The effect of non-fermentable sweeteners on the metabolite profile and sensory properties of water kefirs

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Water kefir, a fermented beverage based on sugared water or juices, became popular for its potential health benefits. The health effect of probiotic products is closely related to the number of viable microorganisms. However, in products with fermentable sugars, viable microbiota can cause changes during storage due to prolonged fermentation. Common techniques ensuring product stability in the food industry may negatively affect microbial viability, which is undesirable for a probiotic product like water kefir. To limit fermentation and preserve viable microbiota in the beverage, non-fermentable sweeteners can replace part of the sucrose, thereby reducing metabolic rates. However, these sweeteners might alter the fermentation process and impact the final metabolite profile and sensory qualities of the final product.

This study aimed to develop water kefirs sweetened with non-fermentable sweeteners and compare their metabolite content and sensory characteristics with sucrose-based water kefir. The products were produced under monitored fermentation conditions, with metabolite and carbohydrate content analysed using gas chromatography with flame ionization detection (GC-FID) and headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME-GC-MS). Sensory attributes were assessed by a trained panel in descriptive sensory analysis.

Partial sucrose substitution resulted in similar organic acid and volatile compound content across different recipes. Significant differences were observed in perceived fizziness and sweet taste, linked to sucrose concentration and the concentration and relative sweetness of other sweeteners. Other taste, odour, and mouthfeel attributes, which could be associated with microbial metabolites, mostly did not show significant differences. This study suggests that using non-sucrose sweeteners in water kefir production is feasible from the perspective of its sensory qualities. Future research could explore carbon dioxide content and consumer preferences for different water kefir recipes.

Keywords: water kefir, sweeteners, xylitol, erythritol, steviol glycosides, volatile compounds, sensory evaluation, fermentation, gas chromatography.

Table of contents

Table of figures

Table of tables

1 Introduction

Humanity has been using fermentation as a process to preserve foodstuffs, increase their nutritional value, and add novel sensory properties to them for more than 10 000 years (Bamforth & Cook, 2019). During fermentation, ingredients are biochemically transformed by microorganisms. Microbial species and strains determine the properties of the final product together with physicochemical properties of the fermentation medium, presence of additives, and fermentation conditions. Some fermentation-based food products have become staples in various geographical regions: for example, soy sauce, alcoholic drinks, sourdough bread, and fermented dairy products (yoghurts, cheeses, quark).

Kefirs represent a group of fermented products that can be divided into milk kefirs and water kefirs. While these two subgroups have similarities, they differ in the substrate used, microbiological composition on a species level, and the fermentation process.

1.1 Water kefir characteristics, origin, and health benefits

Water kefir is a fermented drink produced from sucrose-containing solutions and might contain fruit juices, syrups, extracts, fruit, and other additions (Alves et al., 2021). It is a carbonated, slightly acidic, and slightly alcoholic beverage that is frequently produced with water kefir grains as a starting culture (Lynch et al., 2021).

The history or age of water kefir is not fully clear, while milk kefir is thought to originate from the Caucasus and dates back to at least 2000–1500 BC (Yang et al., 2014). Water kefir and water kefir grains might have originated in several places independently. The first publication describing water kefir is considered to be a British publication by Ward from 1892 (Guzel-Seydim et al., 2021). The grains obtained and described by Ward in 1892 remind of typical water kefir grains: they are “brittle [lumps], like firm jelly” that are usually “the size of a hazelnut” (Ward, 1892). Ward (1892) lists other researchers’ opinions of the origin of water kefir grains, including Italy and the Caucasus area, but discards them as lacking substantial proof. Other authors have reported that similar grains exist in the nature on plant leaves in Mexico and that analogous grains have been found in France (Pidoux et al., 1990). The starter culture and the produced beverage are referred to under various names in different places: “sugar kefir”, “sugary kefir”, “Tibicos”, “Tibi”, “Tibetan mushroom”, “water kefir”, “ginger beer plant”, “Indian sea rice”

(Закирова et al., 2018; Lynch et al., 2021), but no research has been conducted to study the difference between the starting cultures from various regions. For this reason, the beverage and the related starter culture will be referred to as water kefir and water kefir grains, respectively, in this work.

Water kefir is traditionally produced at home, and no starter culture with defined microbial strains has been developed to this moment (Lynch et al., 2021). According to Lynch et al. (2021), consumption of water kefir follows the geographical pattern of its origin: the authors state that the product is commonly consumed in South America, Eastern Europe and Russia. However, water kefirs are gaining popularity in other parts of the world as well, including European countries, as it is now possible to purchase water kefir grains in European online stores (Farmacia Loreto Gallo S.R.L., n.d.; Fermentiamo, n.d.; Ruohonjuuri, n.d.). This might indicate the increasing interest of consumers in this emerging fermented beverage.

Health effects of water kefir are less researched compared to milk kefir, and only separate studies focusing on specific effects of water kefir or its specific metabolite fractions exist at the moment. In previous studies, water kefir has been demonstrated to inhibit growth of pathogenic fungi *Aspergillus flavum* (Gonda et al., 2019), reduce inflammation and formation of granuloma tissue in rats (Diniz et al., 2003), improve hepatic lipid profile in Wistar rats (Rocha-Gomez et al., 2018), and exhibit antioxidant activities (Alsaydi et al., 2013).

As water kefir is different in content from milk kefir, its health effects are not expected to be identical to its dairy counterpart. Lynch et al. (2021) in their review have nevertheless reported that, like in the case of milk kefir, beneficial properties of water kefir can be associated with the presence of potentially probiotic microorganisms. Several species identified in the microbiota of the ready water kefir have been reported to exhibit probiotic properties, for example *Lacticaseibacillus paracasei* and *Lentilactobacillus hilgardii* (Tan et al., 2022). According to the current EU legislation, as also noted by Lynch et al. (2021), neither water kefir nor any other food product has received an approved health claim as a probiotic product in the EU market to this date. Authorising health claims for water kefirs and other probiotic products remains nevertheless possible as the U.S. Food and Drug Administration qualified such a claim for plain yoghurt in 2024 (U.S. Food and Drug Administration, 2024).

1.2 Water kefir production: water kefir grains, production process, ingredients, metabolic transformations

1.2.1 Structure and microbial composition of water kefir grains

Water kefir grains (Figure 1) are translucent, brittle, waxy gelatinous structures of 5 to 20 mm in diameter (Guzel-Seydim et al., 2021; Lynch et al., 2021). As described by Guzel-Seydim et al. (2021) and Lynch et al. (2021), water kefir grains often have tough consistency and irregular, cauliflower-like or “rock salt” shape. The grains are often of white to cream colour, and the colour can be further affected by the colour of the fermentation medium (Guzel-Seydim et al., 2021). Water kefir grains consist of large amounts of water, and they are built from bacterial and yeast cells embedded in an exopolysaccharide environment (Lynch et al., 2021).

Figure 1. Photo of water kefir grains

Water kefir grains represent complex consortia of multiple species of bacteria and yeasts (Table 1). As reported by Patel et al. (2022), the grains have greater microbial density and diversity compared to water kefir. Bacteria present in water kefir grains primarily belong to lactic acid bacteria (LAB), especially bacteria of genera previously described as *Lactobacillus* and *Leuconostoc*, and acetic acid bacteria with *Acetobacter* as the prevailing genus (Fiorda et al., 2017; Lynch et al., 2021). On the level of species, Laureys

and De Vuyst (2017) have suggested *Lactobacillus paracasei*, *Lactobacillus hilgardii*, and *Lactobacillus nagelii*, recently re-classified as *Lacticaseibacillus paracasei*, *Lentilactobacillus hilgardii* and *Liquorilactobacillus nagelii*, respectively (Zheng et al., 2020), to be the key bacterial species in water kefir fermentation. A study review conducted by Lynch et al. (2021) has demonstrated that these species were among the most frequently reported species across different studies, although they were not always detected in water kefir. Water kefir grains can also include other LAB of genera *Leuconostoc*, *Oenococcus*, and *Bifidobacterium* and other bacterial genera (Fiorda at al., 2017). Acetic acid bacteria have been reported to be more abundant in water kefir studies produced under aerobic conditions, however they have been detected in water kefirs in anaerobic conditions as well (Lynch et al., 2021). Among the yeast species, *Saccharomyces cerevisiae* has been reported as most abundant (Fiorda et al., 2017; Lynch et al., 2021). Other species of genus *Saccharomyces* and other yeast genera (*Zygotorulaspora*, *Pichia*, *Hanseniaspora*) are less common for water kefir microbiota (Fiorda et al., 2017; Lynch et al., 2021).

Table 1. Species of yeasts and bacteria discovered in water kefir and water kefir grains

Genus	Species
Lactic acid bacteria	
Lactobacillus [a]	*Levilactobacillus brevis* ***Lacticaseibacillus (Lcb.) casei***, ***Lcb. paracasei***, *Lcb. rhamnosus*, *Lcb. paracasei subsp. tolerans* ***Lentilactobacillus (Llb.) hilgardii***, *Llb. buchneri*, *Llb. parabuchneri*, *Llb. kefiri, Llb. sunkii, Llb. parafarraginis, Llb. diolivorans* *Lactiplantibacillus (Lpb.) plantarum*, *Lpb. pseudoplantarum* *Liquorilactobacillus (Lqb.) hordei*, ***Lqb. nagelii***, *Lqb. satsumensis*, *Lqb. ghanensis* *Lactobacillus (Lb.) helveticus*, *Lb. kefiranofaciens* *Fructilactobacillus fructivorans*, *Secundilactobacillus collinoides* *Schleiferilactobacillus (Slb.) harbinensis, Slb. perolens*
Streptococcus	n.d.
Lactococcus	*Lactococcus (Lc.) lactis*, *Lc. cremoris*
Leuconostoc	*Leuconostoc mesenteroides* subsp. *dextranicum, L. mesenteroides* subsp. *mesenteroides*, *L. citreum*, *L. pseudomesenteroides*
Pediococcus	n.d.
Bifidobacterium	*Bifidobacterium psychraerophilum*, *B. crudilactis*, *B. aquikefiri*
Oenococcus	*Oenococcus oeni*, *O. kitaharae*
Acetic acid bacteria	
Acetobacter	*Acetobacter lovaniensis*, *A. fabarum*, *A. orientalis*, *A. tropicalis*, *A. okinawensis*, *A. indonesisensis*
Gluconobacter	*Gluconobacter liquefaciens*, *G. japonicus*, *G. roseus*, *G. oxydans*
Other bacteria	
	Bacillus cereus, *Klebsiella pneumoniae*, *Enterobacter ludwigii*, *Pseudarthrobacter chlorophenolicus, Zymomonas mobilis*
Yeasts	
Saccharomyces	***Saccharomyces cerevisae***, *S. eubayanus* [b]
Pichia	*Pichia fermentans*, *P. membranifaciens*, *P. cecembensis*
Candida	*Candida californica*, *C. ethanolica*
Hanseniaspora	*Hanseniaspora uvarum*, *H. valbyensis*, *H. vineae*
Other genera	*Brettanomyces anomalus*, *B. bruxellensis*, *Kluyveromyces lactis*, *Lachancea meyersii*, *L. fermentati*, *Meyerozyma caribbica*, *Monosporozyma aerobia*, *Sugiyamaella valdiviana*, *Torulaspora delbrueckii* [b], T. *pretoriensis*, *Yarrowia lipolytica*, *Zygosaccharomyces lentus*, *Zygotorulaspora florentina*,

Modified from Lynch et al. (2021). Species that are suggested as key water kefir species by Lynch et al. (2021) are highlighted in bold. n.d. – not determined;
[a] species of various genera previously considered as genus *Lactobacillus* are put into the same group;
[b] Patel et al. (2022)

Microbial composition and frequency of microbial strata in water kefir grains and liquid exhibit great variation. Lynch et al. (2021) mark that lactic acid bacteria dominate in water kefir in most studies. For example, bacteria of the *Lactobacillus* group or belonging to genera *Bifidobacterium, Leuconostoc*, and *Pediococcus* accounted for up to 50% of microbial metagenome in the metagenomic study of Verce et al. (2019). Verce et al. (2019) did not report identification on acetic acid bacteria, and yeasts (mostly of genus *Saccharomyces* and to a lesser extent genus *Bretannomyces*) represented around 25% of water kefir metagenome. In the study of Patel et al. (2022) involving metagenomics, gram-negative bacteria *Zymomonas mobilis* were reported to be the dominant species in water kefir and water kefir grains. According to the authors, these bacteria that can efficiently perform ethanol fermentation amounted to 72–83% of all detected microorganisms. Patel et al. (2022) reported that the abundance of lactic acid bacteria, acetic acid bacteria, and yeasts (with *Bretannomyces* as a predominant genus) was approximately 20%, below 1%, and 3–7%, respectively. Lynch et al. (2021) also reported that *Z. mobilis* had been previously discovered as the most abundant bacterial species in other studies as well, which allows to assume that this variation in microbial composition can depend on the geographical origin of water kefir grains. In all reviewed studies, however, bacterial count was exceeding the amount of yeast cells, whether it was culture-dependent (Laureys & De Vuyst, 2017; Lynch et al., 2021) or culture-independent studies (Lynch et al., 2021; Patel et al., 2022).

To this moment, development of defined starter cultures is greatly limited, and diverse microbial communities with unknown precise species composition remain the primary starting cultures used in water kefir production (Lynch et al., 2021). Lynch et al. (2021) noted in their review that the species composition of water kefir grains exhibits significant geographical variation and that some water kefir grains may originate from milk kefir grains based on their species composition. Using milk kefir grains for water kefir fermentation has been shown less efficient, however Tzavaras et al. (2022) demonstrated that it is possible to utilise milk kefir grains to produce water kefir by gradually substituting milk-based fermentation medium with a sucrose-based solution. This finding indirectly indicates that some water kefir grains might have a milk kefir origin.

1.2.2 Ingredients and production process of water kefir

Water kefir grains require a source of carbon, nitrogen, and minerals as a minimal condition for fermentation. Lynch et al. (2021) reported that table sugar and brown sugar constitute the most common source of carbon, with brown sugar containing minerals in addition to sucrose. As the authors noted in their review, nitrogen, additional carbon sources, and minerals are usually introduced to the fermentation medium with fresh or dried fruit or their preparations. According to Lynch et al. (2021), dried figs are the most frequently used fruit addition to the substrate, and several studies support better growth of water kefir grains with figs in the substrate compared to other fruit, which might be explained by their micronutrient content. Laureys et al. (2017) in their study demonstrated that calcium and other ions with buffering properties are especially important in water kefir fermentation. Lynch et al. (2021), based on this knowledge, linked the positive effect of dried figs on water kefir fermentation and grain growth to their high calcium content – 162 mg/100 g, which is greater than in dried apricots, raisins, and prunes that follow with 55, 50, and 43 mg of calcium per 100 g, respectively.

Plant juices and saps can serve as a base in water kefir production as well as they represent a suitable medium for the growth of water kefir microorganisms (Randazzo et al., 2016). Compared to a sucrose solution, juices and saps can contain additional valuable nutrients, for example dietary fibre, phenolic compounds, and vitamins. In the recent years, various juices and saps have been studied as media for water kefirs production: for example black carrot, apple, grape, green cabbage (Agirman et al., 2024), chokeberry (Esatbeyoglu et al., 2023), quince, kiwi fruit, prickly pear, pomegranate (Randazzo et al., 2016), melon, strawberry, tomato, onion, carrot, fennel (Corona et al., 2016), and pear juices (Hampton et al., 2021), palm sap (Zongo et al., 2020).

Birch sap is another promising candidate for a medium in water kefir production. It is an odourless and colourless liquid, or a liquid with slight opalescence, derived primarily from silver birch (*Betula pendula*) and downy birch (*Betula pubescens*) during spring (Mingaila et al., 2020). Birch sap has gained increasing recognition as a promising non-wood product in Finland due to the high production potential of birch species (Dubois et al., 2020; Möttönen & Heinonen, 2017). Despite fluctuations in its content (Kallio & Ahtonen, 1987; Mingaila et al., 2020; Staniszewski et al., 2020), birch sap constitutes a satisfactory source of nitrogen and carbon due to its free amino acid and carbohydrate

contents amounting to approximately 100–500 mg/L (Ahtonen & Kallio, 1989) and 0.8–2.6% (w/v), respectively (Łuczaj et al., 2014; Mingaila et al., 2020). Calcium, a potentially important mineral in water kefir production, has been reported as the second most abundant mineral in birch sap, ranging from 14.6 to 43.6 mg/L on average (Bilek et al., 2017). In water kefirs based on diluted birch saps, the calcium content is comparable to the amount that is usually introduced with dried or raw fruit (Laureys et al., 2018; Laureys & De Vuyst, 2017).

Laboratory-scale water kefir production varies in the used substrates, production schemes, and fermentation conditions. Patel et al. (2022) used the following process that consisted of two fermentations (Figure 2): water kefir grains (10.7% w/w) were mixed with a sucrose solution (80.5% w/w) and backslop (8.8% w/w) – the liquid, in which the water kefir grains had been cultivated before. The sucrose solution was prepared by dissolving 5% (w/w) of cane sugar in water. The liquid for water kefir grains cultivation included 10.7% (w/w) of water kefir grains, 78.8% (w/w) of sucrose solution, 8.8% (w/w) of backslop, and 1.8% (w/w) of fig extract (supernatant from the centrifuged mixture of 25% (w/v) homogenised dried organic figs in mineral water). The fermentation was performed for 24 hours at 30°C with access to oxygen, after which the grains were removed by sieving, and the second fermentation continued for 6 hours in tightly closed jars without access to air.

