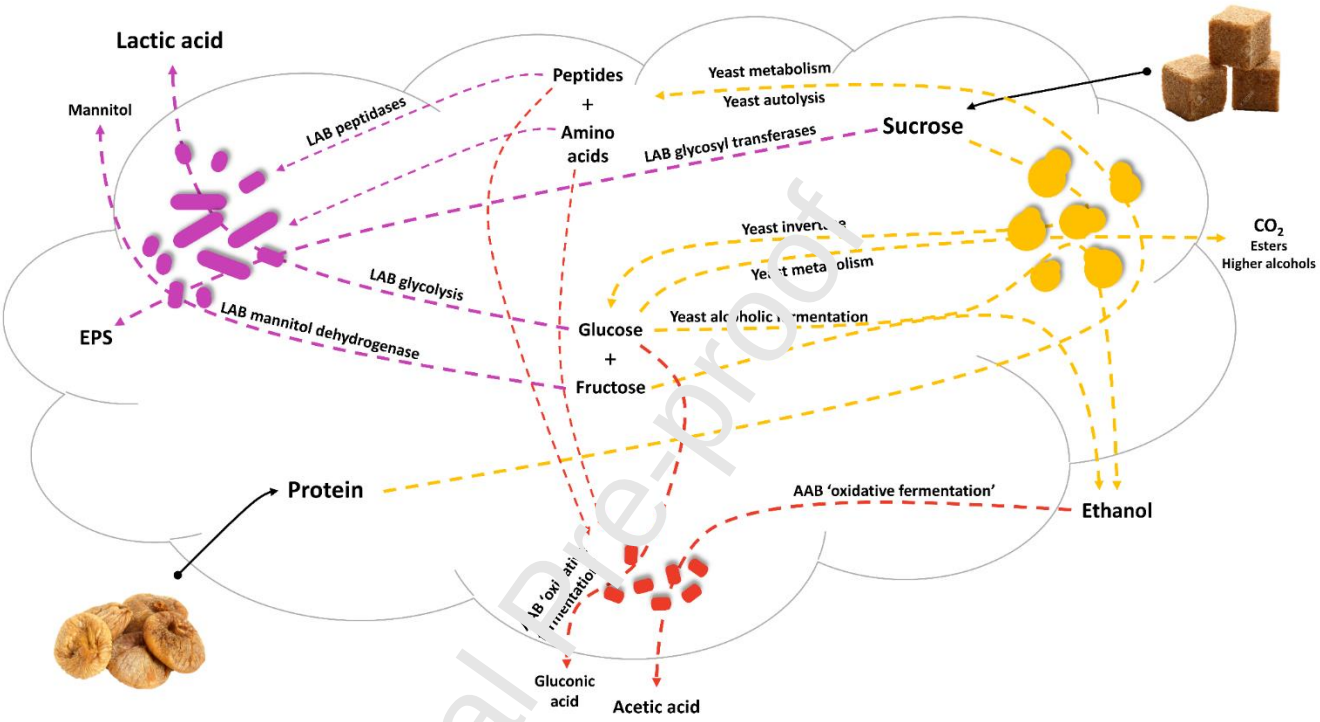

Figure 1: Primary metabolites and interaction between the water kefir microbiota

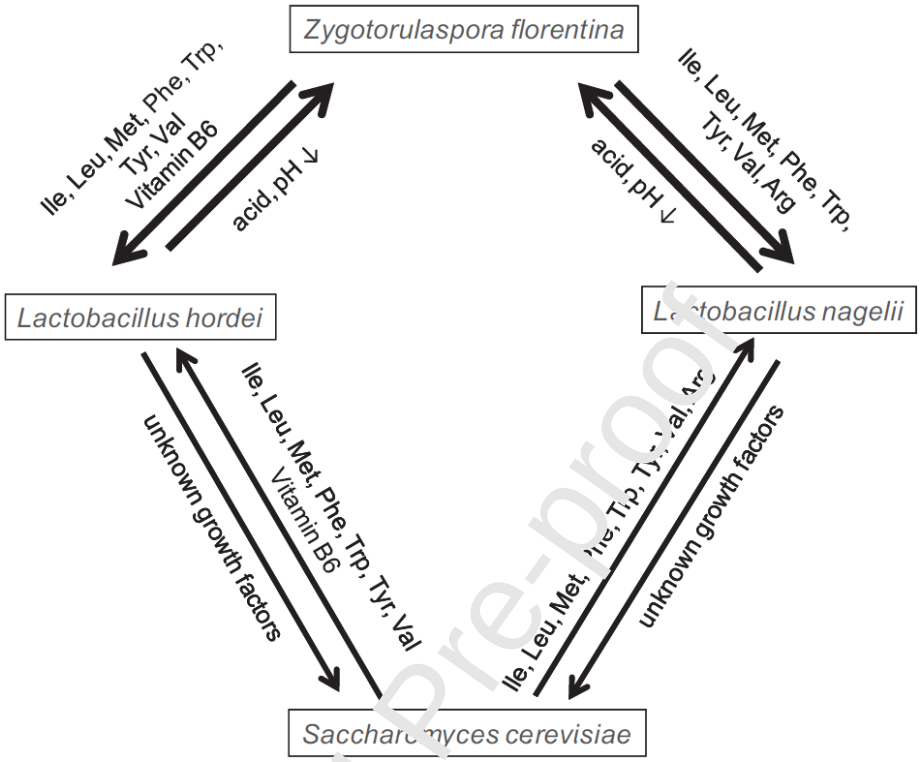

Figure 2: Mutualistic interactions between LAB and yeast in water kefir (source: Stadie et al. (2013))

