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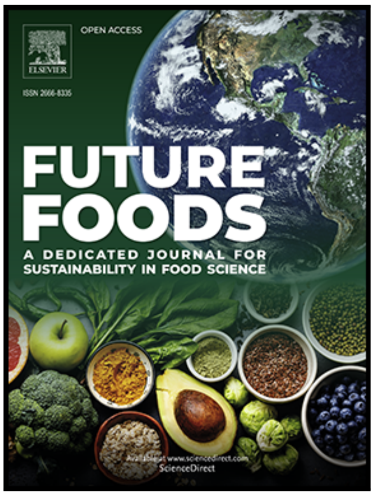

WATER KEFIR, A FERMENTED BEVERAGE CONTAINING PROBIOTIC MICROORGANISMS: FROM ANCIENT AND ARTISANAL MANUFACTURE TO INDUSTRIALIZED AND REGULATED COMMERCIALIZATION

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Highlights

- Water kefir consumption increased during the past years
- Water kefir is suitable for plant-based / vegan / lactose-free diets
- The scaling up of water kefir must me accompanied by scientific monitoring
- Water kefir industrialization should be accompanied by sustainable projection

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ABSTRACT

Throughout the COVID-19 pandemic, there was a demand for natural products able to enhance consumers health. Many people discovered the benefits of fermented products such as milk and water kefir and kombucha. Specifically, water kefir has aroused great interest from people interested in consuming foods that do not come from animals (plant-based and vegan diets) or people allergic to milk proteins or lactose intolerant, while increasing the scientific evidence of water kefir health enhancement. This review deals with the needing for the establishment of quality parameters found in traditional and flavoured water kefir drink, for their implementation in the industrially produced beverage. Such industrialization must seek the sustainable development of this economic activity for the implementation of circular economy guidelines. The benefits and safety of this non-dairy fermented drink have been demonstrated since its ancestral consumption and have been documented by many scientific works around the world. The scientific community must accompany this rapid advance of fermented foods containing probiotic microorganisms, given the changing priorities within the food industry. In addition, the quality parameters for the inclusion of this product in the Codex Alimentarius of many countries must be established, in order to regulate its production on an industrial scale and marketing.

KEY WORDS: water kefir - sugary kefir –probiotic – ancestral beverage –scale-up

INTRODUCTION

Water kefir is an artisanal and ancient fermented beverage, fruity, acidic, sour, and slightly carbonated, with high lactic acid content (up to 2%) and low alcohol content (usually less than 1%), which is obtained after the fermentation of sugary water with water kefir grains (starters), to which dried fruits can be added (Pidoux, 1989; Fiorda et al., 2017). The fermented, filtered and grain-free beverage is known as "water kefir", "sugary kefir" or "acquakefir" (among other regional names), and is the product which is consumed. Grains are called "water kefir grains" (to differentiate them from "milk kefir grains"). Besides, “Tibics” or “Tibetan mushrooms" are other frequent names used (Lynch et al., 2021), despite being equivocal, since such names are also used for milk kefir grains (Chen et al., 2015; Dong et al., 2018).

Water kefir grains consist of a polysaccharide matrix (mainly dextran, and less amount of a levan) where microorganisms are embedded (Pidoux et al., 1988; Fels et al., 2018; Coma et al., 2019). The grains have a jelly and translucent appearance, yellowish to brown, with irregular shapes and sizes ranging from millimetres to a few centimetres (Neve & Heller, 2002). Grains contain lactic acid bacteria (LAB), acetic acid bacteria (AAB), yeasts and sometimes bifidobacteria (Laureys & De Vuyst, 2016; Verce et al., 2019; Pendon et al., 2021). These microorganisms coexist symbiotically in the grains, and some of them can pass through the liquid phase. Water kefir grains are reused for the next fermentation, after filtering the fermented product, which is called the “pitching” process (Verce et al., 2019). If some aliquot of the fermented beverage is added to the new fermentation (in addition to the grains), the process is called “back-slopping”, which is an ancient practice frequently used in diverse types of fermented foods (Garofalo et al., 2020). Fermentation can take place between 20 °C and 37 °C (optimal 20 to 25 °C) for 24 to 72 hs, using between 6 and 10% sucrose and 6 to 30% grains (Laureys et al., 2018). The most used source of sugar for fermentation is raw sugarcane, and the most frequent additives are dried figs or dried grapes (Verce et al., 2019). Nevertheless, due to the high capacity of microorganisms present in water kefir grains to adapt to different substrates, the beverage can be produced from a wide variety of sugar sources (Bueno et al., 2021).

While water kefir is frequently confused with milk kefir in popular knowledge and even in peer-reviewed scientific publications, it is particularly important to note that they are different symbiotic systems (Güzel-Seydim et al., 2021). Firstly, each grain is constituted by a different polysaccharide matrix: the homopolysaccharide α1,6-glucan, synthesized by *Lactobacillus hilgardii*, in the case of water kefir grains (Pidoux, 1989; Fels et al., 2018; Coma et al., 2019), and the heteropolysaccharide glucogalactan named kefiran, synthesized by *L. kefiranofaciens* (Kooiman, 1968; Rimada & Abraham, 2003). Secondly, the disaccharide fermented by microorganisms is also different (sucrose and lactose, respectively), despite the fact milk kefir grains can grow in non-dairy substrates. The ability of water kefir grains to ferment sucrose, make them interesting for the above-mentioned applications. Thirdly, the species found in both kind of grains are not the same (Güzel-Seydim et al., 2021; Lynch et al., 2021).