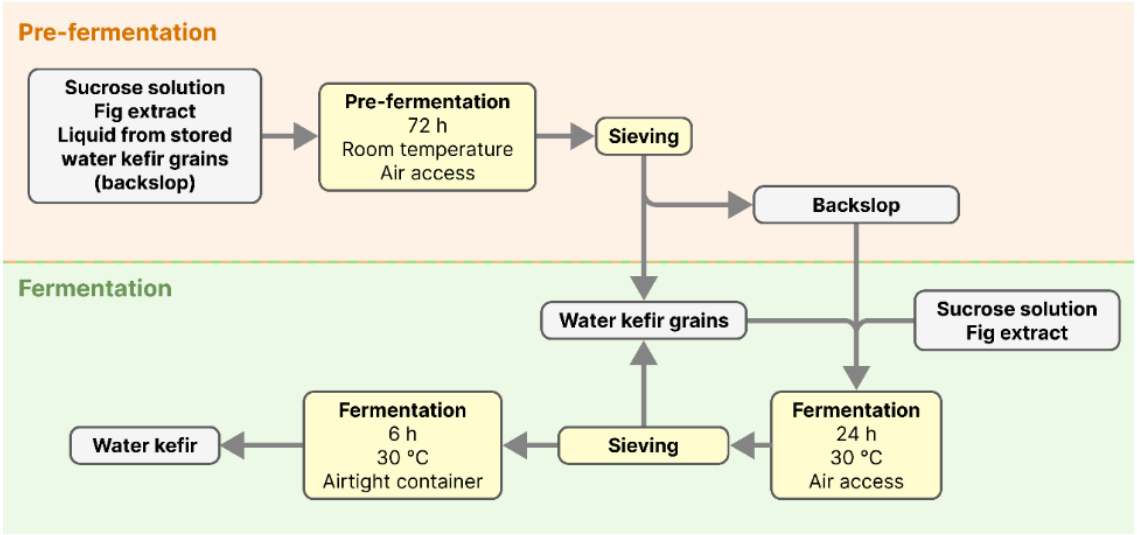

Figure 2. Water kefir production procedure from the study of Patel et al. (2022)

Water kefir grains increase in their mass during fermentation and remain their fermentative ability, which allows for their reuse by storing them in fresh sugar-rich medium or in a lyophilised or deep-frozen form (Lynch et al., 2021). In other studies

reviewed by Lynch et al. (2021), water kefir is produced in a single fermentation stage that continues for 3–8 days at different temperatures with or without access of oxygen.

1.3 Biochemistry of water kefir production: metabolic transformations and their contributions to the sensory qualities of the product

Lactic acid fermentation, ethanol fermentation, and acetic acid fermentation (Figure 3) are dominant metabolic pathways in water kefir production (Lynch et al., 2021). Each fermentation type is typical to a particular group of microorganisms: lactic acid bacteria perform lactic acid fermentation, yeasts perform ethanol fermentation, and acetic acid bacteria perform acetic acid fermentation. All main fermentation pathways start with glucose as substrate, and disaccharides and other monosaccharides are transformed into glucose with the help of microbial enzymes.

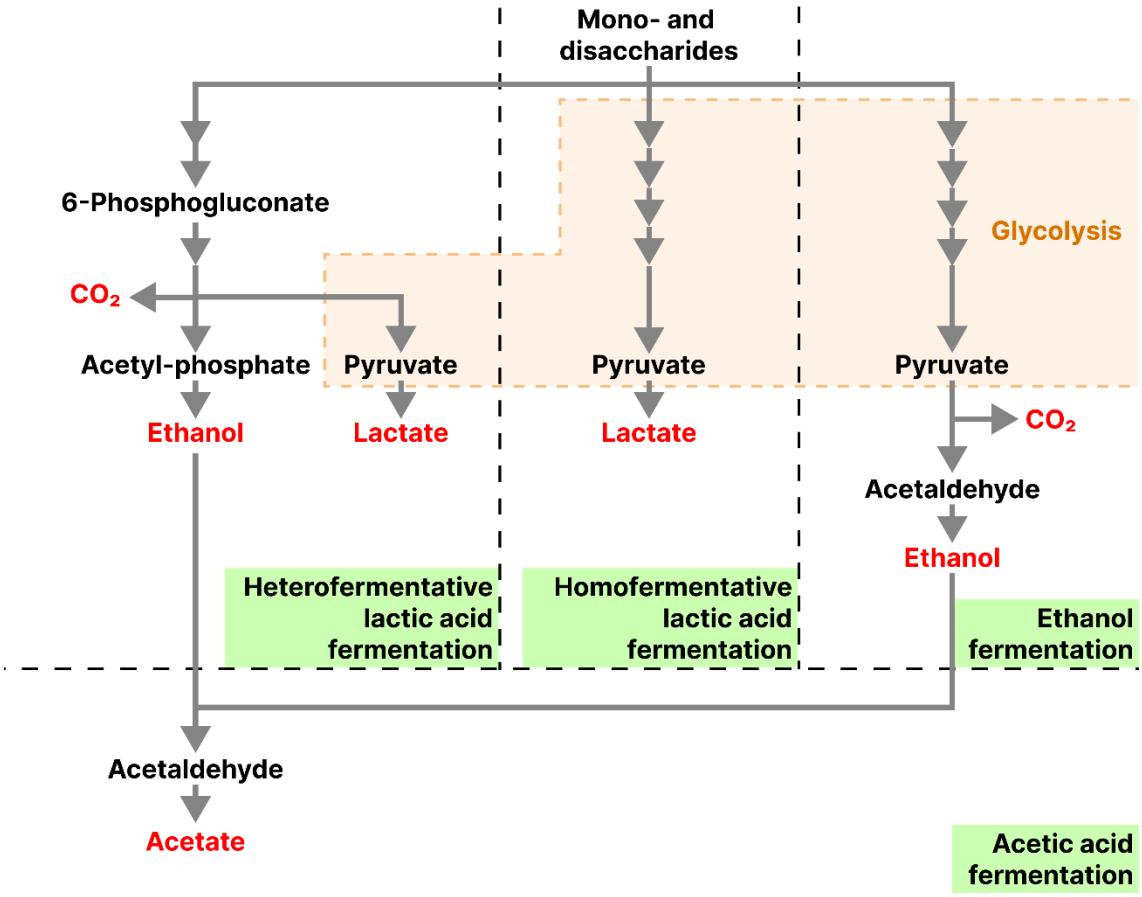

Figure 3. Main fermentation pathways occurring in water kefir production

Lactic acid fermentation can be homofermentative and heterofermentative. In homofermentative lactic acid fermentation (for example, performed by *Liquorilactobacillus (Lqb.) nagelii* from water kefir microbiota, see Table 1), bacteria

metabolise six-carbon monosaccharides and their disaccharides via glycolysis and produce lactic acid as a single product (Axelsson, 2004). Heterofermentative lactic acid fermentation (e.g. in bacteria *Lentilactobacillus (Llb.) hilgardii* and *Lacticaseibacillus (Lcb.) paracasei* in water kefir*)* has 6-phosphogluconate as a key intermediate metabolite, whose further transformation results in production of lactic acid, carbon dioxide, and ethanol (Axelsson, 2004). Lynch et al. (2021) state that the production of lactic acid optimises the pH value of water kefir for water kefir microbiota, preventing the introduction of other microorganisms, including pathogens.

During ethanol fermentation, monosaccharides are transformed into pyruvate via glycolysis, and pyruvate is converted into carbon dioxide and ethanol (Lynch et al., 2021). In water kefir, ethanol fermentation happens mostly in yeasts but also in some bacteria, for example *Zygomonas mobilis* that was reported to be a predominant microbial species in water kefir in some studies (Lynch et al., 2021; Patel et al., 2022). In acetic acid fermentation, ethanol is gradually oxidised to acetic acid.

In addition to products of main fermentation pathways, bacteria and yeasts produce other metabolites that determine the properties of water kefir and water kefir grains. Some lactic acid bacteria produce dextrans – glucose-derived exopolysaccharides (Lynch et al., 2021). According to the authors, most abundant LAB of water kefir *Lqb. nagelii* and *Llb. hilgardii* (see Table 1 for a list of water kefir microorganisms) can produce exopolysaccharides from sucrose, as well as lactic acid bacteria of species *Lqb. hordei*, *Lqb. satsumensis*, *Leuconostoc mesenteroides*, and *Leuconostoc citreum*. LAB polysaccharides ensure the structure and physical unity of water kefir grains and perform a function similar to the function of other exopolysaccharides in biofilm-producing bacteria. They provide structural support for microbial cells and create a separate microenvironment that traps nutrients from the medium and protects the yeast-bacteria consortium from environmental stressors (Nwodo et al., 2012).

Apart from lactic acid fermentation, lactic acid bacteria can transition to other metabolic pathways in different conditions, resulting in a wide range of additional metabolites. If citrate, a major intermediate metabolite of the tricarboxylic acid cycle in yeasts, is present in abundance, while the pH and sugar concentration of the medium are low, heterofermentative LAB of species *Lc. lactis* and *Lc. cremoris* can metabolise citrate into pyruvate (Axelsson, 2004; Zaunmüller et al., 2006). After that, these bacteria can ferment

pyruvate into 2,3-butandedione (diacetyl), 3-hydroxybutanone (acetoin), and 2,3-butanediol (Axelsson, 2004; Zaunmüller et al., 2006). Other water kefir bacteria *Lcb. casei* and *Lcb. paracasei* can switch to production of lactic acid mixed with ethanol, acetic acid, and formic acid when grown in anaerobic conditions with limited sugar content (Axelsson, 2004). According to Zaunmüller et al. (2006), the amount of acetic acid and ethanol produced by heterofermentative lactic acid bacteria depends on the availability of fructose and citrate that can regenerate a cofactor needed for lactic acid fermentation.

Lactic acid bacteria are auxotrophic and require various amino acids, vitamins, and carbohydrates for growth (Whitman, 2009). In the water kefir environment, the nutritional needs of lactic acid bacteria are fulfilled with the help of yeast metabolism. Yeast cells are capable of hydrolysing a wide range of proteins, releasing shorter peptides and free amino acids that lactic acid bacteria can utilise in their growth (Lynch et al., 2021). In addition, high invertase activity of yeasts allows them to quickly convert sucrose into glucose and fructose, which become available to be metabolised by lactic acid bacteria as well (Lynch et al., 2021). Yeast activity therefore enables growth of lactic acid bacteria in the environment of water kefir. Moreover, yeast metabolism can affect the metabolism of lactic acid bacteria, as it was mentioned above in the case of higher citrate concentrations driving diacetyl/acetoin pathway in LAB.

The metabolism of yeast cells depends on external factors as well. In the presence of molecular oxygen, facultative anaerobic yeasts (e.g. *S. cerevisae* in water kefir) tend to utilise carbohydrates in a Krebs cycle with the production of citrate (Teusink & Molenaar, 2017). If the conditions are anaerobic, yeasts metabolise carbohydrate via glycolysis and ethanol fermentation (Teusink & Molenaar, 2017). Teusink & Molenaar (2017) state that carbohydrate concentration is another factor that affects yeast metabolism: higher glucose content induces glycolysis and subsequent ethanol fermentation.

During their growth, yeast cells synthesise and release byproducts of their metabolic activities. In particular, amino acid metabolism in yeasts results in the production and release of various higher alcohols (e.g. propanol, butanol, isoamyl alcohol, phenyl ethanol) and esters (e.g. ethyl acetate, isoamyl acetate, phenyl ethyl acetate, ethyl hexanoate, ethyl octanoate) (Procopio et al., 2011). According to Procopio et al. (2011), higher alcohols and esters are not produced at the same rate: the resulting absolute concentration of higher alcohols sufficiently exceeds esters.

Metabolism of water kefir microbiota contributes to the sensory qualities of the final product (Table 2). All water kefir microorganisms utilise sucrose during their metabolism, which leads to a decrease in the carbohydrate concentration and the associated sweetness sensation. The study of Laureys and De Vuyst (2017) demonstrated that the concentration of fructose increases at the beginning of water kefir production, which might be associated with the higher invertase activity and quicker hydrolysis of sucrose into glucose and fructose compared to the subsequent metabolism rate fructose. The concentration of fructose nevertheless continues to decline after the initial spike, leading to a total amount of residual carbohydrates being less than 1 g/L after 48–144 hours of fermentation (Laureys & De Vuyst, 2017).

Table 2. Microbial metabolites contributing to the sensory qualities of water kefir

Compound		**Related perception**	**Producer**
Ethanol		Alcoholic odour, "burning" mouthfeel and slightly sweet and bitter taste[h]	Yeasts, lactic acid bacteria (less)[a,d]
Organic acids			
	Acetic acid	Tart and sour taste[g], vinegary[c]	Acetic acid bacteria, lactic acid bacteria (less)[a,b]
	Lactic acid	Sour taste, dairy-like[c]; acrid[g]	Lactic acid bacteria[a,b]
	Gluconic acid	Sour taste, mild and clean acidity[c]	*Zygomonas mobilis*[c]
	Citric acid	Tart taste with a "burst" of tartness[g]	Yeasts[a]
Carbon dioxide		"Tingling", "fizzy" mouthfeel, slightly sour[e]	Yeasts (more), lactic acid bacteria (less)[a,b]
Higher alcohols	2-methylbutanol 3-methylbutanol 2-phenylethanol 1-octanol 2,3-butanediol 2-methyl-1-propanol Isoamyl alcohol	Fruity, fermented odour[a,c]	Yeasts (more), lactic acid bacteria (less)[a,c,i]

Compound		Related perception	Producer
Higher aldehydes	2-methylbutanal Benzaldehyde 2-phenylacetaldehyde 3-methylbutanal	Green, fermented odour[a,c]	Yeasts[a,c,i]
Esters	Ethyl acetate 2-phenylethyl acetate Benzyl acetate Furfuryl acetate Isobutyl acetate Isopentyl acetate Isoamyl acetate Ethyl octanoate Ethyl decanoate Ethyl hexanoate	Floral, fruity, sweet, fermented odour[a,c]	Yeasts (more), lactic acid bacteria (less)[a,c,i]
Ketones	2,3-butanediol (diacetyl) 3-hydroxybutanone (acetoin)	Butter, dairy odour[f]	Lactic acid bacteria[d]

[a] Lynch et al. (2021); [b] Axelsson (2004); [c] Patel et al. (2022); [d] Zaunmüller et al., 2006; [e] Clark et al. (2011); [f] Oberman et al. (1982); [g] Da Conceicao Neta et al. (2007); [h] Mattes & DiMeglio (2001); [i] Nsogning Dongmo et al. (2017)

The flavour profile of water kefir is also affected by the produced microbial metabolites. Lactic acid, acetic acid, and citric acid produced mainly by lactic acid bacteria, acetic acid bacteria, and yeasts, respectively, contribute to the sour taste of water kefir (Da Conceicao Neta et al., 2007). Moreover, these organic acids differ in their sensory qualities: Da Conceicao Neta et al. (2007) describe acetic acid as “tart and sour”, citric acid as “tart” and delivering “a ‘burst’ of tartness”, and lactic acid as “acrid”.

Ethanol and carbon dioxide, products of ethanol fermentation and to a lesser extent LAB metabolism, have a specific perception. Carbon dioxide manifests with a slight sour taste and a mouthfeel that is often described as “tingling”, “prickling”, “burning”, “fizzy” or “spritzy” (Clark et al., 2011; Gawel et al., 2020). Ethanol, when ingested, has an irritating, “burning” effect and a slightly sweet and bitter taste (Mattes & DiMeglio, 2001). Mattes & DiMeglio (2001) state that ethanol is more readily perceivable by its odour, which can be called as “alcoholic”. In addition to their own sensory properties, ethanol and carbon dioxide can modify the perception of other tastes, odours, and mouthfeels, for example sweetness, bitterness, astringency (Mattes & DiMeglio, 2001; Clark et al., 2011; Gawel et al., 2020).

Diacetyl and acetoin, which are produced by heterofermentative lactic acid bacteria, contribute to butter- and dairy-like odours (Oberman et al., 1982). Diacetyl is the key aroma compound in dairy products, but it is considered undesirable and related to spoilage in wines when present in concentrations higher than 5–7 mg/L (Bartowsky & Henschke, 2004). Most of other water kefir odours are associated with higher alcohols and esters that come mostly from yeast metabolism (Lynch et al., 2021; Patel et al., 2022). According to the studies of Fiorda et al. (2017) and Patel et al. (2022), floral, fruity, and typical fermented aromas can be attributed to higher alcohols and esters, predominantly resulting from yeast metabolism in water kefirs.