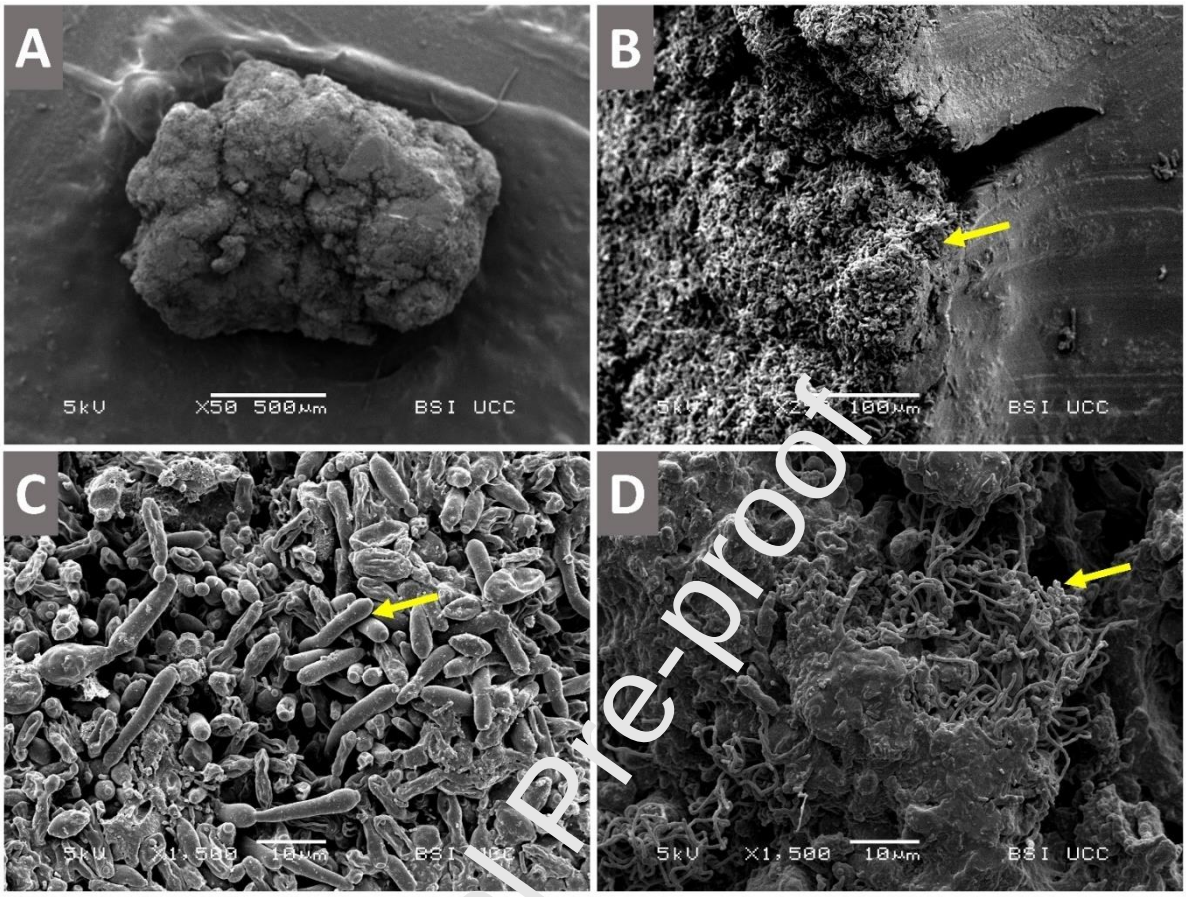

Figure 3: Scanning electron microscopy images of a water kefir grain. A. A water kefir grain (X50); B. A cut water kefir grain showing exposed face of the internal surface (smooth area) and microorganisms on the external surface (X250); C. Rod-shaped and elongated yeast cells on the water kefir grain surface (X1500) and D. Bacilli bacteria on the water kefir grain surface (X1500)