Consumption of water kefir presents a promising alternative for people interested in incorporating fermented beverages into their diet, but who do not want to ingest products of animal origin (plant based diets and vegan diets) or that are intolerant and/or allergic to products of dairy origin (Gamba et al., 2019; Egea et al., 2020; Güzel-Seydim et al., 2021).

The following sections will be focused in three main aspects of water kefir, as summarized in the graphical abstract. First items will deal with the origin and history of water kefir, and the culture hybridization which accompanied its consume around the world. Secondly, some items will deal with present status regarding increment in water kefir consumption, health benefits and characterization of the beverage. Finally, items dealing with aspects related to scaling-up process focused on sustainability such as: use of alternative substrates and applications for grains surplus accompanying industrialization; finally, commercialization and inclusion of water kefir into the Codex Alimentarius of different countries will be also approached as future trends in food regulation.

I PAST

IA- ORIGIN OF WATER KEFIR CONSUMPTION, MIGRATION AND CULTURE HYBRIDIZATION

Although water kefir and milk kefir ought to be named in an unequivocally way, their origin needs to be related. From ancient times, milk kefir has been considered a fermented food with curative properties and promotional activities for health (Shavit, 2008; Oboturova et al., 2022). In the Caucasian region, it is also associated with population longevity (Zourari & Anifantakis, 1988; Cevikbas et al., 1994).

The technology of fermenting different substrates with kefir grains is ancient and simple, and the complexity of its community guarantees the inhibition of contamination by spoilage and pathogenic bacteria, as it has been scientifically proven. It is considered an ancient technology, passed down through generations, as there is evidence of the consumption of milk kefir since the Bronze Age in an organic cheese mass associated with mummies from a Xiaohe cemetery (1450 BC) in Xinjiang, China (Li et al., 2010). Archaeological evidence showed that milk from ruminant animals was fermented by a symbiotic culture of *Lactobacillus kefiranofaciens*, other LAB and yeasts by the semi-pastoral population of the Eastern Eurasia in the Bronze Age (Yang et al., 2014). Milk fermentation allowed the consumption of a final product with lower lactose content than fresh milk and the availability of serum so that both products could serve as sources of proteins, vitamins, and minerals for lactose-intolerant ethnic groups (Wang et al., 1984). In this way, the milk kefir fermentation was kept as cultural practice to the present day (i.e: *Labneh* cheeses, based on filtering kefir are manufactured in the Mid-East region). Xiaohe's kefir cheese is the oldest material evidence on the kefir fermentation and the use of kefir grains became part of Tibetan food culture, thereupon they were known among Europeans as the "Tibetan fungus" or “Tibetan mushrooms" (Yang et al., 2014). Recent studies show that milk kefir was discovered in the water skins of Karachay sherdsmen who shepherded near Kislovodsk. Kefir grains were sacred and considered as a source of wealth for this ethnic group who kept secret the technology of kefir fermentation from other peoples. Kefir grains were delivered to the central region of Russia during XIX century and the first kefir drinking public house was opened in 1884 in Moscow (Oboturova et al., 2022). Milk kefir grains were transported to other continents, encouraging its consumers to experiment with new substrates (goat's milk, cow's milk, etc.).

During the 20th and 21st centuries, the interest in kefir and other fermented products has grown due -in part- to the Russian diaspora which was a turning point in the spread of its consumption beyond Eastern Europe. Recent studies on the Russian diaspora to the United Kingdom, analysed the entry of kefir into that country, considering the transport of special family foods (like milk kefir and kombucha) when migrating, as a strategy to find one's own identity within the context of the receiving culture. Bringing the native "Russian" foods to a new country contributed to reformulating their meaning, making them meaningful to the person or community, but also incorporating them into the cultural heritage of the receiving society (Pechurina, 2020). The kefir introduction in other cultures implies that food practices are "hybridized". This process takes place when the constituent elements of different foods and the ways of making them are modified, for example, using "replacement" ingredients, growing food in the home garden, incorporating traditional foods into the daily diet, or mixing them with traditional foods on holidays. The reproduction of domestic foods in receiving countries both for their own consumption and for sale to other consumers, as a type of "ethnic product" has been considered as an example of the hybridization of food practices (FAO, 2011). While these practices connect people to the home of the past and help maintain established cultural ties, they also produce new sets of meanings during the experience of migration (Pechurina, 2020).

Unlike milk kefir grains, there is no archaeological evidence on the water kefir origin. it seems that there are different possible origins (Waldherr et al., 2010).The first reports on water kefir grains were made by Beijerinck (1889), who associated the water kefir grains with the ginger plants that were brought to Central Europe by British soldiers when the Crimean War was over in 1855. Lutz (1899a,b) described a microbial community denominated "*tibi*", originated in the *Opuntia* sp. leaves and fruits in Mexico. During the twentieth century, these microbial systems were named "sugary kefir grains" (Vayssier, 1978) to differentiate them from the kefir granules that ferment milk (Pidoux, 1989; Bergmann et al., 2010). Multiple denominations of the grains, the fermented beverage and a multiplicity of substrates depending on the country where the kefir is manufactured have been reported, as shown, in Table 1. Regardless there is still a lot of work to be done in order to reveal the different forms of consumption of water kefir in many countries, it is necessary to begin to do this relay, since the names are related to the origin of water kefir consumption in each country, and with the process of culture hybridization. Nowadays, kefir beverages can be obtained in commercial places, but the most common way to obtain and learn to prepare water kefir is still based in personal transmission.