Chemical compounds that play the key role in shaping the flavour of water kefir can be subject to variation. Patel et al. (2022) reported ethanol (up to 1.06 g/L in the final product), acetic acid (up to 11.77 g/L) to be the most dominant compounds in water kefir, with lactic acid and gluconic acid being other important flavour compounds. Among volatile compounds, the authors highlighted higher alcohols (2-methylbutanol and 3-methylbutanol) and higher aldehydes (benzaldehyde, 2-methylbutanal, 2-phenylacetaldehyde, see Table 2). In another study, Laureys and De Vuyst (2014) compared the concentrations of volatile compounds to their threshold values and concluded that esters isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate might be the key volatile compounds in water kefir.

According to the review of Lynch et al. (2021), the role of different microorganisms in the production of different metabolites is not fully understood at the moment. Indeed, the water kefir consortium includes microorganisms of multiple species that are capable of producing the same compounds. The study of Nsogning Dongmo et al. (2017) has demonstrated that lactic acid bacteria *Lactiplantibacillus plantarum*, *Levilactobacillus brevis* (both can be present as minor species in water kefir, see Table 1 in section 1.2.1) can produce a range of aldehydes and higher alcohols. Among such metabolites are 2-methylbutanol and 3-methylbutanol, which have been associated with yeast metabolism and reported to be important contributors to water kefir flavour (Patel et al., 2022). While lactic acid bacteria can produce metabolites usually connected to yeasts, their specific quantitative contribution to the metabolite profile in water kefir remains unclear. In their recent study, Patel et al. (2022) tried to connect water kefir microbiota to specific metabolites and sensory qualities of water kefir. The authors did not discover significant ($p < 0.05$) positive correlation between the number of most LAB species and the

concentration of volatile compound, except for the correlation between *Liquorilactobacillus nagelii* and benzyl acetate. On the other hand, yeasts *Bretannomyces bruxellensis* were associated with 2-phenylethanol, 2-methylbutanol, 3-methylbutanol, and 2-phenylethylacetate (Patel et al., 2022). Another yeast species *Saccharomyces eubayanus* was demonstrated to be in a significant positive correlation with the concentration of 3-methylbutanal (Patel et al., 2022). This indicates that the production of volatile compounds in water kefir can be associated with yeast metabolism, except for a few individual compounds. It has to be added that while various compounds can be reported as present in the water kefir, their concentration might be less than their sensory threshold value, thus factoring in no sensory difference, as it was reported for wine by Varela et al. (2009).

1.4 Product development of organic water kefirs

1.4.1 Preserved microbial viability versus improved product stability

As the potential beneficial health effect of water kefirs previously discussed in this work is associated with the probiotic microbiota of the product (Lynch et al., 2021), manufacturers might have an objective to keep microorganisms in the final product viable in order to maximise the health benefits. Lahtinen (2012) in his article suggests that some beneficial effects of probiotics might be observed with inactivated microbial cells as well, but viable cells are more efficient in most studies. For example, fermented products with viable cells have been found to be more efficient in decreasing allergic reactions (de Water, 1999), to facilitate lactose absorption more efficiently (Lerebours et al., 1989), to have a stronger stimulating effect on the immune system (Ouwehand & Salminen, 1998), to mitigate the symptoms of the irritable bowel syndrome more successfully (Tsuchiya et al., 2004), and to exhibit a stronger immunomodulating effect (Vinderola et al., 2005) compared to products with inactivated microbial cells.

Studies comparing products or mixtures with viable probiotic cells and inactivated microorganisms concern predominantly dairy products, and no similar study has been found regarding water kefirs or water kefir microbiota. Despite that, the beneficial effect of water kefirs can be linked to the viability of their microbiota as well since many microbial species frequently observed in water kefir are associated with positive health effects and found in dairy products as well (see Table 1 in section 1.2.1 for the list of lactic acid bacteria discovered in water kefirs), for example *Lcb. paracasei* (Falfán-Cortés et al., 2022). In addition, Tan et al. (2022) isolated strains of several LAB species, including *Lqb. satsumensis*, *Lcb. paracasei*, *Lqb. hilgardii*, and *Lqb. nagelii*, from milk kefir and water kefir and demonstrated their high potential probiotic activity, for example competitive adhesion to intestinal epithelial cells and exclusion of pathogenic microorganisms. De Filippis et al. (2020) consider the ability to adhere to the intestinal epithelium and to colonise the gastrointestinal tract to be the most important factor in the activity of probiotics. The authors, as well as Tuomola and Salminen (1998) link this ability to the viability and active metabolism of probiotic microorganisms. The connection between possible health benefits of probiotics in water kefir and the viability of related microorganisms creates an objective for manufacturers to keep water kefir microorganisms alive.

However, presence of alive microorganisms is likely to reduce the physicochemical and sensory stability of the ready water kefir due to continuing fermentation. As water kefirs are based on sucrose-containing solutions, they contain substantial amounts of fermentable carbohydrates, predominantly sucrose, glucose, and fructose. Moreover, concentrations of fermentable sugars can remain sufficient for the further fermentation after the beverage has been produced. For example, in the study of Patel et al. (2022), water kefir contained approximately 40 g/L of sucrose at the start of fermentation and approximately 10 g/L of sucrose and 10 g/L of fructose after the end of the 30-hour production cycle. The study of Laureys et al. (2018) showed that water kefirs might contain up to 42.1 g/L of total residual carbohydrates after 72 hours of fermentation. According to the authors, the remaining carbohydrate levels depend on the used substrate and initial sucrose, fructose, and glucose concentration, as well as on nutrient concentration, presence of oxygen, and exhaustion of the starter culture. As the yeasts and bacteria that contribute to the production of the beverage remain viable, they are able to continue fermentation with the residual sugars during storage.

One of the most common ways to prolong the shelf life of foodstuffs and make them more stable is refrigeration. However, although cold storage can reduce the rate of fermentation, the activity of the water kefir microbiota does not stop completely at lower temperatures. Aguilera et al. (2007) state that the general metabolism and expression of most genes are downregulated in *S. cerevisae* at the temperature of 4 °C, but the metabolism does not halt.

Lactic acid bacteria (refer to Table 1 in section 1.2.1 for key microbial species in water kefir) are able to maintain metabolic activity in cold temperatures as well. Most LAB species can grow at 10 °C (Salminen and Wright, 2004), and some bacterial species and strains have been demonstrated to maintain metabolic activity or growth in yoghurts at 4 °C (Rutella et al., 2016). In particular, the authors have established that yoghurts can maintain sufficiently high proteolytic activity after 28 days of cold storage and that the number of colony-forming units for LAB of species *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lacticaseibacillus casei*, *Lacticaseibacillus rhamnosus*, although decreasing, can reach a plato of 10^6–10^8 CFU/g. In addition, Silva et al. (2018) have demonstrated that specific strains of LAB *Weissella viridescens* and *Latilactobacillus sakei* can maintain slow growth at 4 °C.

According to Du Toit & Pretorius (2002), acetic acid bacteria can withstand low temperatures as well, although their growth becomes slower at the temperatures lower than 10 °C. Strains of *Acetobacter*, the most prevalent genus of acetic acid bacteria in water kefir, can be maintained on hard medium at 4 °C for 2 weeks (Staley et al., 2005), which indicates that bacteria of this genus do not immediately lose their viability at cold storage temperatures.

As the microorganisms typically present in water kefirs are reported to withstand the temperatures of cold storage, they can remain maintain metabolic activity in refrigerated water kefirs. Grassi et al. (2022) demonstrated in their study that acetic acid bacteria and yeasts remain viable in kombucha, a beverage of similar nature to water kefir, after 90 days of storage at 4 °C. Although the microbial content of kombucha and water kefirs is not identical, these beverages contain microorganisms belonging to the same genera and species (see microorganisms of water kefir in Table 1 in section 1.2.1), for example acetic acid bacteria of genera *Gluconobacter* and *Acetobacter*, yeasts of genera *Saccharomyces* and *Zygosaccharomyces* (Harrison & Curtin, 2021), and lactic acid bacteria of species *Liquorilactobacillus. nagelii* and *Liquorilactobacillus satsumensis* (Coton et al., 2018). The information about microbiota viability in water kefir during cold storage is lacking in the scientific publications, but the similarity in microbiota and physicochemical factors between kombucha and water kefir provides a reason to assume that the microorganisms in water kefirs can remain viable as well. According to Grassi et al. (2022), LAB do not remain viable after 20 days of cold storage in a kombucha beverage, but this also might be related to their initial smaller representation in the beverage.

Viable and metabolically active microorganisms factor in the physicochemical and sensory instability of water kefir during cold storage as they can change the chemical content and sensory qualities of the beverage over time. Studies of Casarotti et al. (2014) and Jairath et al. (2012) have demonstrated that lactic acid bacteria and yeasts are capable of fermenting substrates in cold storage conditions, resulting in an increase in carbon dioxide pressure in the medium and a decrease in its pH value. Similar physicochemical changes in water kefir can change its sensory qualities as well, namely perceived carbonation and sourness. While individual preferences may influence the acceptability of strong carbonation, excessive carbon dioxide levels can generally be the reason for lower product acceptance (McEwan & Colwill, 1996; Walsh et al., 2014). Bueno et al. (2021) in their study of pitaya- and apple-derived water kefirs discovered that organic

acid and alcohol content differed between freshly prepared beverages and samples that had been stored for 28 days at 4 °C. The authors did not study the sensory acceptance of the beverages after cold storage, and no publications on sensory qualities of water kefirs after cold storage have been found to this date. However, the documented change in the chemical composition of the product during storage provides a reason to expect differences in the sensory perception and acceptability of water kefir.

1.4.2 Reducing post-production fermentation in water kefirs by lowering sucrose content

To prevent the increase of the carbon dioxide concentration and other undesirable physicochemical and sensory changes in water kefir during storage, the fermentation needs to be limited in the final product. Several ways to limit or inhibit fermentation are successfully used in the food industry besides previously discussed refrigeration, including pasteurisation, irradiation, high-pressure treatment, and addition of antimicrobial compounds such as sorbates, benzoates, and dimethyl dicarbonate (Koutchma, 2009; Labbé & Nolan, 2009; Parish, 2009). However, the possibility to implement such methods in a product with alive microbial cells is impossible. These techniques are linked to a nonselective reduction of viable microbiota in the medium and cannot be used in water kefir processing if the aim is to maintain the highest number of probiotic microorganisms possible.

Another possible solution to limit the post-production fermentation in water kefir is to limit the amount of available carbohydrates. The amount of fermentable carbohydrates, as well as other substrates, determines the growth rate and the rate of biochemical reactions in microbial cells. A decrease in the substrate concentration leads to lower metabolic activity and growth rate with a possibility of reaching a plateau (Wang et al., 2004). If the production scheme and the recipe of water kefir are modified so that the ready product contains near-zero amounts of sucrose, glucose, and fructose, the rate of microbial metabolism during cold storage can be expected to be lower. Consequently, restricted metabolism during storage might result in a more stable product that does not change in its sensory qualities over time.

To reduce the amount of fermentable sugars by the end of the production cycle, the amount of sucrose – the sugar added to the fermentation medium in the largest quantity – must be reduced. However, sucrose has a dual of role of a substrate for water kefir microbiota and a sweetener for the final product. For this reason, the reduction in sucrose

content should still allow the yeasts and bacteria in the starter culture to produce metabolites during the production stage. At the same time, water kefir with a reduced amount of sucrose must maintain the acceptable level of sweetness for the optimal taste.

To achieve these two aims, a two-step production process similar to the process used by Patel et al. (2022) (see Figure 2 in section 1.2.2) can be implemented. In the first step, water kefir grains are introduced to the medium consisting of birch sap, water, and a reduced amount of sucrose to metabolise the nutrients in the first round of fermentation with the free access of air. In the second step, the water kefir grains are removed from the medium, non-fermentable sweeteners are introduced, and the medium is transferred into airtight containers for the second round of fermentation. During the second fermentation, the water kefir microbiota metabolises fermentable sugars remaining from the first fermentation and carbonates the beverage making the product ready for consumption.

Splitting the fermentation into two steps in the production process allows to regulate the amount of sucrose in the recipe more easily. It also provides an opportunity to add other sweeteners and flavouring ingredients to the product without a risk of affecting the starter culture as the water kefir grains are removed from the water kefir after the first fermentation. The two-step scheme has been successfully implemented in laboratory-scale water kefir production (Patel et al., 2022), which makes it a viable option for the production of water kefir with reduced sucrose content.

To maintain the satisfactory sweetness of the final product, non-fermentable sweeteners must be used in water kefir. Sweeteners represent a large group of chemical compounds of various structure that differ in their energy value, sweetness intensity, and additional physiological effects. Sweetening compounds can be divided into two groups based on their sweetness intensity: intense sweeteners and natural sweeteners. This division mostly coincides with a division into natural and artificial sweeteners based on their origin. The majority of intense sweeteners are derived from chemical synthesis, whereas most bulk sweeteners are obtained from natural sources (Shankar et al., 2013).

The use of sweeteners in food products in the European Union is regulated by Regulation (EC) No 1333/2008 on food additives. According to the Regulation, a wide range of intense and bulk sweeteners are permitted to be used as sweeteners in food products (Table 3). Most of the permitted sweeteners, however, do not comply with the regulation for organic food products as they are chemically synthesised (Regulation (EU) 2018/848).

According to Regulation (EU) 2018/848 laying down the principles of organic production, the use of food additives and non-organic ingredients with sensory or technological functions must be restricted to a minimal extent and cases of essential need. For this reason, if a manufacturer is aiming at an organic product, they cannot utilise such sweeteners. The focus of this work will be placed on the sweeteners that are allowed to be used in the organic food products now or have a potential to be approved in the future: xylitol, erythritol, and steviol glycosides.

Table 3. Sweeteners permitted for use in the European Union

Sweetener	Sweetness compared to sucrose	Production
Intense sweeteners		
Acesulfame K	200 [a]	Multi-step chemical synthesis [b]
Aspartame	180–200 [a]	Chemical coupling of L-aspartic acid and L- or DL-phenylalanine [c]
Cyclamates	30 [a]	Chemical synthesis [d]
Saccharins	300–500 [a]	Chemical synthesis from e.g. toluene, phthalic acid [e]
Sucralose	600 [a]	Selective chlorination of sucrose [f]
Thaumatin	2000–3000 [a]	Fruit of *Thaumatococcus daniellii* [g]
Neohesperidin dihydrochalcone	1900 [a]	Chemical synthesis [h]
Steviol glycosides	150–450 [h]	Leaves of plant *Stevia rebaudiana*; enzymatic conversion [i]
Neotame	8000 [j]	Reduction alkylation of aspartame [j]
Salt of aspartame-acesulfame	350 [j]	Chemical synthesis [j]
Advantame	7000–47000 [c]	N-alkylation of aspartame [c]
Bulk sweeteners		
Isomalt	0.5 [a]	Enzymatic conversion and hydrogenation of sucrose [k]
Sorbitols	0.5–1.0 [a]	Hydrogenation of glucose [l]
Mannitol	0.7 [a]	Hydrogenation of glucose [l]
Polyglycitol syrup	0.33 [m]	Hydrogenation of starch hydrolysate [n]
Maltitols	1.0 [a]	Hydrogenation of maltose, maltose syrup, or starches [o]
Lactitol	0.5 [a]	Hydrogenation of lactose [p]
Xylitol	1.0 [a]	Hydrogenation of xylose; microbial biosynthesis [q]
Erythritol	0.6–0.8 [a]	Microbial biosynthesis; chemical hydrogenation [r]

[a] Mortensen, 2006; [b] Rymon Lipinski & Hanger, 2001; [c] O'Donnell, 2012; [d] Hunt et al., 2012; [e] Arnold et al., 1983; [f] Grotz & Munro, 2009; [g] EFSA Panel on Food Additives and Flavourings, 2021; [h] González et al., 2014; [i] Kinghorn et al., 2001; [j] O'Donnell, 2006; [k] Sentko & Willibald-Ettle, 2012; [l] Le & Mulderrig, 2001; [m] Evrendilek, 2012; [n] Livesey, 2003; [o] Kearsley & Deis, 2012; [p] Zacharis, 2012; [q] Rafiqul & Sakinah, 2013; [r] Moon et al., 2010

1.4.3 Xylitol, erythritol, steviol glycosides – alternative sweeteners for sucrose in organic water kefir production

Xylitol ((2*R*,3*R*,4*S*)-pentane-1,2,3,4,5-pentol) is a five-carbon polyol that has approximately the same sweetness intensity as sucrose (Ghosh & Sudha, 2012), which is the highest sweetness intensity among polyols (Schiweck et al., 2012). Xylitol can normally be found in small amounts in yeast, lichen, and fungi, fruits, vegetables, and oats (Schiweck et al., 2012). This sweetener is produced industrially by hydrolysis of birch hardwood and wood of other trees with the following hydrogenation of D-xylose

(Schiweck et al., 2012; Grembecka, 2015). Schiweck et al. (2012) report that xylitol can also be theoretically produced in microbial synthesis, but this method found limited industrial applications. This sweetener is widely used in the pharmaceutical and food industries, including production of chewing gums, candies, beverages, and table-top sweeteners (Ahuja et al., 2020)

Xylitol easily dissolves in water at room temperatures (68.8 g per 100 g at 20 °C) and remains stable at high temperatures and in a wide range of pH (Schiweck et al., 2012). This sweetener is considered nutritive as it is metabolised in the human organism and has the energy value of 2.4 kcal/g (Ahuja et al., 2020). Its low glycaemic index (Schiweck et al., 2012) and inhibiting effect on cariogenic bacteria (Ahuja et al., 2020) make it a favourable sweetener in food applications as a sucrose substitute. Xylitol is reported to have no strong aftertastes and to exhibit a noticeable cooling effect (Schiweck et al., 2012). In addition, this polyol has been demonstrated to cause laxative effects when consumed in moderately high amounts. If the ingested dose of xylitol exceeds 30 g, it can cause diarrhoea as xylitol is not fully absorbed in the small intestine, contributing to the osmotic effect in the colon (Ghosh & Sudha, 2012).