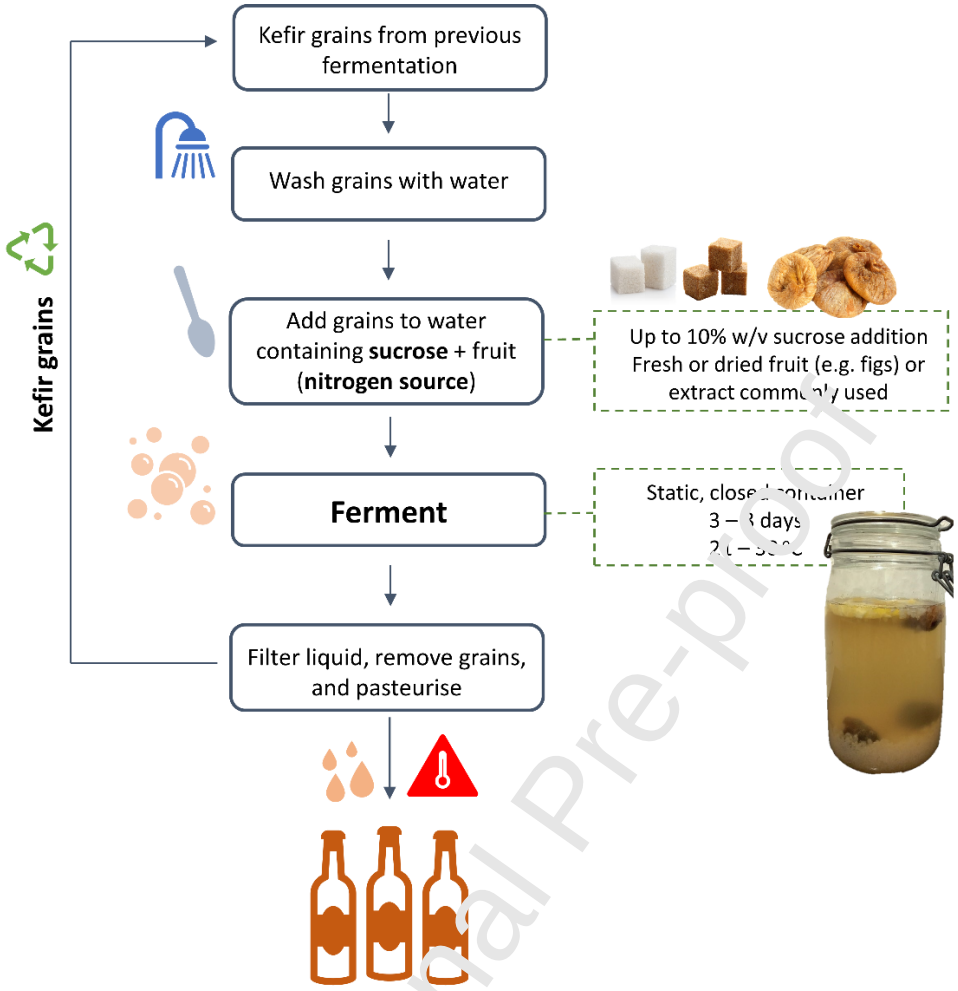

Figure 4: Steps in the production of traditional water kefir

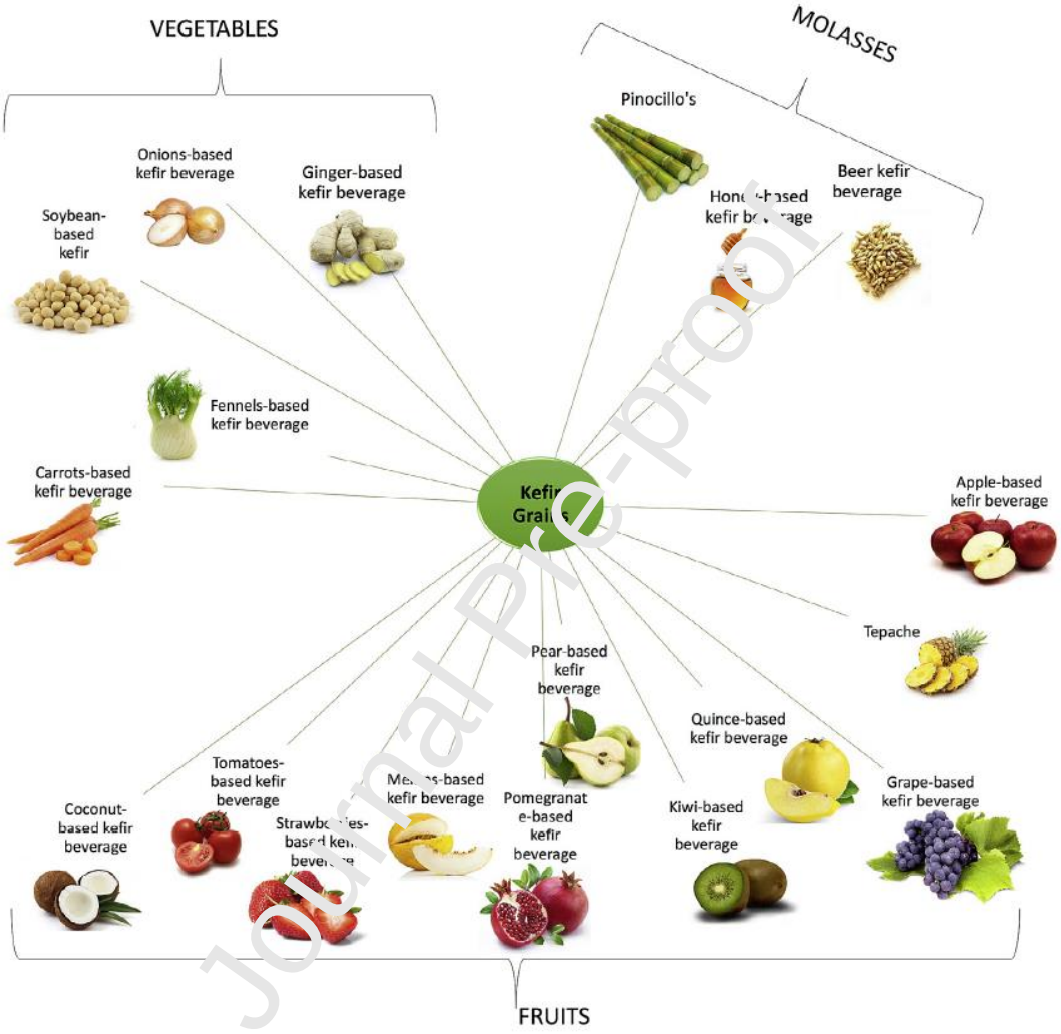

Figure 5: Some untypical substrates that can be used in water kefir preparation (Source: (Fiorda et al., 2017))