Insert Table 1 here

IB - ARTISANAL PRODUCTION PROCESS

The flowchart of the process to produce sugary kefir beverages is illustrated in Figure 1. In this design, water kefir grains are added directly to the water and sugar solution (previously pasteurized and cooled) and incubated at proper temperature. After fermentation, the grains are separated from the medium by filtration through a sterile sieve, washed, dried, and kept in a cooling tank for the next inoculation (Güzel-Seydim et al., 2000; Otles & Çağındı, 2003). This flowchart is applicable to home-made water kefir and to artisanal-made water kefir, although it could be commercialized. The fermented kefir drink is stored at 4 °C and is ready for consumption. The shelf life was not still clearly defined. Since there is still a long way to go regarding food regulation, there are also many quality parameters needed to be defined for the scale-up of this product.

(Insert Figure 1 here)

II - PRESENT

II-A- NOWADAYS WATER KEFIR CONSUMPTION

Beyond ancestral practices exposed before, water kefir consumption (as many other fermented beverages) has increased in the past few years, supported by many scientific studies which have documented that water kefir is a source of probiotic microorganisms and metabolites with potential health benefits (Laureys & De Vuyst, 2014; Zavala et al., 2016; Romero-Luna et al., 2020; Lynch et al., 2021). Currently, the consumption of water kefir has increased, by virtue of its sensory properties, as well as the health benefits associated with its consumption and the advantages that it offers to vegan consumers and / or intolerant to residual lactose in milk kefir (Gamba et al., 2019; Güzel-Seydim et al., 2021). Among the countries with the highest consumption of water kefir are the United States, Mexico, and Canada, in North America; France, Greece, Turkey, Romania, Russia, United Kingdom, Belgium, Netherlands, Norway, Sweden, Spain and Portugal in Europe; and Brazil, Chile, Peru and Argentina in Latin America (Sarkar, 2007; Zhou et al., 2009; Fiorda et al., 2017). In other countries of Central and South America its consumption is widespread. Moreover, during the COVID-19 pandemic, the consumption of natural probiotic-containing foods was suggested as beneficial for improving gut health and, consequently, overall health (Güzel-Seydim et al., 2021). Indeed, consumers global trends such as healthy and ethical consumption are boosting fermentation technology and industry worldwide (Terefe, 2022). Market studies correlate increased water kefir consumption with increased consumers awareness of the benefits of fermentation, and with the knowledge that probiotics may be included in a much wider variety of foods (Lynch et al., 2021). Worldwide, people are more and more alert to the relation between nutrition and health, while the terms “probiotic”, “prebiotic” begin to be used by consumers, and “symbiotic” products market size is expected to witness significant growth over the next 5-years period (Cosme et al., 2022; Web reference 1, see at the end of the bibliographic references). The global kefir market size was $1.23 billion in 2019 and is projected to reach $1.84 billion by 2027 (Web Reference 2).

Although most of the research carried out so far has focused on milk kefir made with milk from cows, sheep, goats, among others (Farnworth, 1999; Garofalo et al., 2015; Prado et al., 2015), it is important to note that it is not consumable for vegans, lactose intolerant and allergic to dairy products. The fermentation of non-dairy substrates with water kefir grains constitutes an interesting alternative to take advantage of the health benefits which it offers, overcoming available probiotic fermented products, such as those mentioned above (Schneedorf, 2012; Fiorda et al., 2017).

II-B - HEALTH BENEFITS

Health benefits of water kefir intake are empirically sustained by centuries of consumption by humans. Much scientific evidence has been written, and recently very complete scientific reviews were published including water kefir biological activity (Gamba et al., 2019; Egea et al., 2020; Lynch et al., 2021; Pendón et al., 2021). In such many time of consumption, microorganisms found in water kefir have shown to be non-pathogenic and moreover, together with the organic acids they produce (and that are found in the fermented product), that they are able to inhibit the growth of pathogenic microorganisms such as *Salmonella* sp., *Shigella* sp. (Koroleva, 1988; Anselmo et al., 2001; Zavala et al., 2016), *Salmonella typhimurium, E. coli* and *Staphylococcus aureus* (Romero-Luna et al., 2020). It has also been shown to inhibit filamentous fungi such as *Aspergillus flavus* (Gonda et al., 2019), *A. ochraceus* (Caro Velez & León Peláez, 2015), *A. niger, Rhizopus* sp. and *Penicillium* sp (Iriquín, 2020, unpublished data). This antimicrobial capacity is related to the acidity of the product, generated by the presence of weak organic acids such as acetic and lactic acids (Caro Velez & León Peláez, 2015; Gamba et al., 2015; Fiorda et al., 2016a).

In addition to possessing the aforementioned antimicrobial activity, water kefir exhibits a positive biological activity (Lynch et al., 2021; Pendón et al., 2021). Some of the reported functional properties are: immunomodulant (Calatayud et al., 2021); antitumoral (Zamberi et al., 2016), antihypertensive (Gamba et al., 2019), antitoxic (Kumar et al., 2021), hepatoprotective (Aspiras et al., 2015), hypocholesterolemic (Rocha-Gomes et al., 2018), hyperglicemic and anti-hyperlipidic (Alsayadi et al., 2014; Koh et al., 2018), antioxidant (Alsayadi et al., 2013; Fiorda et al., 2016b; Darvishzadeh et al., 2021), anti-edematous effects (Moreira et al., 2008), anti-inflammatory (Diniz et al., 2003), anti-ulcerogenic (Rodrigues et al., 2016), healing (Moreira et al., 2008), and other biological activities (Fiorda et al., 2017; Bueno et al., 2021; Lynch et al., 2021; Pendon et al., 2021). In addition, some strains isolated from water kefir shown probiotic properties. Indeed, many microorganisms with probiotic potential have been isolated from water kefir grains or from the fermented beverage (Magalhães et al., 2010; Soccol et al., 2010; Gulitz et al., 2011; Laureys & De Vuyst, 2014; Zavala et al., 2016; Romero-Luna et al., 2020).