Erythritol ((2R,3S)-*meso*-butane-1,2,3,4-tetraol) is a four-carbon polyol with a sweetness intensity of 0.6–0.8 compared to sucrose (Ghosh & Sudha, 2012). According to the review of Grembecka (2015), small amounts of erythritol can be found in nature in various vegetables, fruits, mushrooms and fermented products. While most polyols are industrially produced by hydrogenation of carbohydrates, erythritol is most often produced with microbial synthesis (Rice et al., 2020). Rice et al. (2020) report that the industrial production of erythritol involves yeast and yeast-like species of genera *Torula*, *Moniliella*, *Candida*, and *Yarrowia*. The authors' review also includes the evidence that lactic acid bacteria of genera *Leuconostoc* and *Oenococcus* and LAB previously assigned to genus *Lactobacillus* can produce erythritol in anaerobic conditions.

Erythritol is moderately soluble in water at room temperatures (Schiweck et al., 2012). It is stable at high temperatures and in both acidic and alkaline environments (Schiweck et al., 2012; Rice et al., 2020). Its applications in the food industry include chewing gums, candy products, ice creams, and beverages (Grembecka, 2015). Erythritol is non-glycaemic, i.e. it does not increase blood sugar levels, and has a caloric value of ≤ 0.4 kcal/g as it is not metabolised in the human organism but instead is excreted with urine

and faeces (Schiweck et al., 2012; Rice et al., 2020). The energy value of erythritol is rounded to zero in food labelling in some countries, including the EU (Regulation (EU) No 1169/2011). Erythritol is not metabolised by the oral microbiota, so it is considered non-cariogenic (Schiweck et al., 2012). According to Schiweck et al. (2012), this sweetener does not have an aftertaste and has a significant cooling effect, which is larger than the cooling effect of other polyols. Compared to other polyol sweeteners, erythritol has the lowest laxative effect: it can be ingested in a maximum dose of 0.66 g/kg body weight for males and 0.80 g/kg body weight for females without causing laxation (Ghosh & Sudha, 2012). Despite that, according to the Regulation (EU) No 1169/2011, foods containing more than 10 % of any added polyols, including erythritol, must have the label "excessive consumption may produce laxative effects".

Steviol glycosides are a group of diterpene glycosides with high sweetness intensity that were first discovered in a plant *Stevia rebaudiana*, which is native to certain regions of South America (González et al., 2014). According to the review of González et al., (2014), more than 30 steviol glycosides are known to this date, with stevioside and rebaudoside A being the predominant ones. Extraction and purification of steviol glycosides from the plant material of *S. rebaudiana* remains the main method of industrial production together with enzymatic modification of the obtained glycosides (Goyal et al., 2010).

Steviol glycosides range in their sweetness intensity. Steviol and rebaudoside A, the most common steviol glycosides, are 150–300 and 250–450 times sweeter than sucrose, respectively (González et al., 2014). González et al. (2014) report that these sweeteners are stable in food applications within a pH range of 2–10 and at temperatures of up to 120 °C. Steviol glycosides are extensively used in the food industry, for example as table-top sweeteners and as ingredients in soft drinks, sauces, candies, ice cream, chewing gums and yogurts (Goyal et al., 2010; González et al., 2014). The glycosides represent non-nutritive, non-cariogenic sweeteners with a prominent bitter, astringent aftertaste whose intensity varies among glycosides and depends on the glycoside concentration (Tao & Cho, 2020).

Both erythritol and xylitol are available in the market as organic products (Amanvida, n.d.; Foodin, n.d.; Violey, n.d.; Žaliuomenė, n.d.) and can be used in the production of organic water kefir. Steviol glycosides, however, due to the purification step included in

their production, do not comply with the requirements for organic products. Unprocessed water extracts of stevia leaves, which could potentially organic certification, are not approved for use in food products in the European Union at the moment. Steviol glycosides are nevertheless included in the scope of this work as they represent one of the two intense sweeteners of natural origin currently approved in the EU.

Sweeteners can be added to the water kefir individually or in blends. Using sweeteners in a blend provides a possibility to avoid their unwanted properties if used individually in larger concentrations (e.g., laxative effect of polyols, bitterness and astringency of steviol glycosides). In addition to that, a blend of various sweeteners can help achieve the sensory characteristics usually attributed to sucrose. This can be useful because sucrose, polyols, and steviol glycosides have been reported to have different temporal sweetness profiles (Tan et al., 2019). The study of Tan et al. (2019) demonstrated that the sweetness of xylitol changes after its ingestion similarly to sucrose, but the sweetness of erythritol and stevia extract does not. Erythritol and stevia extract exhibited less intensive sweetness at the beginning compared to sucrose when they were used in amounts that theoretically match the sweetness intensity of sucrose. In another work on sweeteners, Mora et al. (2023) studied the development of sweetness and bitterness over time for erythritol and several individual steviol glycosides, among other compounds. In the study of Mora et al. (2023), erythritol and steviol glycosides demonstrated higher sweetness intensity peaks at the beginning compared to sucrose, which does not follow the findings of Tan et el. (2019). However, both the studies of Mora et al. (2023) and Tan et al. (2019) are valuable as they show that there is a difference between steviol glycosides, erythritol, and sucrose in their sweetness profiles. Moreover, the observed difference between the two studies regarding steviol glycosides can be attributed to different glycosides. Pure individual glycosides were used in the study of Mora et al. (2023) and stevia extract representing a blend of various glycosides was used in the work of Tan et al. (2019).

Mora et al. (2023) in their study discuss that blending sucrose substitutes with sucrose mostly made their sensory profiles more similar to sucrose. This effect can be desired in the food industry as the familiarity can factor in higher acceptability of the product with added sweeteners. Although Mora et al. (2023) did not study the effect of different sweetener blends, a similar effect can be expected, especially if xylitol is used as one of the sweeteners as its sweetness profile was considered the most similar to sucrose by Tan et al. (2019).

Water kefir production with non-fermentable sweeteners is an understudied field as no information regarding use of sweeteners in water kefir production has been found in publicly available scientific literature. However, satisfactory results have been reported for home production of other fermented beverages with xylitol, erythritol, or stevia extracts: beer, cider, and mead (Badger & Blade, 2022; DIY Hard Cider, n.d.; Homebrew Talk - Beer, Wine, Mead, & Cider Brewing Discussion Forum, 2023; Oculyze, n.d.). The reported success of the individual attempts at the production of fermented beverages with sweeteners provides the basis for the development of water kefirs and their potential commercial production.

1.4.4 Potential effects of non-fermentable sweeteners on water kefir sensory qualities

As water kefir grains are complex consortia consisting of multiple species of bacteria and yeasts, properties of the final product depend on many factors, including the composition of the fermentation medium (Laureys & De Vuyst, 2014; Laureys & De Vuyst, 2017; Laureys et al., 2018). In particular, Laureys et al. (2018) have demonstrated that the volatile profile of water kefir changes depending on the contents of the medium. Addition of erythritol, xylitol, and steviol glycoside to the fermentation medium may influence abundance and metabolism of water kefir microorganisms as well.

No studies regarding the effect of sweeteners on water kefir microorganisms and sensory qualities were found in the preparation of this work. However, individual studies focused on the effect of sweeteners on complex microbial consortia in other environments. Gardana et al. (2003) have demonstrated that specific steviol glycosides might exert a weak inhibiting effect on different groups of bacteria from human gastrointestinal tract. According to the authors, stevioside is associated with a slight decrease in the number of anaerobic bacteria and lactobacilli – major microbial group in water kefir (see Table 1 in section 1.2.1), and rebaudoside A is associated with a decrease in the number of total aerobic bacteria (particularly coliform bacteria) and bifidobacteria. According to the review of Ruiz-Ojeda et al. (2019), erythritol has not been proven to impose any effect on gut microbiota in humans in clinical trials. Xylitol has been demonstrated to increase the relative abundance of *Bifidobacterium* genus in the gastrointestinal tract (GIT) of mice (Uebanso et al., 2017). In the same study, xylitol has been also shown to increase the absolute and relative number of bacteria belonging to phylum Firmicutes (Bacillota).

Firmicutes is a vast bacterial group that includes, among others, bacteria previously identified as *Lactobacillus* – key component of water kefir microbiota.

The effect of steviol glycosides and xylitol on GIT microorganisms allows to form the assumptions about its effect on water kefir microbiota. The gastrointestinal tract represents a complex environment with a large number of different microbial species. Although the microbial composition of water kefir and GIT are different, these findings allow to assume that the sweeteners in question might affect the dynamics of multi-species systems, such as water kefir. Moreover, some water kefir species *Lcb. casei*, *Lcb. paracasei*, *Lcb. rhamnosus*, and *Lactiplantibacillus plantarum* can be a part of human gut microbiota as a sporadic component together with *Levilactobacillus brevis* (Walter, 2008).

In addition to a possible effect on the frequencies of different microbial groups in water kefir, the use of sweeteners might affect their metabolic pathways. Xylitol and erythritol, although not fermentable by lactic acid bacteria or yeasts, have been reported to be involved in their metabolic pathways. Various strains of yeasts and lactic acid bacteria have been demonstrated to produce small amounts of erythritol (Rice et al., 2020). He et al. (2021) reported that various strains of *S. cerevisae* can also produce xylitol. Metabolic reactions are often reversible and involve a series of feedback loops, where an increase in the concentration of specific metabolites can accelerate or inhibit preceding reactions (Ferrell, 2002; Sauro, 2017). Therefore, the addition of compounds like xylitol and erythritol, which can appear in the metabolic pathways of water kefir microorganisms, may lead to the downregulation or upregulation of individual biochemical reactions.

The potential effects of xylitol, erythritol, and steviol glycosides on water kefir are not well understood and have not been extensively researched. However, studies on the impact of sweeteners on microorganisms in other environments suggest that the addition of sweeteners could alter the ratio of different microorganisms and affect their metabolism. This alteration may lead to changes in the concentration of the metabolites that contribute to the sensory qualities (taste, odour, fizziness, other mouthfeel) of water kefir, for example volatile compounds and organic acids. Consequently, these changes in the metabolite profile might modify the sensory properties of water kefir compared to its sucrose-based counterpart. Therefore, there is a need to study whether the addition of

sweeteners can affect the metabolite profile and sensory qualities of water kefir as it will allow for the development of commercial water kefirs with sweeteners.

1.5 Aim of the study

The following aim and objectives have been set for the thesis work:

- to develop recipes of water kefirs with non-sucrose sweeteners and to assess their metabolite profile and sensory qualities compared to sucrose-based water kefir:
    - to select appropriate sweeteners from the range of natural sweeteners with a potential creation of an organic product that would comply with the EU legislation;
    - to produce water kefirs based on the developed recipes and to obtain steady, reproducible products based on the created recipes;
    - to assess the metabolite profiles and sensory qualities of water kefirs produced with sucrose and sucrose-sweetener blends;
    - to determine whether water kefirs produced with different sweeteners differ in their volatile compound, sugar, and organic acid content after production and after a period of cold storage;
    - to discover whether the reduction of sucrose and addition of non-sucrose sweeteners affects the sensory perception of the water kefirs after their production and after a period of cold storage.

The practical part of thesis work was done in a cooperation with the company Lapin Maria Oy – manufacturer of condiments and beverages, which is interested in the production of water kefirs and has developed a model system and recipes for kefir production. The cooperation involved joint determination of the research question and the scope of work and exchange of the original and newly developed water kefir recipes, ingredients, and results.

2 Materials and Methods

The work was conducted on the premises of the Food Sciences Unit at the University of Turku. The study consisted of water kefir production, analysis of sugar, acid, and volatile compound content, and sensory evaluation (Figure 4).

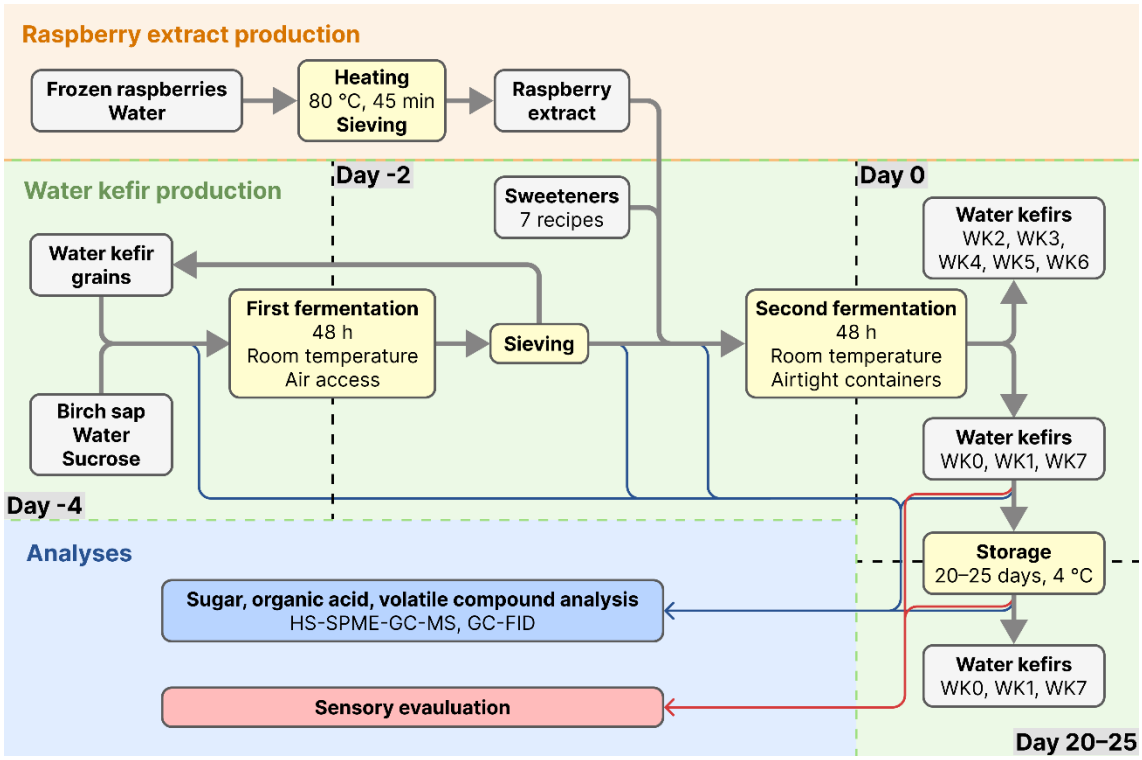

Figure 4. Flow chart of the study design including water kefir production, storage, and analyses. Yellow blocks refer to the stages in water kefir production and storage, grey blocks refer to products, recipes and ingredients, blue and red blocks and arrows refer to analysis methods at different time points. Day -4, day -2, day 0, and day 20–25 refer to days before or after the end of production (the end of the second fermentation). More information on water kefir production can be found in section 2.2, more information on analysis methods used in the work can be found in sections 2.3—2.5

2.1 Materials in prototype production

The water kefir grains used in the current work were courtesy of Lapin Maria Oy (Finland).

Other materials used in the work included frozen raspberries (Sirogoino Company, Serbia), xylitol (Foodin Oy, Finland), erythritol (Foodin Oy, China), steviol glycosides (Govinda Natur GmbH, Germany), birch sap (Nordic Koivu Oy, Finland), sucrose (Nordzucker AG, Germany).

2.2 Product development and water kefir production

New recipe development was based on the original recipe for raspberry-flavoured water kefir provided by Lapin Maria Oy and used in the research development of the company. The recipe was adapted to the aims of the thesis work so that it would allow for addition of various sweeteners. The final baseline recipe used in the current work was the following (see Figure 4, block Water kefir production):

Water and birch sap were mixed in a ratio 9:1 (v/v), after which 3% (w/v) of sucrose was added and mixed until dissolved. 4% (w/v) of water kefir grains were added to the solution, and the mixture was left to ferment at room temperature in a covered container with free air access for 48 hours. After the first fermentation, the water kefir grains were drained for their reuse in further water kefir production.

To produce raspberry extract, frozen raspberries and water were mixed in a ratio 1:1 and heated at 80 °C for 45 minutes. The mixture was then drained through cheesecloth twice to remove pulp and seeds. The raspberry extract was added to the water kefir in a ratio 1:9 (v/v) together with sweeteners according to the developed recipes (Table 4) and mixed until homogenous. The water kefir was then divided into airtight 250 ml glass bottles and left for the second fermentation at room temperature for 48 hours. After the second fermentation, the production was considered finished, and the products were stored at 4 °C for 20–25 days.