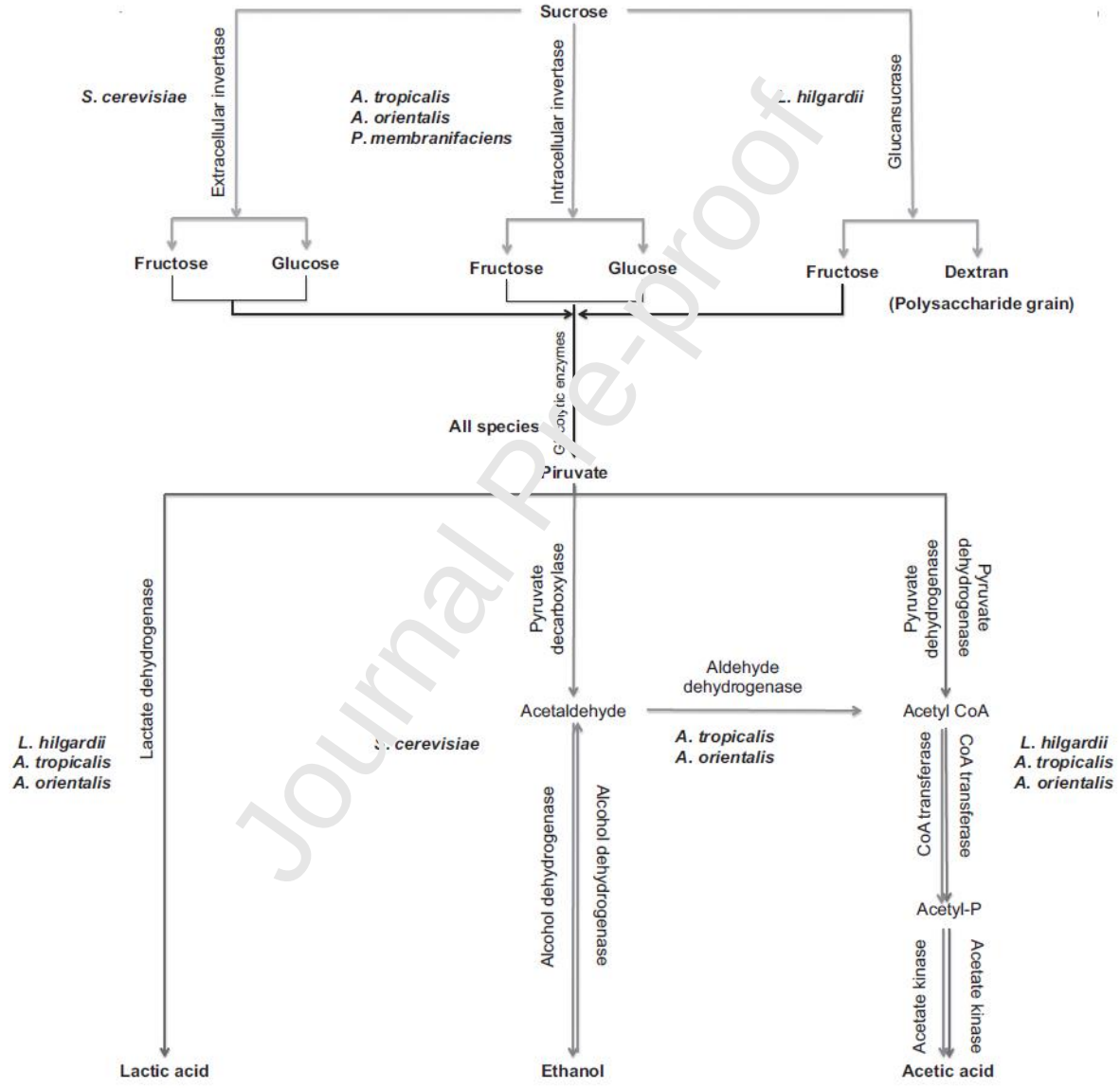

Figure 6: Hypothetical flux of carbon during a WK fermentation from sucrose (source: Martínez-Torres et al., 2017)

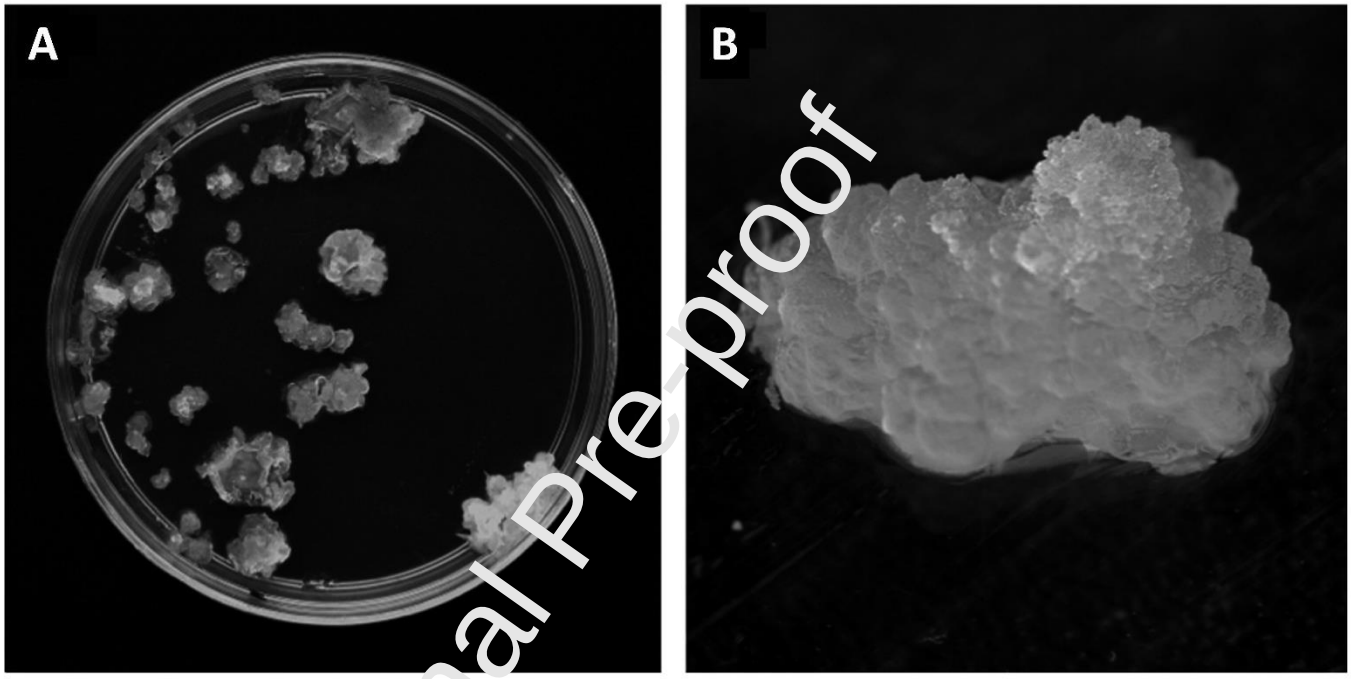

Figure 7: Colonies of L. hilgardii *on sucrose-casein peptone solid medium (A), showing the kefir granule-like colony structure (B)*

Highlights

- Water kefir is a sparkling, slightly acidic fermented beverage produced by fermenting a solution of sucrose, to which dried fruits have been added, with water kefir grains.
- Lactic acid bacteria, yeast and acetic acid bacteria are the primary microbial members of the sugary kefir grain.
- Which species predominate with the kefir grain appears to be dependent on the geographical origin of the grains and the fermentation substrate and conditions.
- Purported water kefir health benefits are related to the presence of potentially probiotic lactic acid bacteria.
- Water kefir is seen as a vegan alternative to milk kefir and purported health benefits related to the presence live microorganisms.
- As water kefir increases in popularity as a beverage there is a need for a thorough understanding of the biology and dynamics of water kefir.