Health benefits could be attributed to the beneficial microorganisms found in the consumed fermented drink, as to their metabolites (organic acids and oligosaccharides), as to both (synergic effect). Moreover, biological activity could be elicited by microorganisms or their metabolites in a direct way, or by an indirect way, via the stimulation of intestinal microbiota (Simonelli et al., 2021). At any case, more investigations are still needed to understand the multiple health benefits associated to water kefir.

IIC - CHARACTERISTICS OF THE BEVERAGE

As stated before, the starter culture of water kefir beverages consists of "water kefir grains", which constitute a complex symbiotic ecosystem of microorganisms embedded in a matrix of exopolysaccharide produced by bacteria. This exopolysaccharide is made up of glucose units, joined by bonds α-1,6 with branches in junctions α-1,3 in a smaller percentage. Chemically, it is a branched α-1,6 dextran, with a molecular weight varying between 450-2000 kDa (Fels et al., 2018). No proteins or lipids have been described in the composition of the grain (Coma et al., 2019). This exopolysaccharide is completely insoluble in water. The microbial groups embedded in the water kefir grain are lactic acid bacteria (LAB) (10^7-10^8 CFU/g grain; mainly *Lactobacillus* sp., *Lactococcus* sp., *Leuconostoc* sp. and *Streptococcus* sp.), acetic acid bacteria (AAB) (10^6-10^7 CFU/g grain) and yeasts (10^6-10^7 CFU/g grain). A list of taxonomic groups that are described in the literature as common genera and species that can be found in water kefir grains is given in Table 2.

(Insert Table 2 here)

In general, it has been reported that the LAB population exceeds that of yeasts in both milk kefir grains and water kefir grains (Lynch et al., 2021). However, some studies have reported the existence of similar populations or have shown the opposite, i.e. predominance of the yeast population over LAB. The diverse microbial profile of water kefir grains has been attributed to the various geographic regions from where it was originated. Variations in the microbial population from the same grain have recently been demonstrated by massive sequencing studies on water kefir beverages obtained through successive fermentations on different substrates (Gamba et al., 2021). Regarding yeasts (commonly *Saccharomyces* sp., *Kluyveromyces* sp., *Pichia* sp. and *Candida* sp.), they constitute an extremely diverse group, which contributes to the formation of aroma and flavour precursors during the fermentation and maturation processes. The geographical origin of the grain determines the microbial profile. For example, in the case of yeasts, some studies showed that the predominant yeast population is *S. cerevisiae* over *K. marxianus* (Lu et al., 2014); while in other works *S. cerevisiae* has been the secondary population, being *Zygotorulaspora florentina* the dominant one (Gulitz et al., 2011).

In the case of LAB, the domain of *Lb. hordei* and *Lb. nagelii* and a secondary microbiota formed by *Leuconostoc mesenteroides* and *Ln. citreum* in water kefir grains has been reported (Gulitz et al., 2011). Using a combined approach of dependent and independent culture methods, the predominance of *Ln. mesenteroides* in water kefir fermented with brown sugar was reported, followed by *Lb. hordei* and *Lb. mali* at lower levels (Hsieh et al., 2012). *Lb. perolens, Lb. parapharraginis, Lb. diolivorans* and *Oenococcus oeni* have also been reported (Zanirati et al., 2015).

It was recently determined that the microbial composition of the grain presents differences with the fermented beverages obtained during 7 fermentative cycles in different substrates (brown sugar, purified molasses and high-quality molasses). In the grain, the concentration of *Lb. casei / paracasei* ranges between 31 and 63%, *Lb. hilgardi / diolivorans* between 14 and 46%, and *Lb. nagelii* from 2 to 19% (Gamba et al., 2021). The same variations have been found in the case of milk kefir grains and milk kefir beverages (Marsh et al., 2013a; Korsak et al., 2015); while, on the contrary, other researchers reported a high abundance of *Zymomonas* sp., alcohol-producing bacteria, both in the water kefir grain and in the fermented beverage (Marsh et al., 2013b). The composition of water kefir can be affected by the substrates used and, consequently, the chemical and sensory characteristics of the final drink.

The fermented product is a drink with a certain degree of turbidity and carbonation, fruity, acidic, sour, and slightly carbonated, with low alcohol content and high content of lactic and / or acetic acid, containing microorganisms. The microbial groups that can be found in the product (fermented at 20 °C after 72 hs) are described in Table 3.

(Insert Table 3 here)

The microbial diversity of water kefir has been studied for some time (Pidoux, 1989; Fiorda et al., 2017). In recent years, studies carried out with molecular biology techniques have allowed insight into the diversity of bacteria and yeasts of water kefir. The predominant species of bacteria found in the drink are *Lb. paracasei, Lb. kefirii, Lb. parabuchneri* and *Acetobacter lovaniensis;* among the yeasts predominate *S. cerevisiae* and *K. lactis* (Fiorda et al., 2017).

Recently, the variation in the bacterial population of the grain and the fermented beverage was determined by 16S high-throughput sequencing. In both, grains and water kefir drinks, the phylum *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* were determined, with the highest proportions being the phylum *Firmicutes* (21–99%) and *Proteobacteria* (0–78%). It was established that kefir beverages present differences in the proportion of their composition with respect to the grain from which they are obtained, depending on the type of fermentation substrate used, such as brown sugar, purified molasses and high-quality molasses. Additionally, the fermented beverage with the same grain can vary its composition as successive fermentations are made on each substrate (Gamba et al., 2021).