Table 4. Water kefir recipes developed in the current thesis work (see Figure 4 for the flow chart of the thesis work). All recipes contained the same ingredients before the first fermentation

Ingredient	Recipe							
	WK0	WK1	WK2	WK3	WK4	WK5	WK6	WK7
First fermentation								
Water, v/v	90%	90%	90%	90%	90%	90%	90%	90%
Birch sap, v/v	10%	10%	10%	10%	10%	10%	10%	10%
Water kefir grains, w/v	4%	4%	4%	4%	4%	4%	4%	4%
Sucrose, w/v	3%	3%	3%	3%	3%	3%	3%	3%
Second fermentation								
Medium from first fermentation, v/v	90%	90%	90%	90%	90%	90%	90%	90%
Raspberry extract, v/v	10%	10%	10%	10%	10%	10%	10%	10%
Erythritol, w/v		5%	5%	5%	5%			4%
Xylitol, w/v		3%		1%				
Steviol glycosides, w/v		0.01%	0.02%	0.01%	0.01%	0.02%	0.02%	
Sucrose, w/v	5%				1%		1%	1%

Weights and volumes are given per final volume at a given fermentation stage

The fermentation during water kefir production was monitored by measuring pH and °Bx values before the first fermentation, after the first fermentation, before the second fermentation, and after the second fermentation. In addition, pH and °Bx values were measured in the products pre-selected for further analyses after 1, 5, and 20–25 days of cold storage. All measurements were made in biological triplicates.

2.3 Sugar and organic acid analysis

Sugars and organic acids of water kefirs were analysed as trimethylsilyl (TMS) derivatives with a gas chromatograph (GC-2010Plus, Shimadzu Corp., Japan) equipped with a flame ionisation detector and the auto injector/auto sampler AOC-20i/AOC-20s (Shimadzu Corp., Japan), using a method described previously in the study of Kelanne et al. (2019) with slight modifications. An aliquot of 850 µL of water kefir was taken, to which 50 µL of sorbitol (5.007 g/L, Fluka Biochemika, Germany) and 50 µL of tartaric acid (5.006 g/L, Sigma-Aldrich, USA) were added as internal standards, in addition to 50 µL of water. The mixture was then filtered with regenerated cellulose (RC, Whatman™ Puradisc™, Pall Corp., USA) or water-wettable polytetrafluoroethylene (wwPTFE, Pall Corp., USA) syringe filters of with a pore diameter of 0.2 µm. After that, an aliquot of 300 µL was taken from the filtrate, and it was evaporated at 50 °C under a nitrogen stream

for approximately 30–40 minutes until dry and kept in a desiccator with silica overnight. TMS derivatives of sugars and acids were prepared by adding 500 µL of Tri-Sil reagent (hexamethyldisilazane:trimethylchlorosilane:pyridine in a ratio 2:1:10, Thermo Fisher Scientific Inc., USA) to the sample, after which it was shaken vigorously with a vortex mixer (Vortex-Genie, Springfield, MA, USA) for 5 min. After that, the sample was incubated at 60 °C for 30 min and then cooled to room temperature. Pre-prepared external standards for glucose, fructose, sucrose, xylitol, malic acid, citric acid, lactic acid, and ascorbic acid were used to identify and quantify sugars and organic acids in the samples.

The analysis of the samples was performed with a DB-WAX polyethylene glycol capillary column (30 m length × 0.25 mm inner diameter; 0.25 µm film thickness; Agilent, USA). A sample of 1 µL was injected automatically via a split/splitless injector. The analysis was conducted under the following conditions: helium as carrier gas, flow rate 1.9 mL/min; temperatures of the injector and detector 210 °C and 290 °C, respectively; column temperature as 150 °C for 2 min, raised to 210 °C at a rate of 4 °C/min, raised to the final temperature of 275 °C at a rate of 40 °C/min, 275 °C for 5 min. The analysis was completed for water kefir samples before and after the first fermentation, before and after the second fermentation step, and after 20–25 days of storage at 4 °C. Analyses of water kefir samples were conducted in biological triplicates and technical duplicates.

Organic acids and sugars were identified by comparing their retention time with the retention time of external standards. Concentrations of the identified compounds were quantified from their peak areas, peak areas of the internal standards, and corresponding correction factors. The correction factors for the quantification were calculated from the concentrations and peak areas of external and internal standards.

2.4 Volatile profile analysis

Volatile compound content of the water kefirs was analysed in biological triplicate using headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME-GC-MS). Two millilitres of each sample were placed in a 20 mL glass vial and mixed with 0.2 g of sodium chloride. After that, 10 µL of 4-methyl-2-pentanol solution ($0.08075 \cdot 10^{-5}$ g/mL in methanol) were added as an internal standard. A 2 cm DVB/CAR/PDMS fibre (50/30 µm, Supelco, USA) was used for the extraction of volatile compounds from the headspace. First, the fibre was conditioned at 250 °C, after

which it was incubated in the sample for 10 min, followed by 30 min of extraction at 45 °C.

After the extraction, the microextraction fibre was immediately transferred to the injection port of Trace 1310 gas chromatograph equipped with TSQ 8000 EVO mass spectrometer (Thermo Fisher Scientific, USA) to be thermally desorbed in splitless mode at 240 °C for 3 min. The volatile compounds were separated in the sample with the help of a DB-WAX polyethylene glycol capillary column (60 m length × 0.25 mm inner diameter × 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA) and helium as the carrier gas at a flow rate of 1.6 mL/min. The temperature of the column was set to 50 °C for 3 min at the beginning. After that, the column was heated to 220 °C at a rate of 5 °C/min and kept at 220 °C for 8 min. Mass spectra were detected in electron impact mode at 70 eV, with am m/s scan range from 33 to 300. The MS transfer line and ionisation source temperatures were 220 °C and 240 °C, respectively.

The volatile compounds were identified by matching the mass spectra obtained from the samples with the standard U.S. National Institute of Standards and Technology (NIST) 14 library. In addition to that, the retention indices (RIs) of the identified volatile compounds were calculated through co-injection with an alkane mixture (C_7-C_{21}, Sigma-Aldrich, USA). The RIs were then compared against the RIs reported for those compounds in the literature and the NIST Webbook (https://webbook.nist.gov/chemistry, accessed on 8–29 April 2024) as a method to confirm the identification.

2.5 Descriptive sensory analysis

To assess whether a difference in the sweetener composition of water kefir can affect its sensory perception by a potential consumer, a descriptive sensory analysis was conducted. The procedure consisted of panel recruitment, three training sessions and two sensory evaluation iterations. As the sensory evaluation of the product prototypes involved ingestion of the samples and thus intervened with the physical integrity of participants, ethical review was requested and passed for the study.

2.5.1 Panellists

Panellists were recruited for the sensory evaluation from the Food Sciences unit and other units of the University of Turku. The invitation to participate and the link to the sign-up

form were sent in the Moodle area for Master's degree students of the Food Sciences unit at the University of Turku. In addition to that, the invitation and the link to the sign-up form were sent in private messages and group chats. In total, 12 panellists were recruited for the study.

2.5.2 Trainings

To prepare the panellists for the sensory evaluation, three training sessions were organised. All trainings included water kefirs of various recipes that had been stored for various amounts of time.

In the first training session, the aims and basic instructions of sensory evaluation were explained to the panellists. The panellists were introduced to water kefirs, provided with reference solutions, and asked to complete a questionnaire in Compusense software (version 24.0.26998) for the subsequent attribute generation. The questionnaire included optional free comment questions and mandatory check-all-that-apply questions with attributes that were used in the sensory evaluation of water kefirs in previous studies. The answers to the questionnaire were discussed with participants to generate sensory attributes and evaluation procedures for the sensory evaluation sessions in this work. The list of references was reduced to align with the remaining attributes (Table 5).

Table 5. References provided in the training sessions and sensory evaluation sessions

Attribute	Compound/product	Concentration/dilution [a]
Odour		
Sweet	Isoamyl alcohol	0.081 g/L
Alcohol	Apple cider (Mighty Hard Cider 5.5%, Olvi, Estonia)	–
Fruity	Raspberry juice concentrate (Ekströms, Orkla Foods Sverige Ab, Sweden)	1:5 in water
Rancid	Butyric acid	0.048 g/L
Vinegar	Acetic acid	1% (v/v)
Taste		
Sweet	Sucrose	40.0 g/L in water
Sour	Citric acid	0.8 g/L in water
Red berry	Raspberry extract produced in the current work	1:7 (v/v) in water
Mouthfeel		
Dryness	Tonic water (Schweppes Indian Tonic, Sinebrychoff Oy Ab, Finland)	–
Fizziness	Mineral water (Vichy, Kotimaista, Refresco Finland Oy, Finland)	–

[a] The concentrations provided in the table were used in the third training session and two sensory evaluation sessions

When the sensory attributes were chosen and defined, the participants had the second training session that focused on the evaluation of water kefir samples with the chosen attributes on linear scales. The panellists were asked to assess the intensity of specific attributes in a Compusense questionnaire consisting of linear scales. In addition, the questionnaire included several categorical questions asking whether a particular attribute is present in the water kefir, as it had been decided with the panellists in the first training session. The training participants were provided with reference solutions for the attributes in evaluation. After the panellists answered the questionnaire, the attributes and answers were discussed with the participants to determine whether some attributes should be removed or modified or whether the concentration of particular reference compounds should be changed.

After the second training session and analysis of answers, the attributes and reference solutions were revised. A new questionnaire with the revised questions was created in Compusense for the third training session, which followed a similar procedure to the previous session.

2.5.3 Sensory evaluation sessions

The descriptive analysis included two sensory evaluation sessions with an identical procedure. The panellists evaluated six water kefir samples made with three recipes: WK0, WK1, WK7. The production of three samples ended on the day of the sensory evaluation, and three samples had been stored at 4 °C for 20–25 days after their production. The newly produced samples were put in the temperature of 4 °C for 1 hour before the evaluation to minimise the temperature difference between the samples.

During each sensory evaluation session, panellists were asked to evaluate the samples in a Compusense questionnaire consisting of categorical questions (odour: vinegar, rancid; dry mouthfeel) and linear scales (odour: sweet, alcohol, total intensity; taste: sweet, sour, red berry, total intensity; fizziness). The questionnaire was created based on the feedback and data from three training sessions. In addition, optional free comment text sections were added to the questionnaire sections, so that the panellists could put their findings about other sensory attributes there. A preference question was added to the questionnaire as well to provide insights for Lapin Maria Oy. During evaluation, the panellists were provided with reference solutions of specific concentrations that were selected during training sessions (see Table 5). The solutions served as reference points for the sensory

attributes and represented the right-edge anchor point in linear scales (10 on the scale from 0 to 10). To minimise the sensory exhaustion of the participants, they were provided with plain crackers and water.

2.6 Statistics

The concentrations of malic acid, citric acid, citric acid, ascorbic acid, glucose, fructose, sucrose, and xylitol of different water kefirs at the same time point were statistically analysed with a multifactor ANOVA and Tukey honest difference test as post hoc analysis. The analysis was conducted in IBM SPSS Statistics software (version 29.0.0.0). The criterion for statistical significance was set to $p < 0.05$.

Numerical data regarding the sweet obtained from the sensory evaluation (sweet odour, fruity odour, alcohol odour, total odour intensity, sweet taste, sour taste, red berry taste, total taste intensity, fizziness) for water kefirs before and after cold storage were analysed from were with a multifactor ANOVA and Tukey honest difference test as post hoc analysis. To compare water kefirs with the same recipe before and after cold storage, unpaired Student's t-test with Bonferroni-Hochberg post hoc correction was conducted for the numerical data. The categorical data (vinegar odour, rancid odour, dryness) were analysed with Pearson's chi-squared test. The criterion for statistical significance in all tests was set to $p < 0.1$. The analysis was conducted in IBM SPSS Statistics software (version 29.0.0.0) and in the python environment (version 3.19.2).

The numerical data from 10 participants that participated in two sensory evaluation sessions were used to assess panel performance in the sensory evaluation. Panel performance analysis was conducted in PanelCheck software (version 1.4.1) and included building plots for correlation loadings, means and standard deviations, and average scoring of attributes by the panellists.

3 Results

3.1 Product development

The product development stage included several iterations of recipe development with sucrose, erythritol, xylitol, and steviol glycosides used individually or in blends. Sweetener concentrations were selected based on their reported relative sweetness compared to sucrose, other information in scientific publications, and findings from the

student's previous work. In the result, 7 product recipes with different sweetener blends were created (refer to Table 4 in section 2.2). All recipes contained the same ingredients during the first fermentation step, and different sweeteners were added to the water kefirs before the second fermentation. This was done to limit the difference in recipes to sweeteners and maintain water kefir grains in the same fermentation medium in all recipes before they were removed.

After recipe development, 5 water kefirs recipes (WK0, WK1, WK2 WK3, and WK7; refer to Table 4 in section 2.2 for composition) were pre-selected to be produced for the further metabolite analysis and sensory evaluation based on their sensory qualities. During the monitored production of water kefirs according to five recipes, all water kefirs exhibited an expected decrease in pH and °Bx values during fermentation (Figure 5). As the first fermentation stage was the same for all recipes, a large batch of medium intended for all recipes underwent the first fermentation step in triplicate and then was split into smaller batches corresponding to separate recipes. For this reason, the first two data points in pH and °Bx graphs are identical for all five recipes, with the initial pH and °Bx being 6.66±0.01 and 3.00±0.00, respectively. During two days of the first fermentation, the pH decreased to 4.18±0.03 and the °Bx value became 2.77±0.06, which indicates the successful fermentation of the medium during the first fermentation.

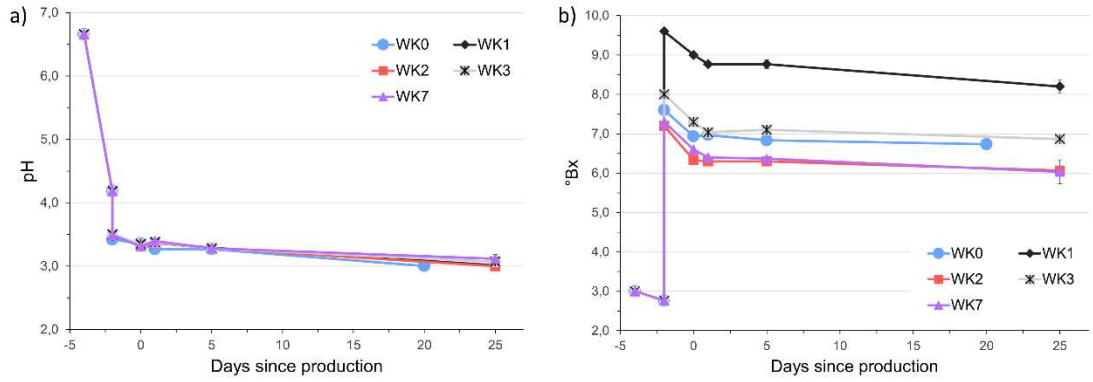

Figure 5. Average **a)** pH and **b)** °Bx values of water kefirs during production (days -4, -2, 0) and cold storage (days 5, 20, 25). Vertical lines represent standard deviation. Refer to Figure 4 for the study design and Table 4 for the recipes

After the first fermentation, the raspberry extract and different additional sweeteners were added to water kefirs according to the recipes. The addition of ingredients was expected to change the pH and °Bx values in water kefirs, due to which their measurements were taken before and after the ingredients were added. As the ingredients were added to the

water kefirs on the same day as the end of the first fermentation stage (the time point of -2 days in Figure 5), two data points exist in the graph for the same time point.

The addition of acidic raspberry extract further reduced the pH in all water kefirs to similar values of 3.42–3.50. Similar pH values were expected in water kefirs before the second fermentation as the same amount of the same raspberry syrup was added to all recipes and the used sweeteners do not affect the pH. Since different amounts of different sweeteners were added to different water kefirs, they contributed to a varying increase of the °Bx value in different recipes. After the ingredients were added, the °Bx value of the water kefirs was in the range of 7.20–9.60 with the lowest value in water kefir WK2 and the highest value in water kefir WK1.

After the second fermentation, the pH of the water kefirs was in the range of 3.31–3.35, showing a decrease of approximately 2–5%. A lack of a large decrease can be explained by the fact that lactic acid bacteria that represent the bacterial majority in water kefirs do not tolerate environments with pH values lower than 3.5–4 and therefore do not actively metabolise the sugars (Shan & Jelen, 1990; Adamberg et al., 2003). Acetic acid bacteria, in comparison to LAB, can grow at pH values of 3 and 4 (Sengun & Karabiyikli, 2011) and might continue metabolising available carbohydrates in the acetic acid fermentation.

In contrast, the °Bx value demonstrated a slight decrease of approximately 9–12% across all water kefirs, which can be attributed to metabolic activity of yeasts and their sugar consumption, accompanied by the residual activity of LAB or acetic acid bacteria. However, the absolute °Bx value cannot be directly linked to the sucrose content of the samples as the analysed water kefir samples included other dissolved sweeteners.