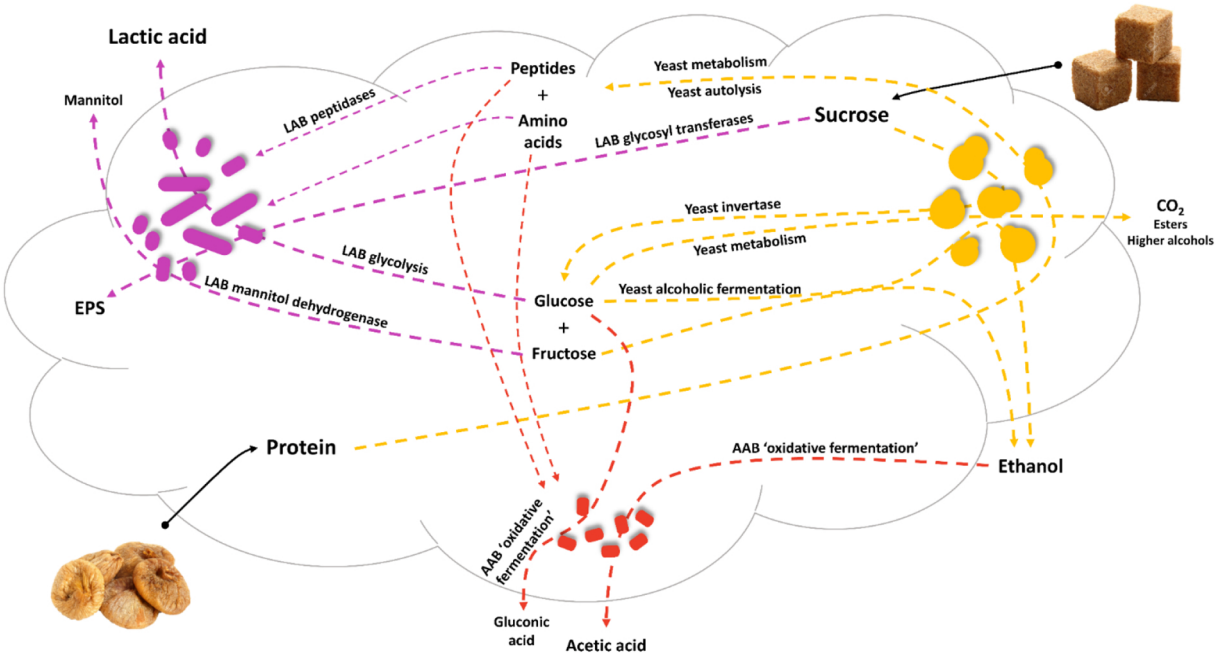
Lactic acid
Mannitol
LAB peptidases
Peptides
+
Amino acids
Yeast metabolism
Yeast autolysis
Sucrose
LAB glycosyl transferases
Yeast invertase
Yeast metabolism
CO_2
Esters
Higher alcohols
LAB glycolysis
EPS
LAB mannitol dehydrogenase
Glucose
+
Fructose
Yeast alcoholic fermentation
Protein
AAB 'oxidative fermentation'
Ethanol
AAB 'oxidative fermentation'
Gluconic acid
Acetic acid

Figure 1

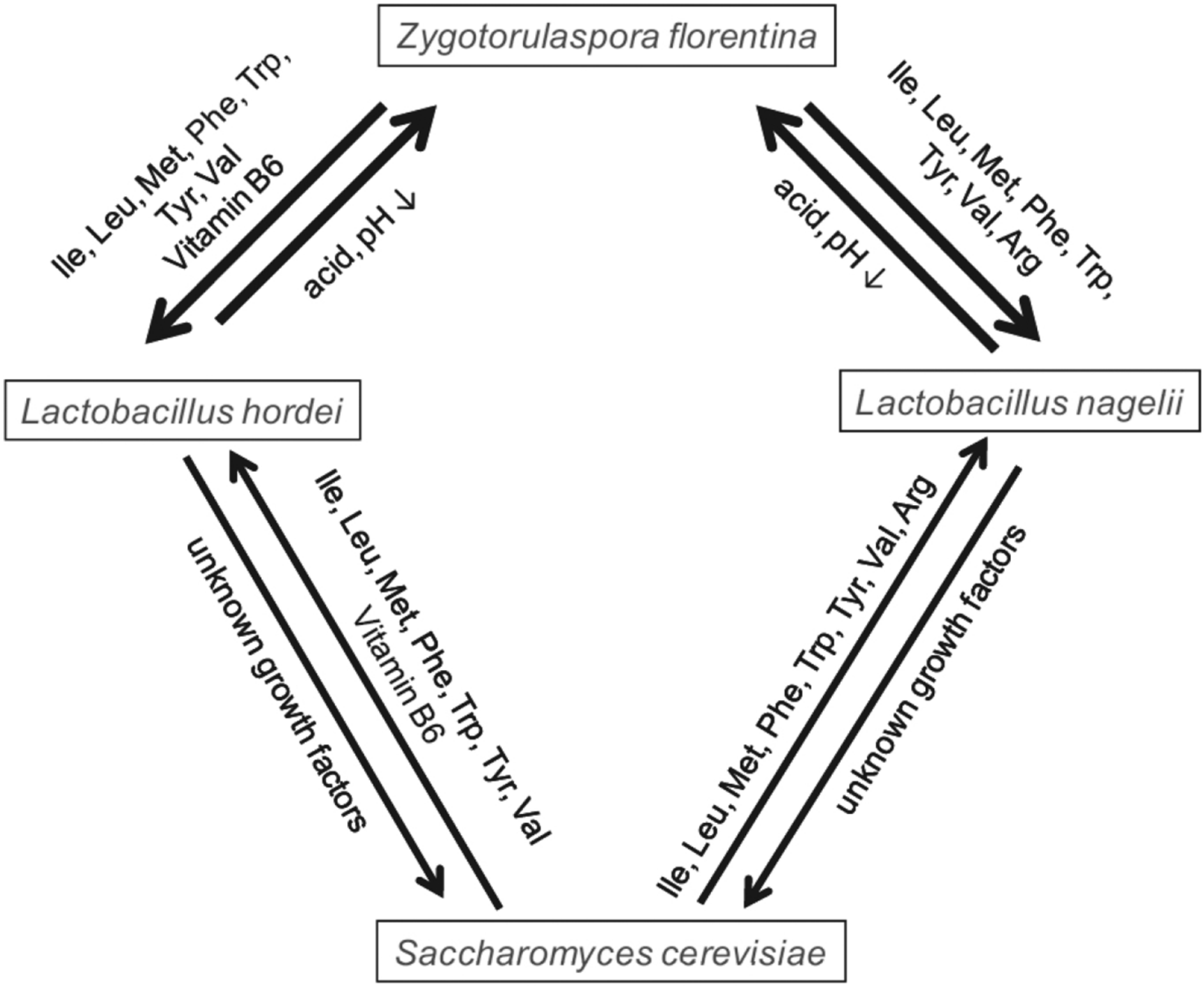
Zygotorulaspora florentina
Ile, Leu, Met, Phe, Trp, Tyr, Val Vitamin B6
acid, pH ↘
Ile, Leu, Met, Phe, Trp, Tyr, Val, Arg
acid, pH ↘
Lactobacillus hordei
Lactobacillus nagelii
unknown growth factors
Ile, Leu, Met, Phe, Trp, Tyr, Val Vitamin B6
Ile, Leu, Met, Phe, Trp, Tyr, Val, Arg
unknown growth factors
Saccharomyces cerevisiae