The phylum *Firmicut*es, which includes different species of LAB, has been determined both in kefir grains and in the fermented drink obtained with brown sugar, purified molasses and high-quality molasses. The specie *Liquorilactobacillus nagelii* was determined in the highest proportion in kefir beverages obtained with brown sugar from 33 to 71% and with purified molasses from 64 to 84% (Gamba et al., 2019, 2021). However, in kefir made with high-quality molasses, the pattern changes, with proportions of *Lentilactobacillus hilgardii/diolivorans* and *Lacticaseibacillus casei/paracasei* between 12 - 44% and 5 - 19% respectively, higher than that corresponding to *Lb. nagelii,* of 3 to 6%. Despite the variations between the substrates used, the species *Lb. nagelii, Lb. hilgardii/diolivorans* and *Lb. casei/paracasei* play a central role in the formation of lactic acid from water kefir. In addition, other studies have shown the presence of similar bacteria in water kefir in different countries (Fiorda et al., 2017), although different substrates are used like brown sugar solutions (Pidoux, 1989; Miguel et al., 2011) and sucrose with figs and lemon slices (Gulitz, et al., 2011).

The phylum *Proteobacteria* increases in the kefir beverage from 22% to 78% of the total bacterial population between cycles 1 and 7 of successive fermentation with high quality molasses, demonstrating that this is a suitable substrate for this phylum, which includes genera associated with the production of acetic acid from kefir.

Within the AAB, *A. lovaniensis* constitutes the largest percentage of bacteria, increasing from 62% to 75% between fermentation cycles 4 and 7, indicating that this species contributes to the greater production of acetic acid in this substrate (Gamba et al., 2021). This is corroborated by a high count of this species, around 10^7 CFU/mL after seven fermentative cycles on this substrate (Gamba et al., 2019). Contrary to what happens in the beverages obtained with high quality molasses, in those from purified molasses, the phylum *Proteobacteria* is not detectable by following several fermentative cycles, being *Acetobacter* and *Gluconobacter,* the main affected genera of acetic acid bacteria (AAB). This variation is related to the composition of the substrate. Purified molasses is a poorer substrate than high quality molasses in terms of micronutrient content, essential for acetic acid fermentation. In kefir from brown sugar there is a decrease in *A. lovaniensis,* from 14% to 1% between fermentation cycles 1 and 7 (Gamba et al., 2021). In conclusion, water kefir beverages are microbiologically diverse depending on the sweet substrates used.

The afore mentioned microorganisms present in the grains, consume sugars, and produce various metabolites. At the beginning of the fermentation process, the substrate consists of approximately 90% sucrose, 6% reducing sugars and 1.5%

minerals (K, Ca, P, Mg, Na, Fe, Mn, Zn and Cu) (Martínez-Torres et al., 2017). When fermentation is finished, the main final products found are ethanol, lactic acid, acetic acid, and other metabolites such as mannitol, glycerol, esters, and other organic acids (Fiorda et al., 2017). Among the prevalent aromatic compounds, ethyl acetate, isoamyl acetate, ethyl hexanoate, ethyl octanoate and ethyl decanoate have been detected. The latter are fruit esters that have an organoleptic impact on the final product (Laureys & De Vuyst, 2014). In the fermented product (using sugary water with additives) there have been found a total of 134 different volatile compounds (Corona et al., 2016). The fermented product (24 hs 20 °C) has mostly lactic acid (2%), followed by acetic acid (1.5%) and ethanol (1%). Furthermore, the sucrose content decreases at 24 hs (in fermentation at 20 °C) to 50% of the initial value, while the glucose and fructose content increases (Magalhães et al., 2010). At the same time, an exopolysaccharide (product of the bacteria metabolism) is released to the medium. The exopolysaccharide found in the fermented beverage was identified as a levan (fructose polymer) (Fels et al., 2018).

The final acidity values depend on the activity of the microorganisms due to the temperature, the fermentation time, and the proportion of granules with which the sugary liquid is inoculated. The pH of the product fermented for 24 hs at 20 °C is close to 4; the total titratable acids (g/100 ml) are 0.07, and total soluble solids: 4.1°Brix (Magalhães et al., 2010). Using panela as a substrate, the pH after 24 hs of fermentation is between 4 and 6, decreasing with the increase in temperature (Caro Velez & León Peláez, 2015). These values may be different when fruits or vegetables are added to the substrate prior to the inoculation. For example, it is described that the pH can vary between 3.6 and 5 when strawberry or onion are used as an additive, respectively. The ethanol content may also vary up to 3% (v/v) when additives containing additional sugars are used, e.g. carrots, melons or strawberries. In the same manner, the lactic acid and acetic acid contents vary up to 4.8% (with carrot and melon) and 1.9% (with carrot), respectively (Corona et al., 2016). A total of 134 different volatile compounds were identified when using different substrates for water kefir fermentation (Corona et al., 2016). Volatile acid values confirm the metabolic heterogeneity (homofermentative and heterofermentative species) of active LAB in kefir products. Acetic acid contributes to providing a pleasant taste and plays a role in the inhibition of undesirable microorganisms (Puerari et al., 2012) and could be decisive for the sensory evaluation of fermented products that have a unique refreshing taste, aroma, and texture, depending on its quantity (Duarte et al., 2010).