Water kefir WK0 was produced for the second time due to a mistake with the used ingredients. As all samples were scheduled for sensory evaluation at the same time, water kefir WK0 was stored at 4°C for 20 days instead of 25 days. During the cold storage (days 0–25 in Figure 5), the pH and °Bx values in all water kefirs exhibited a slight reduction to the average values of 3.00–3.12 and 6.03–8.20, respectively. The continuing decline of pH and °Bx values can signify the continuing metabolic activity at slower rates, resulting in the production of more organic acids and the decrease in fermentable sugar concentrations in the products. As the °Bx value remained relatively high, this can potentially signify substantial amounts of dissolved solids in all studied product

prototypes. However, like in previous measurements, the absolute °Bx value does not directly point at sucrose content in the recipes.

At the end of the product development stage, three recipes were selected for the metabolite analysis and sensory evaluation: WK0, WK1, and WK7 (see Table 4 for the ingredients). These recipes were selected as they represent the baseline recipe with sucrose, a recipe with a combination of all three non-fermentable sweeteners substituting all sucrose added before the second fermentation, and a recipe with a decreased amount of sucrose and its part substituted with another sweetener. The choice of the recipes was also based on the feedback on the sensory properties of the water kefirs provided by the supervisors of the current work.

3.2 Sugar and organic acid content of water kefirs

GC-FID chromatograms of all water kefirs demonstrated few unidentified peaks at all timepoints (Figure 6). In addition, although erythritol had a distinctive peak in chromatograms of water kefirs that contained it, it was not quantified due to the absence of its external standard (Figure 6b).

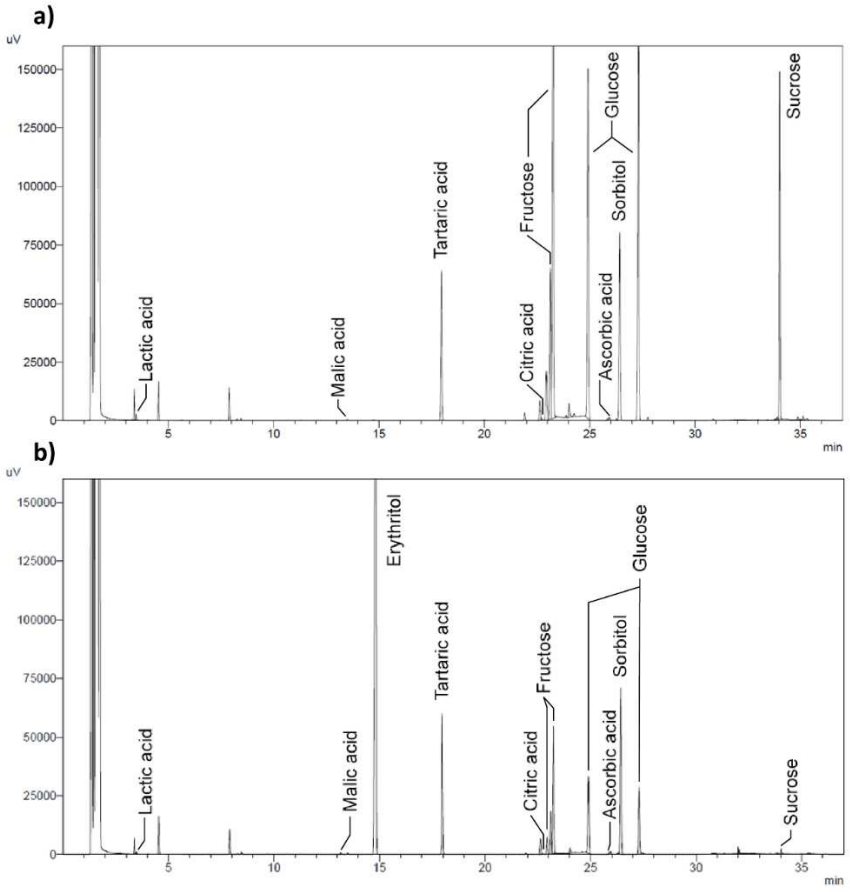

Figure 6. Examples of GC-FID chromatograms: water kefirs **a)** WK0 and **b)** WK7 after 20 and 25 days of cold storage, respectively. Sorbitol and tartaric acid were used as internal standards

The fermentation medium consisting of water, birch sap, and sucrose did not contain xylitol, malic acid, or citric acid before the first fermentation (Table 6). After the first fermentation (day -2), water kefir medium contained small amounts of citric acid and higher amounts of all analysed compounds, except for sucrose, which decreased from 33.40±1.60 g/L to 13.25±0.63 g/L, and xylitol, which remained at near-zero levels in water kefirs without added xylitol. The changes in the carbohydrate and organic acid concentrations indicate the metabolic activity of the water kefir microbiota during the first stage of fermentation.

The addition of raspberry syrup and sweeteners before the second fermentation stage (day -2 before second fermentation in Table 6) expectedly increased the concentration of sucrose and xylitol in the corresponding recipes. The amount of sucrose in water kefirs WK0 and WK7 raised to 55.55±10.76 g/L and 18.02±1.70 g/L, respectively. However, according to the recipes, the water kefirs were mixed with raspberry extract in a ratio 9:1 after the first fermentation, and 5% and 1% (w) of sucrose were added to the water kefirs

WK0 and WK7, respectively. As the water kefir medium contained 13.25±0.63 g/L of sucrose after the first concentration, the concentration of sucrose before the second fermentation was expected to be higher than 55.55±10.76 g/L in water kefir WK0 and 18.02±1.70 g/L in water kefir WK7. The same discrepancy was observed for water kefir WK1; although xylitol was added in the amount of 3% of weight per final volume, the observed concentration was 17.69±2.45 g/L. Such differences from the expected values can be explained by errors in the measurement of ingredient weight during water kefir production and/or measurement errors associated with the chromatography procedure.

Organic acid concentrations point at the chromatography measurement errors as well. As the same amount of identical raspberry extract and specific sweeteners were added to all products before the second fermentation, organic acid concentration in all water kefirs was expected to be identical for all products before the second fermentation. However, the GC-FID with the subsequent statistical analysis demonstrated a significant difference in lactic acid, citric acid, and ascorbic acid content for the products before the second fermentation (-2 days before second fermentation in Table 6). The observed results provide a reason to assume that they were caused by an observational error, which is further supported by the fact that the observed organic acid concentrations were low and close to the detection threshold of the used method.

Table 6. Sugar and organic acid content (g/L, mean ± SD) in water kefirs with sucrose and non-sucrose sweeteners 4 days and 2 days before the end of production, on the day of production, and 20–25 days since production

Compound	Day -4 (before first fermentation)	Day -2 (after first fermentation)	Day -2 (before second fermentation)			Day 0 (after second fermentation)			Day 20–25		
			WK0	WK1	WK7	WK0	WK1	WK7	*WK0*	*WK1*	*WK7*
Glucose	0.24±0.04	4.41±0.26	5.36±0.65 [a]	4.02±0.51 [b]	4.15±0.42 [b]	8.77±1.28 [d]	2.74±0.17 [e]	4.33±0.83 [f]	22.52±2.14 [g]	1.59±0.61 [h]	7.08±2.82 [i]
Fructose	0.11±0.05	3.95±0.81	4.86±0.93 [a]	3.69±0.66 [b]	3.69±0.27 [b]	9.61±1.86 [d]	3.53±0.62 [e]	4.05±1.03 [e]	29.42±3.76 [g]	2.68±0.97 [h]	8.58±1.86 [i]
Sucrose	33.40±1.60	13.25±0.63	55.55±10.76 [a]	8.35±0.93 [c]	18.02±1.70 [b]	52.22±5.86 [d]	6.70±0.76 [e]	12.29±3.96 [f]	5.86±1.13 [g]	0.21±0.11 [h]	0.39±0.22 [h]
Xylitol	ND	ND	0.012±0.029 [a]	17.69±2.45 [b]	0.020±0.031 [a]	ND	22.60±1.81 [d]	0.076±0.039 [e]	ND	24.03±1.88	ND
Lactic acid	0.051±0.002	0.111±0.010	0.107±0.016 [a]	0.071±0.016 [b]	0.087±0.009 [ab]	0.151±0.017 [d]	0.090±0.007 [e]	0.055±0.034 [e]	0.133±0.020 [g]	0.075±0.017 [h]	0.059±0.008 [h]
Malic acid	ND	ND	0.043±0.008 [a]	0.055±0.012 [a]	0.049±0.007 [a]	0.042±0.005 [d]	0.059±0.012 [e]	0.046±0.010 [de]	0.044±0.008 [g]	0.058±0.008 [h]	0.057±0.006 [h]
Citric acid	ND	0.065±0.025	0.072±0.008 [a]	0.075±0.012 [a]	0.112±0.021 [b]	0.086±0.009 [d]	0.087±0.018 [d]	0.092±0.016 [d]	0.142±0.021 [g]	0.695±0.052 [h]	0.082±0.014 [g]
Ascorbic acid	0.121±0.001	0.164±0.001	0.112±0.016 [a]	0.157±0.022 [b]	0.271±0.043 [c]	0.101±0.018 [d]	0.266±0.021 [e]	0.141±0.031 [f]	0.184±0.020 [g]	0.210±0.022 [g]	0.283±0.045 [h]

ND – not detected

Significant differences ($p < 0.05$) between the recipes are indicated with different superscripts (a, b, c for day -2; d, e, f for day 0; g, h, i for day 20–25. Refer to Figure 4 in Chapter 2 for further details on the production stages

During the second fermentation, sucrose, glucose, and fructose concentrations continued to alter in the water kefirs (day 0 in Table 6). The sucrose content decreased in water kefirs WK7 and WK1, reaching 12.29±3.96 g/L and 6.70±0.76 g/L, respectively. Sucrose content in the sucrose-based water kefir WK0 remained higher and comprised 52.22±5.86 g/L, indicating little change in the amount of sucrose in water kefir WK0 during the second fermentation. Compared to the product before the second fermentation, water kefir WK0 exhibited an increase in the concentration of glucose and fructose from 5.36±0.65 g/L and 4.86±0.93 g/L to 8.77±1.28 g/L and 9.61±1.86 g/L, respectively. Such a change was not observed in the water kefirs WK1 or WK7 during the second fermentation stage. The amount of xylitol in the water kefir WK1 (22.60±1.81 g/L) at the end of production was slightly greater than before the second fermentation (17.69±2.45 g/L), but the statistical analysis did not show a significant difference. Xylitol concentration was likely not affected by the activity of water kefir microorganisms, and the observed variability in concentrations can be attributed to measurement errors.

The organic acid content of the water kefirs did not undergo significant changes in any of the recipes during the second fermentation. The content of ascorbic acid, malic acid, and lactic acid was shown to differ among three recipes (Table 6, day 0 since production), but their concentrations were close to detection limits, similar to the values before the second fermentation. Due to this, the discovered statistical difference between the recipes might be attributed to the real difference in organic acid concentration between different water kefirs as well as to the observation error inherent in the analysis procedure.

After 20–25 days of storage at 4 °C, sucrose, glucose, and fructose content changed in water kefirs created with all three recipes (Table 6, day 20–25). During the cold storage period, sucrose concentration in water kefir WK0 decreased from 52.22±5.86 g/L to 5.86±1.13 g/L. Sucrose content in the water kefirs WK1 and WK7 reached similar levels of 0.390±0.223 g/L and 0.206±0.109 g/L, respectively. This indicates that microbial metabolism of sucrose continued in the water kefirs during cold storage. In addition, the results demonstrate that sucrose was fully metabolised in the products with lower amount of added sucrose within the 25 days of cold storage. Levels of glucose and fructose, although decreased, remained at non-zero levels and differed between all water kefirs, with the lowest concentration observed in the water kefir WK1 and the highest in the water kefir WK0. This demonstrates that all three water kefirs still contained fermentable

carbohydrates in sufficient amounts for the fermentation to continue after 20–25 days of cold storage.

Lactic acid, malic acid, and ascorbic acid content remained at similar, low levels after cold storage (Table 6, day 20–25), but a significant difference in lactic acid and malic acid content was observed between water kefir WK0 and water kefirs WK1 and WK7. The amount of citric acid increased during cold storage in water kefir WK1 and became equal to 0.695±0.052 g/L.

3.3 Volatile compound profile of water kefirs with sucrose and other sweeteners

61 volatile compounds were discovered in total with the HS-SPME-GC-MS method (see full list in Appendix I). The discovered compounds belong to higher organic acids and their esters, aldehydes, ketones, higher alcohols, and terpenoids. Most terpenoids were present in the samples before the first fermentation and did not occur in water kefirs after it. 2,4-di-tert-butylphenolethyl acetate, 2-methylbutanal, and ethanol were other prominent compounds in water kefirs before the first fermentation. After the first fermentation, the range of volatile compounds found in the water kefirs enlarged and included ethanol and isoamyl alcohol, which became the most prominent metabolites in the beverage. The samples also contained small amounts of acetaldehyde, 2-methylbutanal, isoamyl acetate, methyl octanoate, ethyl decanoate, phenylethyl alcohol, and acetic acid.

More volatile compounds were identified in water kefirs after the raspberry extract and sweeteners were added to the beverages between the fermentations (Figure 7a). While ethanol, isoamyl alcohol, and 2,4-di-tert-butylphenol remained the most prominent metabolites, octanoic acid, nonanoic acid, *n*-decanoic acid, α-ionol, β-ionone, acetoin, and ethyl dodecanoate became new compounds in the water kefir samples. The chromatograms also demonstrated higher intensity for the peaks corresponding to methyl octanoate, ethyl decanoate, and acetic acid.

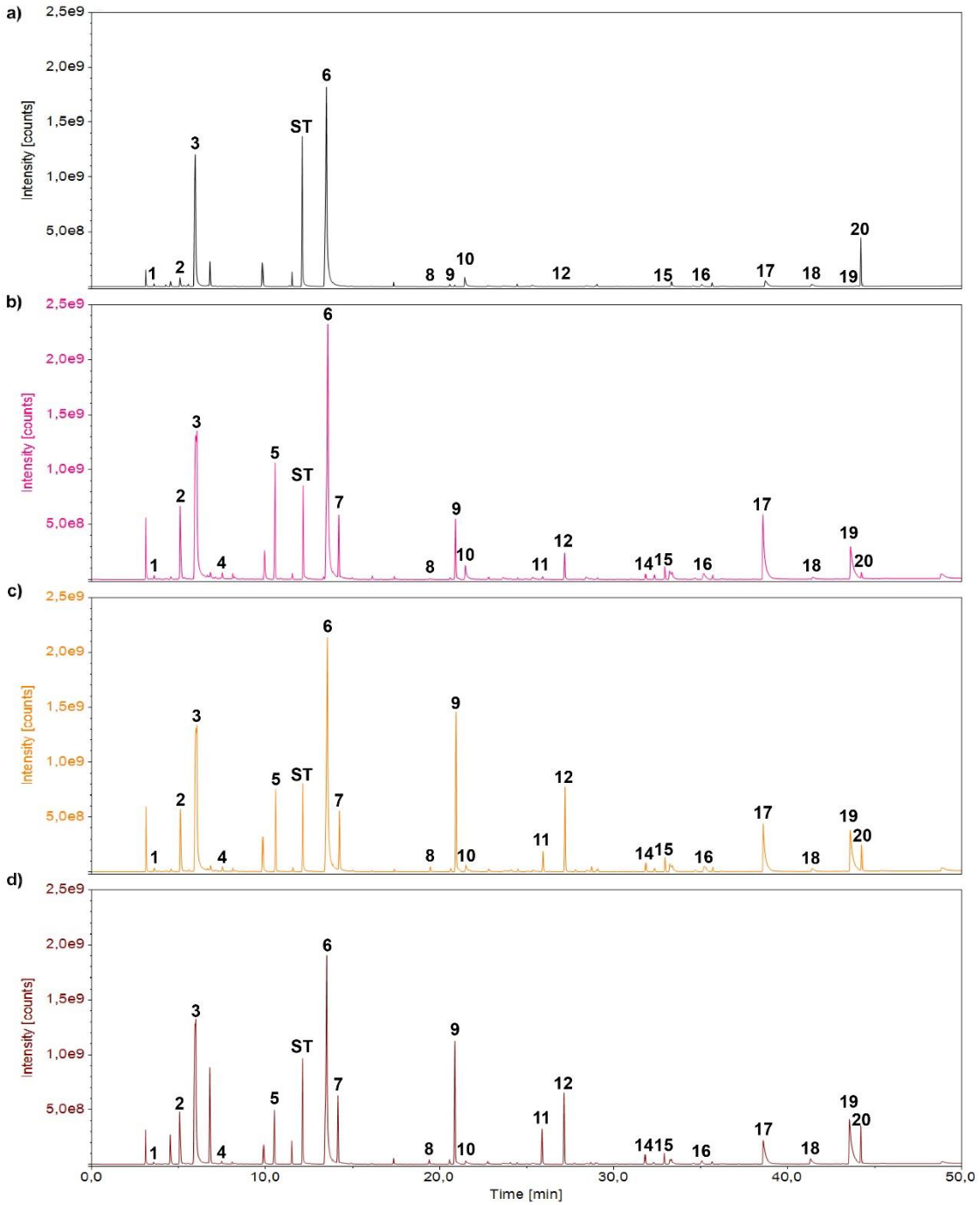

Figure 7. HS-SPME-GC-MS chromatogram of **a)** water kefir WK0 before the second fermentation and water kefirs **b)** WK0, **c)** WK1, and **d)** WK7 after production. ST – internal standard (4-methyl-2-pentanol). Refer to Table 7 for the list of metabolites corresponding to other peaks

The volatile profile of the water kefirs at the end of production demonstrated a change in the ester and organic acid content in comparison with the samples before the second fermentation (Figure 7b–d). The GC-MS chromatograms included higher levels of some esters that were detected in the water kefirs before the second fermentation: ethyl acetate,

ethyl octanoate, methyl decanoate, ethyl decanoate. Some esters identified after the second fermentation were not present in the product before, namely isobutyl acetate, isoamyl acetate, ethyl hexanoate, methyl octanoate, ethyl 9-decenoate, methyl hexadecanoate, ethyl dodecanoate. Some of the newly discovered compounds became one of the most abundant in chromatograms, for example isobutyl acetate, isoamyl acetate, and methyl octanoate.