Figure 2

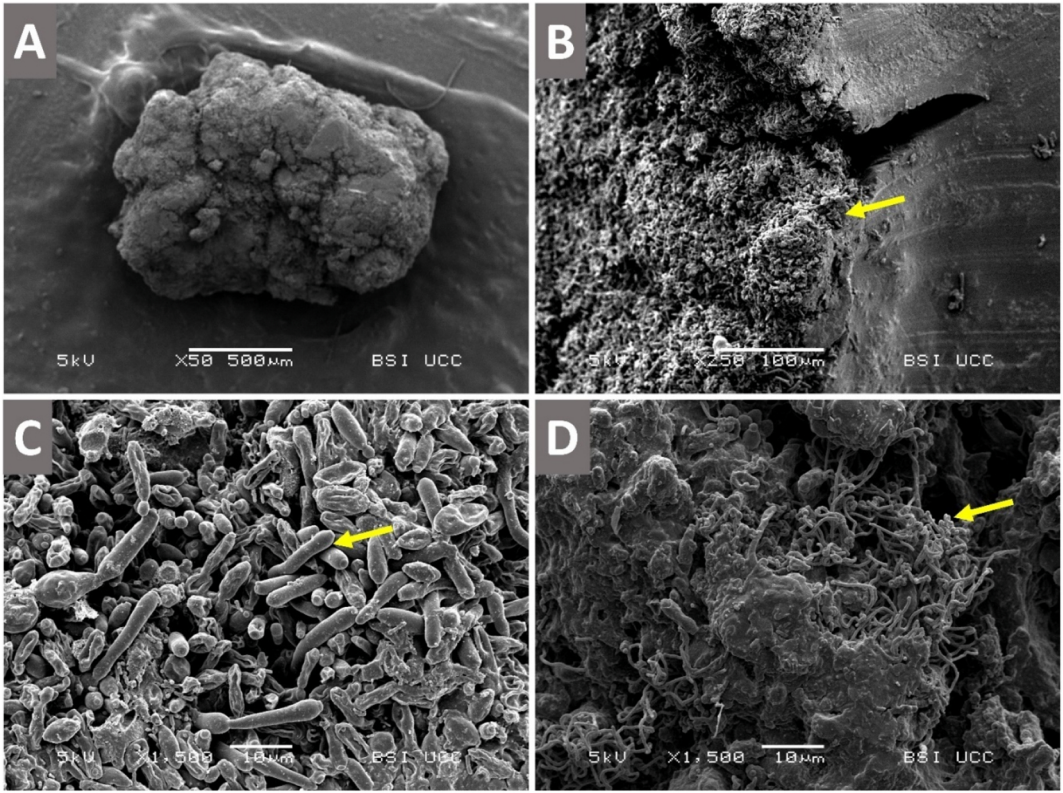
A
5kV X50 500µm BSI UCC
B
BSI UCC
C
5kV X1,500 10µm BSI UCC
D
5kV X1,500 10µm BSI UCC

Figure 3

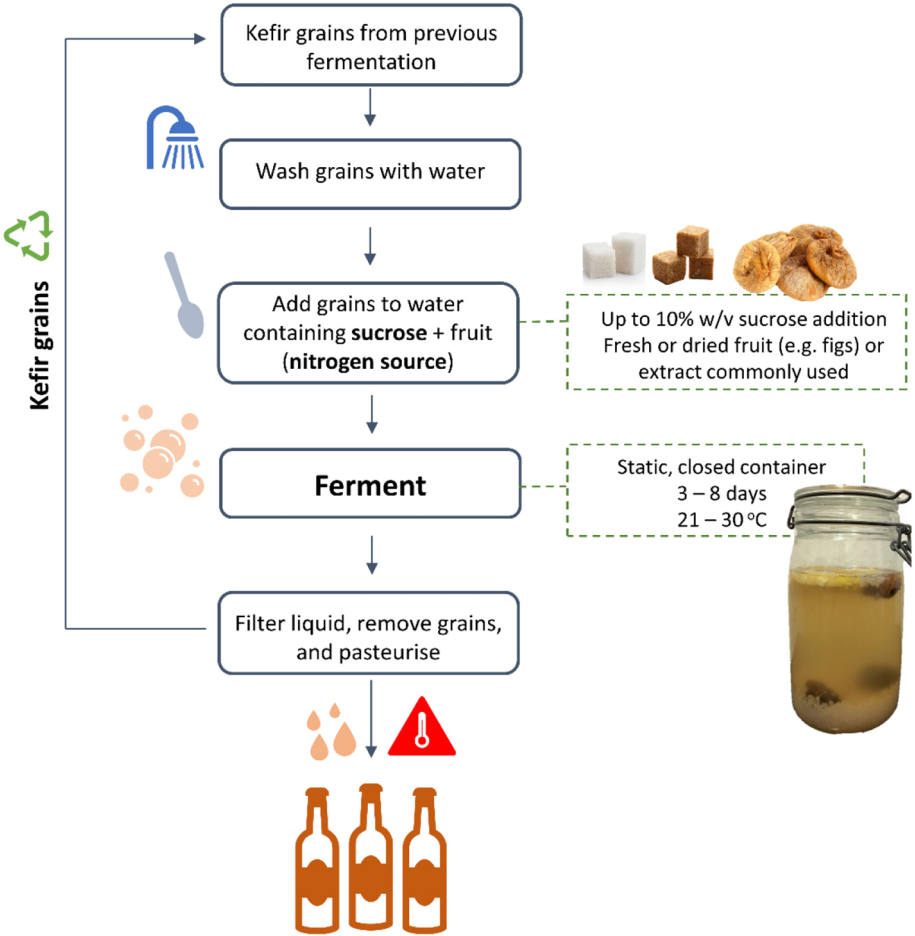
Kefir grains from previous fermentation
Wash grains with water
Add grains to water containing **sucrose** + fruit (**nitrogen source**)
Up to 10% w/v sucrose addition
Fresh or dried fruit (e.g. figs) or extract commonly used
Ferment
Static, closed container
3 – 8 days
21 – 30 °C
Filter liquid, remove grains, and pasteurise
Kefir grains