III – TRENDS

III-A- ALTERNATIVE SUBSTRATES

A great variety of substrates has been used to prepare water kefir. Nevertheless, molasses from sugarcane juice are the most commonly used substrates to make this product. The granules are added to suspensions made with the dry juice of the cane known as "Piloncillo" in Mexico (cone shape) or "panela" in Colombia (disk shape); "*chancaca"* in Peru, "*molasses" in* Brazil and Japan, cane honey, "*mascabado*" sugar, usually including figs or lemon slices (Ward, 1892; Lutz, 1899a, b; Rubio et al., 1993; Gulitz, et al., 2011; Caro-Vélez & León-Peláez, 2014; Magalhães et al., 2010; Laureys & De Vuyst, 2014; Fiorda et al., 2016a, 2016b; Gamba et al., 2019).

Beyond the traditional raw sugarcane described before, the versatility of the water kefir granule to ferment different substrates is evidenced in early and recent studies that show the manufacture of water kefir with alternative sources of carbohydrates to explore new flavours related to regional availability. The use of alternative substrates leads to the production of functional beverages with different metabolic compounds, thus different sensorial characteristics (Fiorda et al., 2017), and different biological properties.

The number of scientific reports where water kefir grains are used for the fermentation of alternative substrates has increased in the last years. The addition of fruits, and vegetables such as carrot, fennel, melon, onion, tomato, and strawberry (Corona et al., 2016), "*yacon"* (Veeck et al., 2018), pumpkin (Koh et al., 2018) was also described. Recently, the addition of coconut extract (Alves et al., 2021), soy whey (Azi et al., 2021), Russian olive fruit (Darvishzadeh et al., 2021), apple (Velázquez-Quiñones et al., 2021), red pitaya (Bueno et al., 2021), Cornelian cherry, hawthorn, red plum, roseship and pomegranate (Ozcelik et al., 2021), and beet (Paredes et al., 2022) manifest the interest in the screening of new substrates for innovative fermented beverages.

As can be noted, kefir grains have a high capacity to adapt to different food substrates and can be used to produce a wide variety of fermented beverages (Bueno et al., 2021). Fermenting sugary substrates that come from various plant sources makes water kefir an interesting application for the use of alternative sources of sugars (such as bagasse, fruit peels, and/or "orujos", among others). Thus, water kefir could be considered as a fermented beverage capable of giving greater added value to by-products of the food industry that still contain fermentable sugars. Although scientific studies are still needed to prove this, the use of by-products coming from the food industry can be considered as a possibility of substrate for fermentation, with the consequent value added, generating a product with high nutritional value and ability to increase consumers health.

III B - GRAINS SURPLUS APPLICATIONS

Although problems with the growth of grains have been reported (Laureys et al., 2017; Lynch et al., 2021), the fact of producing kefir at an industrial level brings with it the increase in the volume of grains (surplus). Projecting a sustainable development of this industry, and thinking about the favouring of the circular economy, possible applications for the surplus of grains are mentioned below.

Firstly, it must be considered the possible biological activity that the polymers could elicit. Indeed, they could be expected to have biological properties since there is enough scientific evidence in previous studies using analogous polysaccharides. In fact, dextrans and levans are promising biomolecules to be used as functional food additives (Freitas et al., 2011). In general, dextrans are recognized as bioactive molecules with high potential to be considered prebiotics (Sarbini et al., 2013; de Paiva et al., 2016; Calatayud et al., 2021). Alternative uses for exopolysaccharides isolated from water kefir grains related to their technological properties includes film formation properties (Coma et al., 2019) and as a baking hydrocolloid (Hermann et al, 2016). Grains were used as a starter to produce a fermented Japanese-Sausage (Wu et al., 2018) and vinegar (Terpou & Mantzourani, 2019). On the other hand, water kefir grains were used for the treatment of food processing wastewaters, offering a potential application for a preliminary and turning organic matters into value-added metabolites (Sarikkha et al., 2014; Maldonado et al., 2020). Also, water kefir grains were used to retain heavy metal ions dissolved in aqueous solution (Volpi et al., 2019). Thus, it can be said that grains surplus produced by water kefir scaling up process can be managed in a sustainable and efficient resource use.

III C - COMMERCIALIZATION AND REGULATORY APPROVAL

As said before, worldwide consumption of water kefir increased during the last years (Güzel-Seydim et al., 2021). Despite homemade water kefir being the most frequent source of consumption, many semi-industrialized commercial water kefir could be found in some markets of the world. Sometimes it is sold as an “artisanal beverage” which is not regulated by food legislation. Nevertheless, trends in the last decades, mainly related to regulations on probiotic strains, also reached water kefir, thus needing the incorporation to food legislation in the countries where it is consumed.

Regarding international regulations (EU, FDA -NO GRAS-, CODEX), water kefir is approved in several legislations. For instance, in Australia water kefir could be found under the following normative: Standard 2.6.2 Non-alcoholic beverages and brewed soft drinks (Web Reference 3), classified in the Brewed soft drink category. The product must be elaborated by a fermentation process made from water with sugar and one or more extracts or infusions of fruit or vegetables; it should not contain more than 1.15% alcohol by volume.

Considering the United States and FDA legislation, it can be observed that, just as in the Argentine Food Code, milk kefir is regulated in the Code of Federal Regulations, Title 21: Food and Drugs PART 131—MILK AND CREAM, Subpart B—Requirements for Specific Standardized Milk and Cream (Web Reference 4), where kefir cultured milk is specifically listed. In parallel, the Enhancing Retailer Standards in the Supplemental Nutrition Assistance Program (SNAP) regulation (Web Reference 5) considers, according to the Agency's policy, plant-based dairy alternatives, including water kefir as one of the products that may present a plant-based option. As for the European Union, water kefir is currently commercialized as a probiotic under food safety legislation, although it is not literally in the food codex. Recently, in Argentina, it has been requested the inclusion of water kefir in the codex, pending final decision of the evaluation committee (Web Reference 6). In some European countries, such as Belgium for example, it is produced industrially and commercialized in the market (Thylbert Company). In Italy, it is marketed by the company BioNova. In the United States, KeVita, Sunny Culture and GT's Living Foods companies have recently incorporated the water kefir line into their products.