Table 7. The most abundant volatile compounds presented in Figures 7 and 8. Refer to Appendix I for the full list of the compounds that were detected in water kefir samples

Compound	Number of the peak in Figure 7 and Figure 8	Retention index according to NIST 14 library
Acetaldehyde	1	702
Ethyl acetate	2	888
Ethanol	3	932
Isobutyl acetate	4	1012
Isoamyl acetate	5	1123
Isoamyl alcohol	6	1209
Ethyl hexanoate	7	1233
Methyl octanoate	8	1385
Ethyl octanoate	9	1435
Acetic acid	10	1449
Methyl decanoate	11	1593
Ethyl decanoate	12	1639
Ethyl 9-decenoate	13	1694
Methyl hexadecanoate	14	1804
Ethyl dodecanoate	15	1843
Phenylethyl alcohol	16	1907
Octanoic acid	17	2060
Nonanoic acid	18	2170
n-Decanoic acid	19	2276
2,4-Di-ter-butylphenol	20	2321

The content of volatile organic acids changed in the water kefirs as well, although to a smaller extent than esters. Hexanoic acid, 3-decenoic acid, and dodecanoic acid were not detected in the product before the second fermentation but were detected when the second fermentation was complete. In addition, the chromatograms of water kefirs demonstrated larger peaks for octanoic acid and nonanoic acid after the second fermentation.

All three recipes shared a similar volatile profile at the end of production. All water kefirs contained the same set of volatile compounds with a certain degree of quantitative variation within the same recipes and between different recipes. Semi-quantification of the compounds was not included in the scope of the current work, due to which

estimations about relative amounts of volatile compounds in different recipes were not made.

After the cold storage, no major changes were found in the volatile profile of water kefirs (Figure 8). The only change in the compound range was the detection of an ester ethyl 9-decenoate that was not present in the water kefirs before the cold storage. Other compounds detected in the water kefirs after 20–25 of cold storage were present in the products before.

Water kefirs created with different recipes showed no difference in the range of detected volatile compounds. The most prominent volatile compounds in all water kefirs after the period of cold storage were ethyl acetate, ethanol, isoamyl alcohol, ethyl hexanoate, ethyl octanoate, ethyl decanoate, octanoic acid, and *n*-decanoic acid. The intensity of the peaks corresponding to individual compounds demonstrated no visible difference between the recipes without the semi-quantification of the identified chemicals.

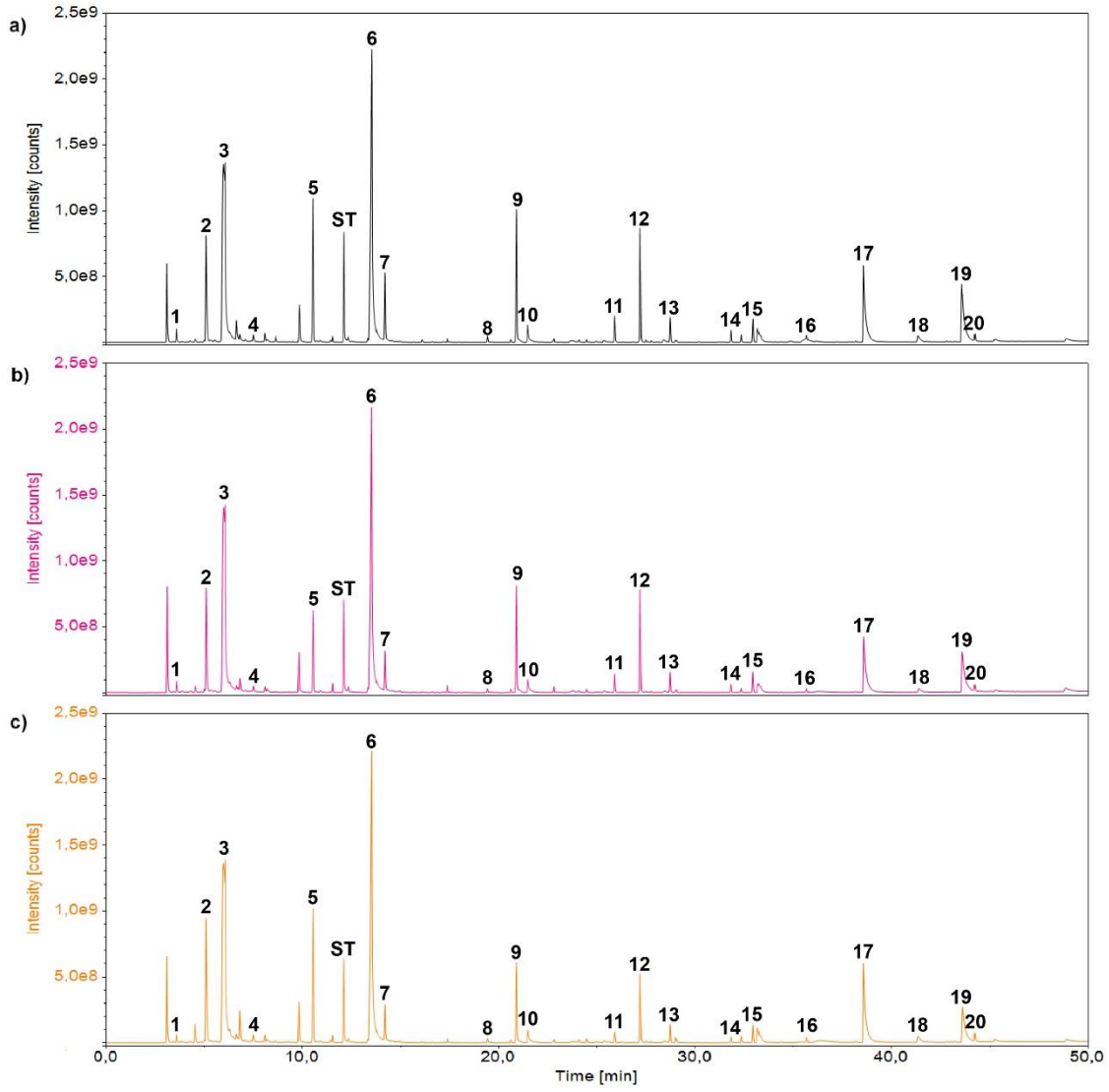

Figure 8. HS-SPME-GC-MS chromatogram of water kefirs **a)** WK0, **b)** WK7, and **c)** WK1 after 20–25 days of cold storage. ST – internal standard (4-methyl-2-pentanol). Refer to Table 7 for the list of metabolites corresponding to other peaks

3.4 Sensory evaluation of water kefirs

Twelve panellists aged 24–43 years participated in the descriptive sensory analysis. 2 participants did not participate in the second sensory evaluation session, due to which the results of 10 panellists were analysed in this work.

3.4.1 Panel performance during sensory evaluation

To assess whether panel performance could influence the obtained results of sensory evaluation, panel performance evaluation was conducted for 10 people that participated in two evaluation sessions. The panel performance evaluation was based on numerical

data obtained from linear scale attributes. It included correlation loading graphs, analysis of mean and standard deviation, and profile plots.

The correlation loading graphs demonstrated that sweet taste and fizziness had the highest degree of explained variance and the highest degree of agreement among the panellists (Figure 9). In a correlation plot, compact clusters of data points located closer to the outer ellipse indicate agreement among participants in the assessment of a particular attribute and a higher ratio of data to noise. Such a pattern was observed for the sweet taste and fizziness in the evaluation of water kefirs. In other attributes, sourness had the closest panel agreement and explained variance to the fizziness and sweet taste. This indicates that the sweetness, fizziness, and sourness were likely understood by the panellists similarly. A possible lack or presence of significant difference between water kefir recipes is less likely to be associated with insufficient panel performance for these attributes.

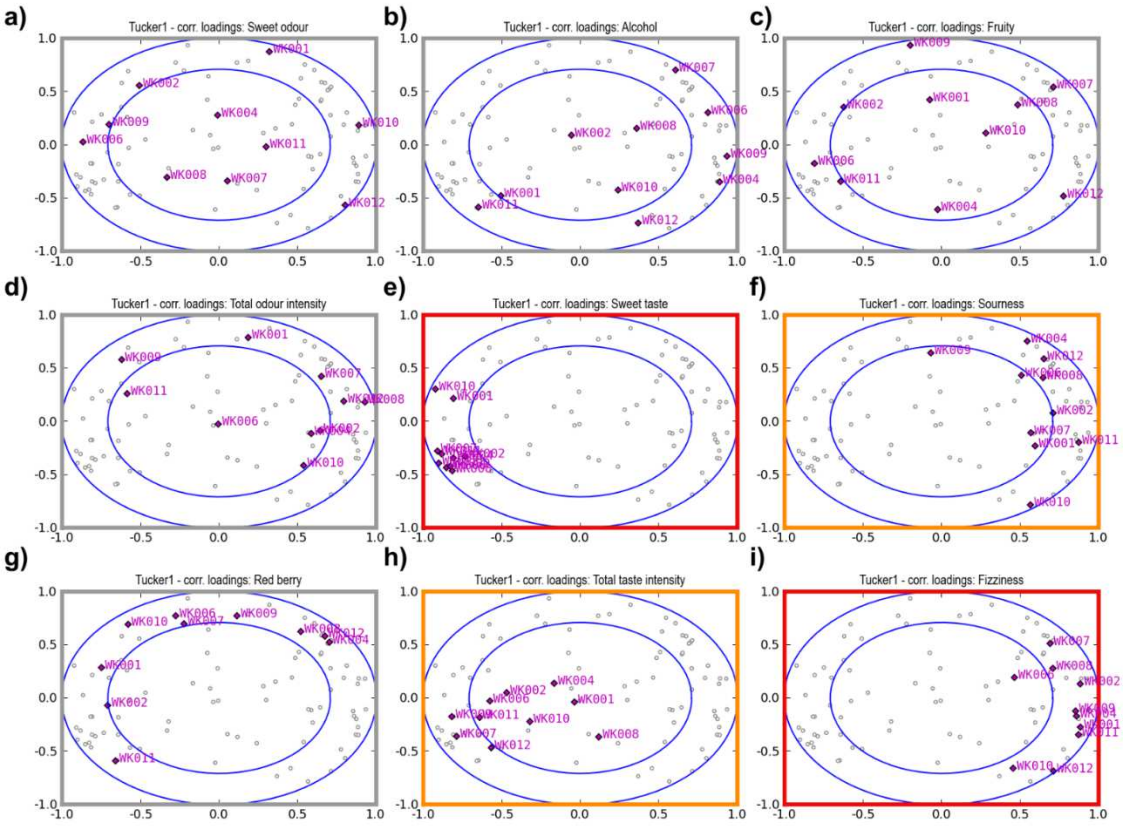

Figure 9. Correlation loading plots representing the extent of agreement explained variance in the evaluation between the panellists (n = 10) for **a)** sweet odour; **b)** alcohol odour; **c)** fruity odour; **d)** total odour intensity; **e)** sweet taste; **f)** sour taste; **g)** red berry taste; **h)** total taste intensity; **i)** fizziness. Value points represent average evaluation scores from two sensory evaluation sessions for each panel participant

The rest of evaluated sensory attributes (all odour attributes, red berry taste, total taste intensity) demonstrated less agreement in evaluation among the panellists (data points lying on bigger distances from each other in a graph) and/or less explained variance (data points located within the inner ellipse in the graph). Such panel performance might influence the results of the sensory evaluation. If the participants evaluate the same samples differently, this can increase variation within one sample. The increased variation can affect the statistical testing and lead to a false conclusion that the difference between recipes does not exist. Panel performance can be suboptimal for several reasons. The panellists can use the attribute scales differently (for example, different parts of scales or ranges of different widths), or they can understand the selected attributes differently, due to which they will be less able to reproduce their answers for the same sample in two different iterations or to discriminate different samples.

To estimate the effect of the different use of scale on the evaluation results, the plots for mean and standard deviation values were compared between different panellists for all numerical attributes (Figure 10). Mean values and standard deviation can be linked to the particular part and range of the linear scale, respectively, that a particular panel participant tended to use while evaluating the samples. According to the obtained values and plots, the panellists exhibited less variation in mean scores and standard deviations when evaluating sweet taste, total taste intensity, and fizziness. This can indicate that their use of scales was more consistent as a whole panel for the attributes in question. In the evaluation of sweet and fruity odour attributes, total odour intensity, and sour taste, more panellists differed in the level of the scale that they used and the range of their scores. The panellists demonstrated the biggest variation in the used part of the scale and the range of their answers when they were evaluating the alcohol odour and red berry taste. This indicates that the scoring of the participants was inconsistent with other panellists for the attributes in question.

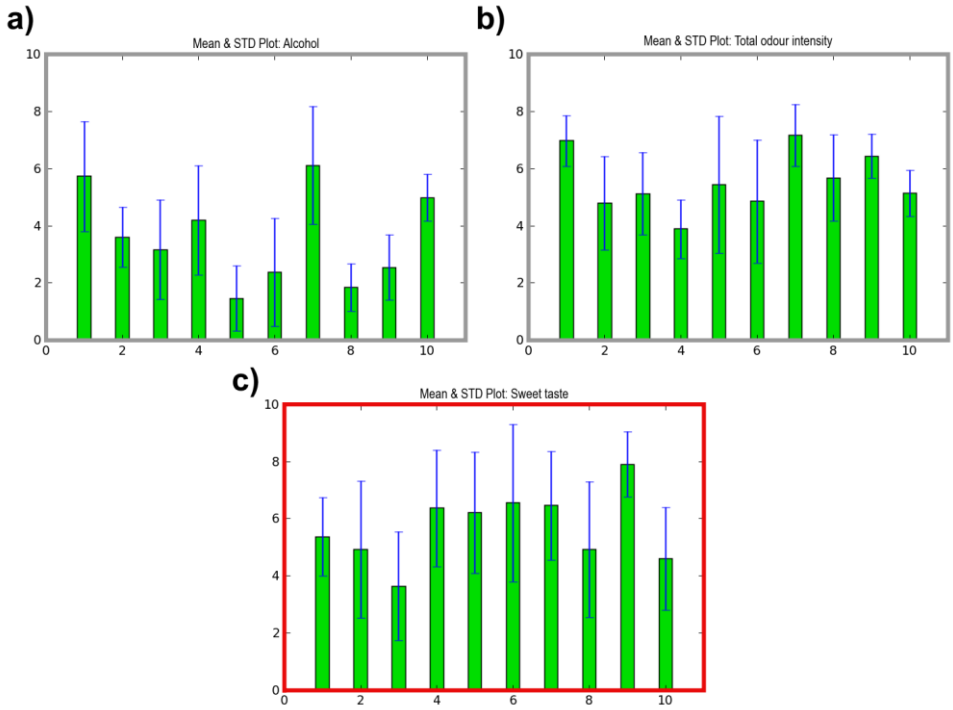

Figure 10. Examples of mean and standard deviation graphs for attribute scoring done by all panellists (n = 10) in two sensory evaluation sessions of **a)** alcohol odour; **b)** total odour intensity; **c)** sweet taste. Horizontal axes represent individual panellists, vertical axes represent evaluation scores

The degree of agreement among the panellists in sample ranking was assessed by creating profile plots for each attribute, in which horizontal axes represent water kefir samples in a fixed order and vertical axes represent evaluation scores (Figure 11). The scores of an individual panellist are connected with a line, and comparison of the lines allows to estimate how much the panellists agree in the evaluation of the samples. If the lines follow one trend, the panellists put the same samples on the same places regarding the intensity of an attribute in question. Similar to mean and standard deviation plots, sensory evaluation participants exhibited more uniform ranking of the samples in the evaluation of the sweet taste, sour taste and total taste intensity. Scoring of fizziness and total odour intensity demonstrated more variation, but an agreeing trend could be nevertheless located. In the evaluation of sweet odour, alcohol odour, fruity odour, and red berry taste, the panellists exhibited the least agreement regarding sample ranks according to their attribute intensities.

The lack of agreement in samples ranking and the differences in the use of scales among the panellists in the descriptive analysis could be caused by their varying understandings of attribute definitions and evaluation procedures. Although the panellists participated in the definition of sensory attributes and were provided with the references that they had

considered appropriate, this might have not ensured a similar understanding of the attribute definitions between the panellists.