Figure 4

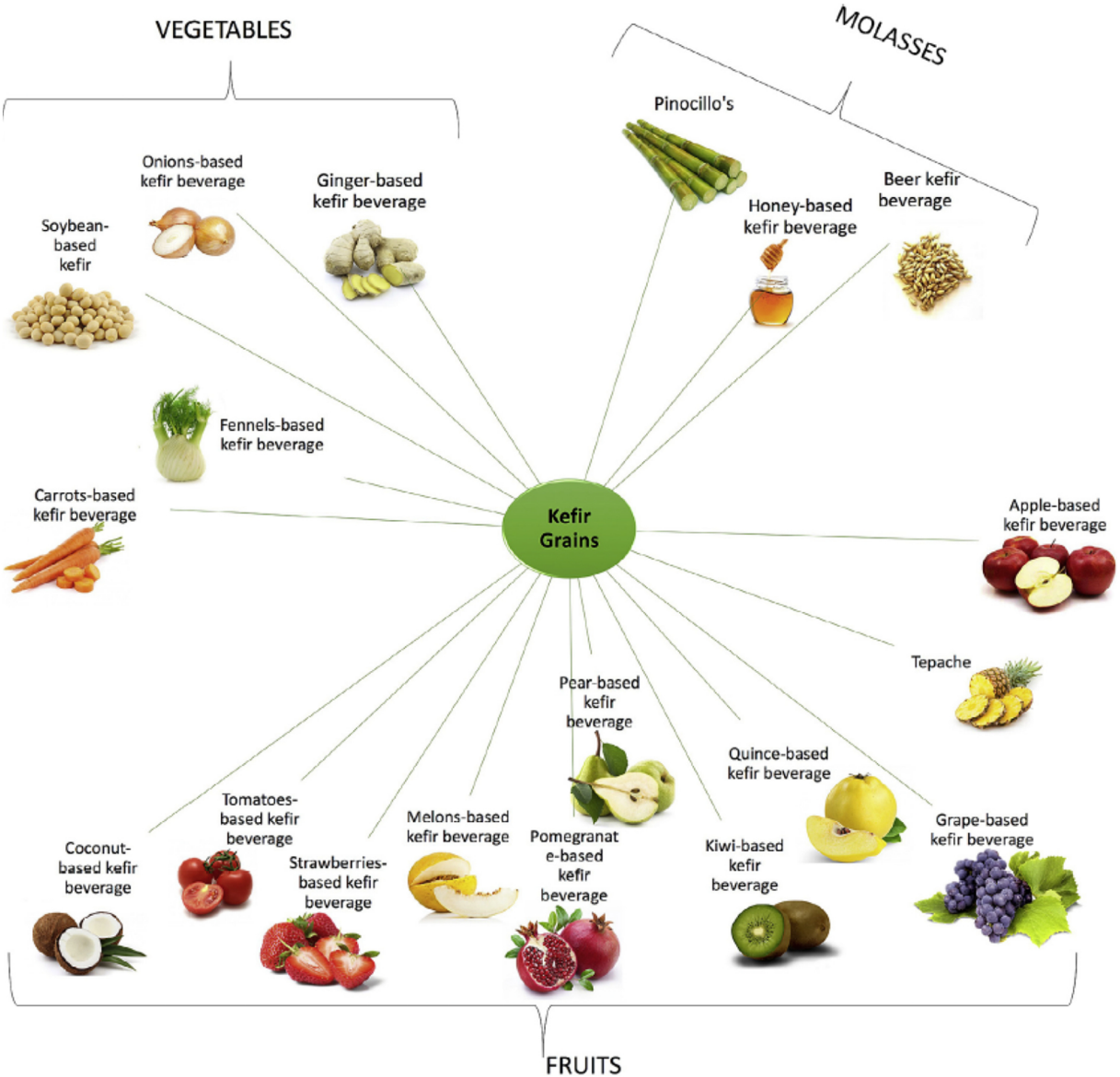
VEGETABLES
MOLASSES
Onions-based kefir beverage
Ginger-based kefir beverage
Soybean-based kefir
Pinocillo's
Honey-based kefir beverage
Beer kefir beverage
Fennels-based kefir beverage
Carrots-based kefir beverage
Kefir Grains
Apple-based kefir beverage
Tepache
Pear-based kefir beverage
Quince-based kefir beverage
Grape-based kefir beverage
Tomatoes-based kefir beverage
Melons-based kefir beverage
Pomegranate-based kefir beverage
Kiwi-based kefir beverage
Coconut-based kefir beverage
Strawberries-based kefir beverage
FRUITS