CONCLUSIONS

Sugary kefir is an ancestral beverage whose security and health benefits are ensured after a long time of consumption. Water kefir was mainly produced in artisanal way (except from developed countries such as Belgium Australia, and United States of America, where industrialized kefir can be found in markets since many, years ago). In the rest of the world -presumably because of the COVID19 pandemic- there is an increasing interest on the consumption of fermented foods containing probiotic microorganisms. Concomitantly, there is an increasing interest in consumption of non-dairy fermented foods (plant-based diets and vegan diets, cow's milk allergies, non-tolerant to lactose), so water kefir hast the potential application of be consumed as a promising alternative of probiotics consumption for such diets.

On the other hand, as exposed in the present review, water kefir could be produced by the addition of many different substrates, since kefir grains have a high capacity to adapt to different food substrates (Bueno et al., 2021). This fact makes water kefir a tool for adding value to by-products of food industry such as bagasse or pomace. Variations on kefir microbiota when using different substrates is scientifically demonstrated. So, scientists should accompany the trend of water kefir industrialization and commercialization, by the characterization of the products in such scaling-up processes to keep the probiotic characteristics of the microorganisms present in the fermented beverage made in an artisanal scale. The projection of sustainable production regarding grain biomass surplus is another challenge to be boarded. Thus, research needs are related to the characterization of different water kefir beverages around the world, using different substrates and to attend the passage form artisanal to scale up and the inclusion in the Codex Alimentarius of different countries.

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FIGURE CAPTIONS

Table 1: Grain (G) and Fermented beverage (FB) denominations and substrates used in the kefir manufacture.

Table 2: List of species of bacteria and yeasts described in the literature as found to be isolated from water kefir rains or fermented products.

Figure 1

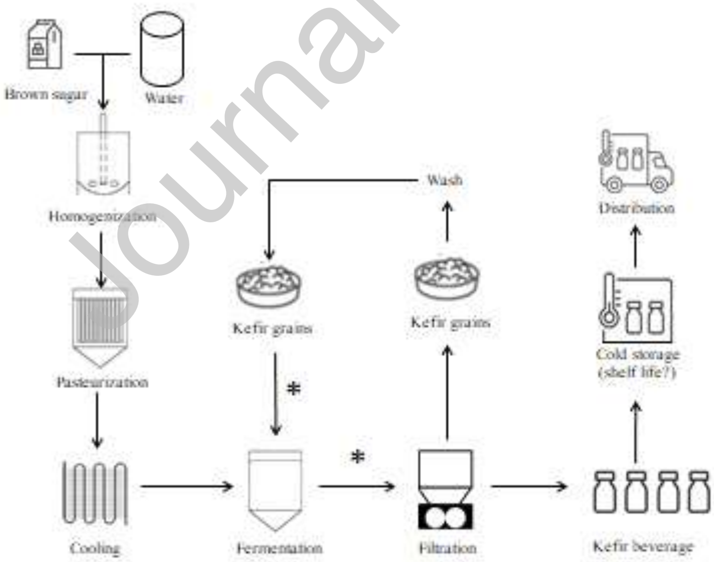

Figure 1: Flow chart of water kefir industrial production. (*) means the different stages where additives can be incorporated to the beverage. Adapted from Fiorda et al., (2017).

Table 3: values of the maximum and minimum CFU found in the fermented product, using the specific media: de Man, Rogosa and Sharpe Agar (MRS), Acetic Acid Bacteria Agar (AAB) and Yeast extract-Glucose-Chloramphenicol Agar (YGC), for LAB, AAB and yeasts, respectively (Pidoux, 1989; Caro Velez & León Peláez, 2015). .

FIGURES AND TABLES

Grain (G) and/or fermented beverage (FB) Denomination	Country[1]	Fermentation substrate	Reference
Colonche (FB) "Tibi" (G)	Mexico	Nopal Prickly pears and fruits juice	Lutz 1899a,b. Ulloa & Herrera, 1978.
"Arroz de indio" or "Indiecitos" (G) (“Indian rice” and “Little Indians" respectively)	Colombia (Antioquia department)	“aguapanela” (solution prepared with the dry cane syrup, known as “panela”) solution	Zapata M., oral communication, 2020. Caro Velez & León Peláez, 2015.
Tbicos (G) Granillo (G) Tibi-complex (G) Tepache de tibicos (FB) Vinagre de tibicos (FB)	Mexico (comes from the *Opuntia* sp.)	Piloncillo (sugar cane extract), pineapple, brown sugar and cinnamon solutions	Daker & Stacey, 1938. Macott & Terrés, 1952. Ulloa & Herrera, 1981. Martínez-Torres et al., 2017. Romero-Luna et al., 2017. Velázquez-Quiñones et al., 2021.
Sugary kefir (FB)	France (Angers Area)	Sugar solutions amended with figs and lemon	Leroi & Pidoux, 1993.
Californian bees (G) Japanese Beer Seeds (G) California Bees’ Beer (G) American Kephir (G) Bees (G)	USA (Kentucky, Texas, New Jersey, Tennessee, Philadelphia), Canadá (Ontario)	Cornmeal or wheat flour with sweetened water Sweetened water with cream of tartar and pieces of ginger	Kebler, 1921.

BeBées (G)			
Ginger beer plants (G)	British Isles	Sugar solutions amended with ginger	Ward, 1892.
Kefir d'uva (FB)	Italy (southern Italy)	Grape solutions	Gaware et al., 2011.
Ginger Beer (FB)	Greece (Corfu)	Ginger crystals	Daker & Stacey, 1938. Ward, 1892.
Tangawizi (Swahili word for ginger) (FB)	East Africa (mainly Kenya and Tanzania)	Ginger	Açık et al., 2020.

[1]Country where the denomination is referred.

Table 2: species of bacteria and yeasts found in water kefir grains described in bibliography

Species	Reference
Bacteria	
Acetobacter fabarium	Gulitz et al. (2011)
Acetobacter orientalis	Gulitz et al. (2011)
Acetobacter lovaniensis	Gamba et al. (2019)
Acetobacter indonesiensis	Gamba et al. (2019)
Acetobacter tropicalis	Gamba et al. (2019)
Bifidobacterium psychraerophilum	Gulitz et al. (2011); Laureys & De Vuyst (2014)
Bifidobacterium aquikefiri	Laureys & De Vuyst (2016)
Lactobacillus brevis	Pidoux (1989); Waldherr et al. (2010)
Lactobacillus hilgardii	Pidoux (1989); Galli et al. (1995)
Lactobacillus casei ssp. *casei*	Pidoux (1989)
Lactobacillus casei ssp. *rhamnosus Lactobacillus casei* ssp. *pseudoplantarum*	Galli et al., (1995)
Lactobacillus diolivorans	Gulitz et al. (2011)
Lactobacillus buchneri	Pidoux (1989); Galli et al. (1995)
Lactobacillus fructivorans	Galli et al. (1995)
Lactobacillus fermentum	Corona et al. (2016)
Lactobacillus harbinensis	Laureys & De Vuyst (2014); Gamba et al. (2019)
Lactobacillus hordeii	Gulitz et al. (2011)
Lactobacillus kefiranofaciens	Zanirati et al. (2015)
Lactobacillus kefiri	Corona et al. (2016)
Lactobacillus mali	Zanirati et al. (2015)
Lactobacillus nagelli	Gulitz et al. (2011); Fiorda et al. (2016a); Gamba et al. (2019)
Lactobacillus paracasei	Magalhães et al. (2010)
Lactobacillus parafarraginis	Gamba et al. (2019)
Lactobacillus perolens	Zanirati et al. (2015)
Lactobacillus plantarum	Pidoux (1989); Angelescu et al. (2019)
Lactobacillus satsumensis	Gamba et al. (2019), Bueno et al. (2021).
Lactobacillus collinoides	Galli et al. (1995)
Lactococcus lactis ssp. *Cremoris*	Galli et al. (1995)
Lactococcus lactis ssp. *Lactis*	Pidoux (1989)
Leuconostoc citreum	Monar et al. (2014)
Leuconostoc mesenteroides ssp. *mesenteroides*	Pidoux (1989); Galli et al. (1995);
Leuconostoc mesenteroides ssp. *dextranicum* *Enterobacter hormachei*	Moinas et al. (2004); Waldherr et al. (2010)
Leuconostoc holzapfelii	Monar et al. (2014)
Lysinibacillus sphaericus	Fiorda et al. (2016a)
Gluconobacter frateuri	Pidoux (1989)
Oenococcus kitaharae	Zanirati et al. (2015); Gamba et al. (2019)
Oenococcus oeni	Zanirati et al. (2015)
Oenococcus sicerae	Verce et al. (2020)
Oenococcus aquakefirii	Verce et al. (2020)
Yeasts	

Sacharomyces bayanus	Waldherr et al. (2010)
Sacharomyces cerevisiae	Galli et al. (1995); Franzetti et al. (1998); Moinas et al. (2004); Laureys & De Vuyst (2014); Fiorda et al. (2017)
Sacharomyces florentinus	Waldherr et al. (2010)
Sacharomyces pretoriensis	Galli et al. (1995)
Zygosacharomyces florentinus	Galli et al. (1995); Franzetti et al. (1998); Moinas et al. (2004)
Zygosacharomyces fermentati	Gulitz et al. (2011); Kurtzman (2011)
Zygotorulaspora florentina	Gulitz et al. (2011)
Hanseniaspora valbyensis	Pidoux (1989); Galli et al. (1995); Neve & Heller (2002)
Hanseniaspora vinae	Pidoux, (1989); Neve & Heller (2002)
Hanseniaspora yalbensis	Franzetti et al. (1998)
Kloeckera apiculata	Pidoux (1989); Galli et al. (1995); Neve & Heller (2002)
Candida lambica	Pidoux (1989); Franzetti et al (1998)
Candida valida	Pidoux (1989); Franzetti et al. (1998)
Dekkera bruxellensis	Laureys & De Vuyst (2014)
Pichia membranifaciens	Gamba et al. (2019)
Pichia kudriavzevii	Gamba et al. (2019)
Pichia occidentalis	Gamba et al. (2019)

Other species: *Issatchenkia orientalis; Lanchancea: L. fermentati, L. meyercii; Kluveromyces: K. lactis, K. marxianus; Kazachstania: K. aerobia, K. unispora; Hanseniaspora uvarum, Brettanomyces bruxellensis.* (Fiorda et al., 2016a; Gulitz et al., 2011; Magalhães et al., 2010; Puerari et al., 2012; Bueno et al., 2021).

Figure 1: in a separated file

Table 3

Microbial group	Maximum (CFU/ml)	Minimum (CFU/ml)
Lactic acid bacteria	$9.0*10^7$	$2.8*10^4$
Acetic acid bacteria	$3.22*10^6$	$7.0*10^2$
Yeasts	$4.8*10^7$	$4.77*10^5$