Figure 5

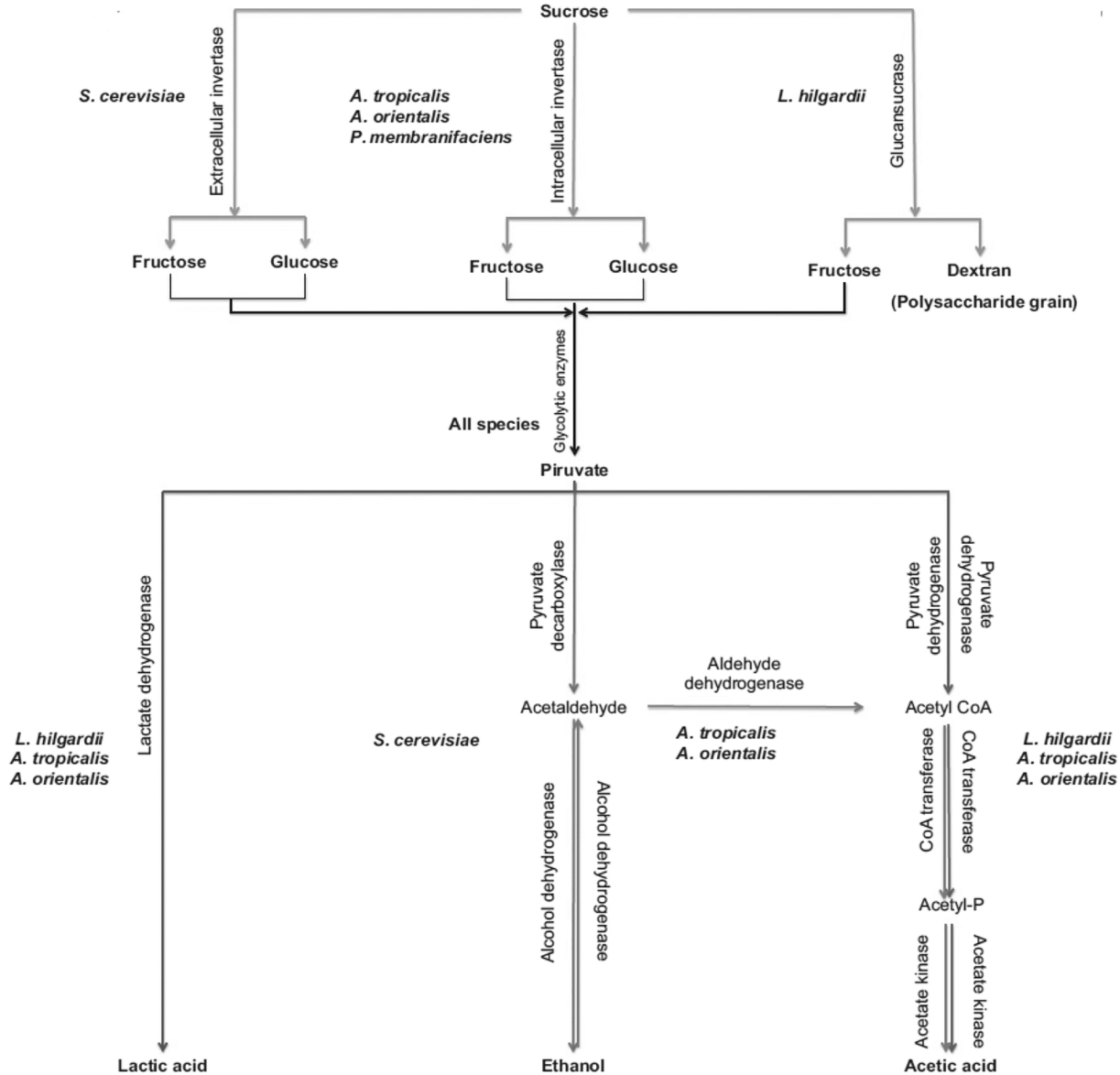
Sucrose
S. cerevisiae
Extracellular invertase
A. tropicalis
A. orientalis
P. membranifaciens
Intracellular invertase
L. hilgardii
Glucansucrase
Fructose
Glucose
Fructose
Glucose
Fructose
Dextran
(Polysaccharide grain)
All species
Glycolytic enzymes
Piruvate
Lactate dehydrogenase
Pyruvate decarboxylase
Pyruvate dehydrogenase
Pyruvate dehydrogenase
Aldehyde dehydrogenase
Acetaldehyde
Acetyl CoA
L. hilgardii
A. tropicalis
A. orientalis
S. cerevisiae
A. tropicalis
A. orientalis
L. hilgardii
A. tropicalis
A. orientalis
Alcohol dehydrogenase
Alcohol dehydrogenase
CoA transferase
CoA transferase
Acetyl-P
Acetate kinase
Acetate kinase
Lactic acid
Ethanol
Acetic acid

Figure 6

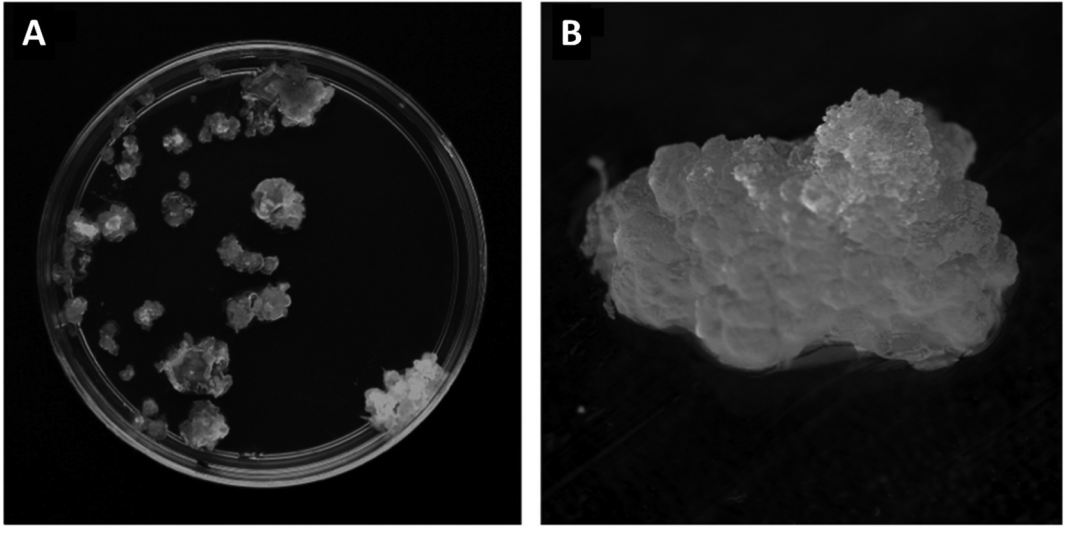

Figure 7