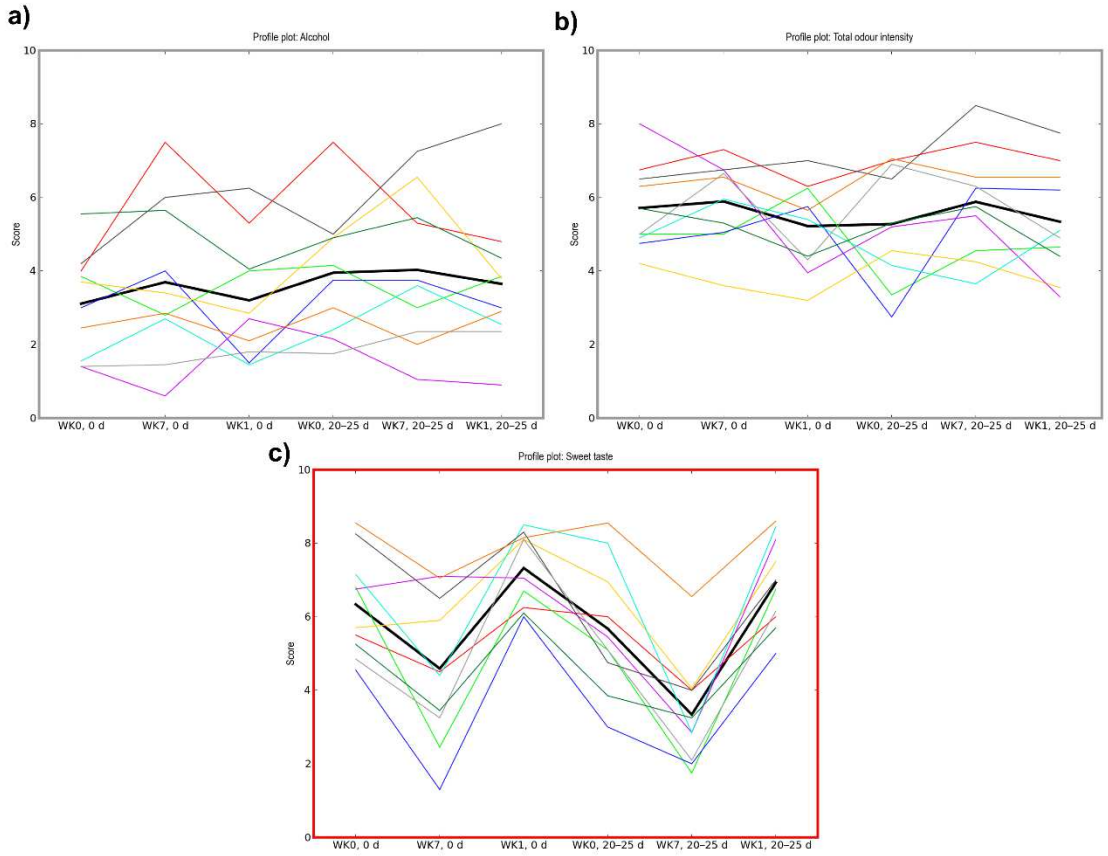

Figure 11. Examples of profile plots representing average scoring of **a)** alcohol odour; **b)** total odour intensity; **c)** sweet taste by panellists (n = 10) in two sensory evaluation sessions. Horizontal axes represent six water kefir samples, vertical axes represent evaluation scores, lines connect average scores of individual panellists from two sessions. The thick black line represents average evaluation scores across the panel

Panel performance could also be affected by the time between the two evaluation sessions. The break between two sensory evaluation sessions constituted three weeks without additional training sessions. As most of the panellists did not participate in sensory evaluation trainings before, they could forget the references, attribute definitions, or evaluation procedures.

3.4.2 Sensory qualities of products with sucrose and non-fermentable sweeteners

No statistically significant difference in odour attributes was discovered by the panellists between different water kefirs recipes neither before nor after cold storage (Figure 12). Sweet odour, alcohol odour, fruity odour, and total odour intensity were at similar levels in the samples before and after cold storage (Table 8).

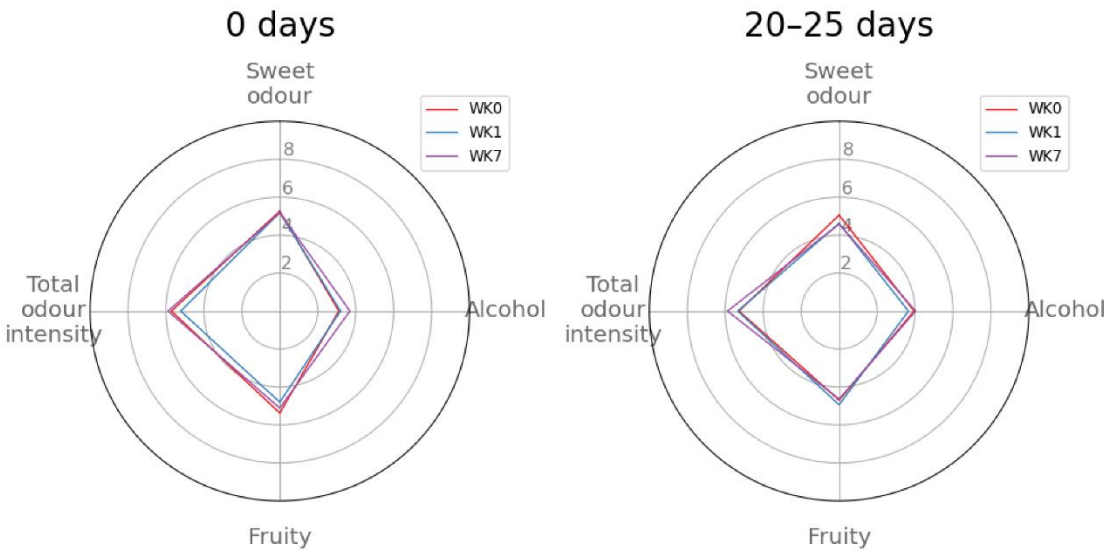

Figure 12. Perceived odour of water kefirs after production (0 days) and after 20–25 days of cold storage, mean scoring values by panellists

Recipes exhibited no statistical difference in most sensory attributes related to taste and mouthfeel either (Figure 13). Sensory evaluation revealed statistically significant ($p < 0.1$) difference in sweet taste between water kefir WK7 and water kefirs WK0 and WK1 before cold storage (4.59±2.21 versus 6.34±1.75 and 7.33±1.39, respectively; see Table 8). After cold storage, the panellists again scored water kefir WK7 as significantly less sweet than water kefirs WK0 and WK1 (3.36±1.86 versus 5.68±2.26 and 6.93±1.47, respectively). In addition, the participants perceived water kefir WK0 as significantly fizzier than water kefir WK1 after cold storage (7.55±1.84 versus 5.71±2.55). Water kefir WK7 with added sucrose and erythrose was also perceived as more sour (average scoring 4.15±2.26) compared to water kefirs WK0 and WK1 (average scorings 2.58±1.80 and 2.60±1.53, respectively) before cold storage, but this difference was not observed after 20–25 days of cold storage. In contrast, the water kefirs exhibited a difference in total taste intensity only after the period of cold storage: water kefir WK1 was perceived as significantly more intense in taste with an average scoring of 6.93±1.47 compared to water kefir WK7 with an average scoring of 3.34±1.86.

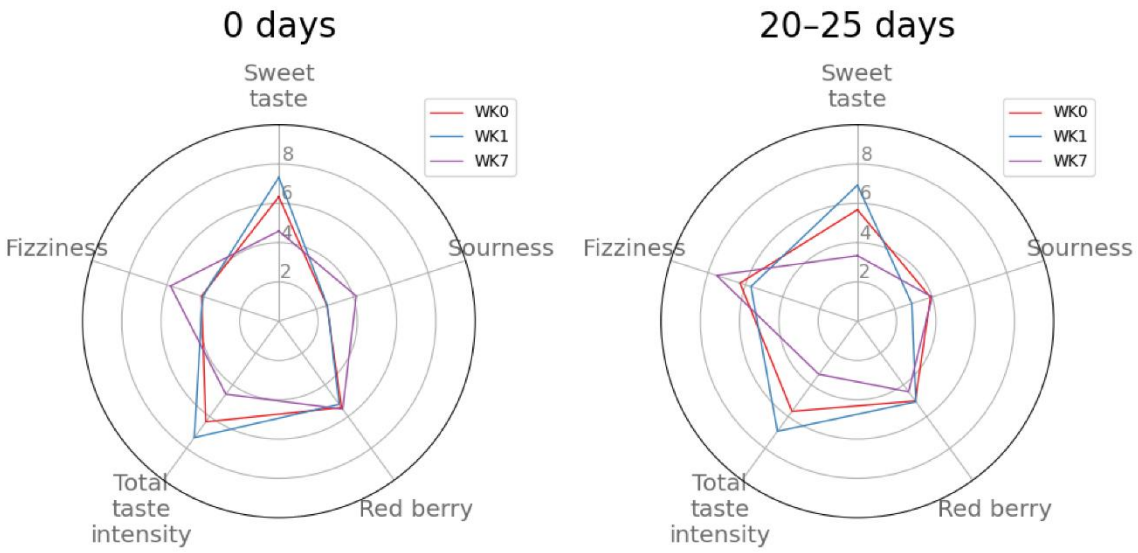

Figure 13. Perceived taste and mouthfeel of water kefirs after production (0 days) and after 20–25 days of cold storage, mean scoring values by panellists

Each water kefir recipe did not exhibit a significant difference ($p < 0.1$) in most sensory attributes before and after cold storage. The water kefirs differed only in their perceived fizziness: water kefirs WK0 and WK7 were perceived as fizzier after 20–25 days of cold storage (4.14±2.32 and 5.81±1.77 before cold storage versus 6.29±2.45 and 7.55±1.84 after cold storage, respectively).

Table 8. Attribute scoring in water kefirs with sucrose and non-sucrose sweeteners before and 20–25 days of cold storage

Attribute	Before cold storage			After cold storage		
	WK0	WK1	WK7	WK0	WK1	WK7
Odour						
Sweet odour	5.27±2.08 ^{a}	5.19±2.29 ^{a}	5.18±2.22 ^{a}	5.06±1.78 ^{d}	4.62±1.93 ^{d}	4.58±2.27 ^{d}
Alcohol	3.11±1.90 ^{a}	3.2±1.78 ^{a}	3.70±2.28 ^{a}	3.95±2.18 ^{d}	3.65±2.23 ^{d}	4.03±2.42 ^{d}
Fruity	5.38±1.83 ^{a}	4.8±1.58 ^{a}	5.11±2.04 ^{a}	4.70±1.58 ^{d}	4.94±1.90 ^{d}	4.64±2.26 ^{d}
Total odour intensity	5.71±1.49 ^{a}	5.22±1.55 ^{a}	5.89±1.39 ^{a}	5.28±1.87 ^{d}	5.34±2.15 ^{d}	5.88±1.85 ^{d}
Taste and mouthfeel						
Sweet taste	6.34±1.75 ^{a}	7.33±1.39 ^{a}	4.59±2.21 ^{b}	5.68±2.26 ^{d}	6.93±1.47 ^{d}	3.34±1.86 ^{e}
Sour taste	2.58±1.80 ^{a}	2.60±1.53 ^{a}	4.15±2.26 ^{b}	3.92±1.97 ^{d}	2.92±1.86 ^{d}	4.00±1.76 ^{d}
Red berry	5.43±1.88 ^{a}	5.23±1.73 ^{a}	5.53±1.86 ^{a}	5.02±1.79 ^{d}	5.07±1.83 ^{d}	4.44±1.84 ^{d}
Total taste intensity	6.34±1.75 ^{a}	7.33±1.39 ^{a}	4.59±2.21 ^{a}	5.68±2.26 ^{de}	6.93±1.47 ^{d}	3.34±1.86 ^{e}
Fizziness	4.14±2.32 ^{a}	4.08±2.57 ^{a}	5.81±1.77 ^{b}	6.29±2.45 $^{de\,*}$	5.71±2.55 ^{d}	7.55±1.84 $^{e\,*}$

Significant differences ($p < 0.1$) between the recipes are indicated with different superscripts (a, b, c for water kefirs before cold storage; d, e, f for recipes after 20–25 days of cold storage)
Significant difference ($p < 0.1$) for the same water kefir recipe before and after 20–25 days of cold storage is indicated with an asterisk *

Out of three categorical sensory attributes (rancid odour, vinegar odour, and dry mouthfeel), only dryness exhibited significant difference (Figure 14). Panellists experienced the dry mouthfeel in water kefir WK7 significantly ($p < 0.1$) more often in comparison to water kefirs WK0 and WK1 on the day they were produce. Dryness in water kefir WK7 was perceived as "slightly noticeable" or "present" in 95% of the answers, while the participants marked dryness as "slightly noticeable" or "present" in 55% and 50% of answers in water kefirs WK0 and WK1, respectively. After 20–25 days of cold storage, the significant ($p < 0.1$) difference in dryness remained only between water kefir WK7 (90% of answers were "slightly noticeable" and "present") and water kefir WK1 (participants perceived dryness in 45% of answers).

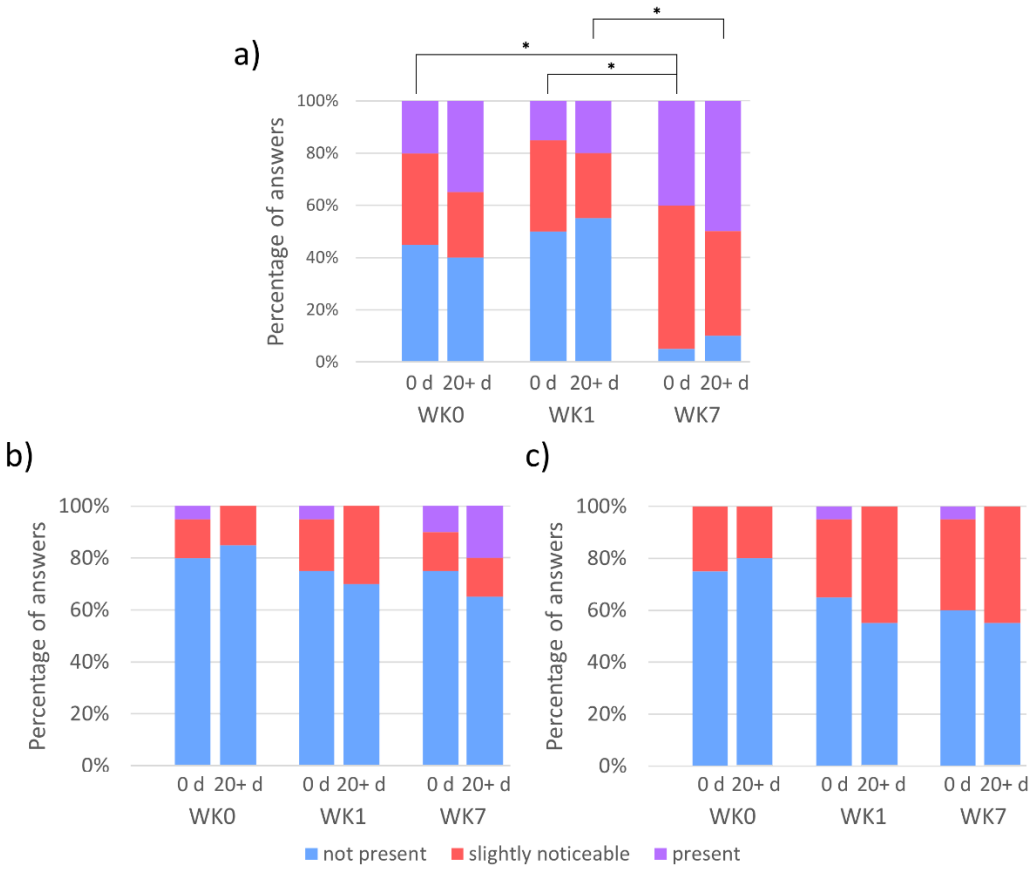

Figure 14. Perceived **a)** dryness, **b)** rancid odour, and **c)** vinegar odour of water kefirs with different sweeteners before cold storage and after 20–25 days. Bars represent the percentage of answers "not present", "slightly noticeable", and "present" among participants. * – $p < 0.1$

4 Discussions

During the work, five water kefir with different sweetener compositions were produced under monitoring of their pH and °Bx values (see Figure 5). The decrease in pH values followed the results previously reported in the scientific literature, for example the studies of Laureys & De Vuyst (2017), Laureys et al. (2018), Patel et al. (2022). These results were an initial indication of the fact that the reduction of sucrose content to 3% (w/v) does not interfere with the metabolism of water kefir microbiota. The addition of acidic raspberry extract also decreased the pH value of the fermentation medium, and for this reason it is more difficult to assess the contribution of microbial metabolism to the pH of the product after the raspberry extract was added. At the same time, an approximately 10% reduction in the pH value of the produced water kefirs during cold storage indicated the possible continuing metabolic activity of yeasts and/or bacteria in water kefir. This goes in line with the study of Bueno et al. (2021), in which the authors observed a 5.25–9.15% decrease in pH for fruit-based water kefirs after 28 days of cold storage. To limit the possible effect of additional ingredients on pH measurements of the water kefir, the pH values of such ingredients can be adjusted in the further studies.

4.1 Effect of sweeteners on the organic acid content of water kefir

The reduction of sucrose content and addition of sweeteners to water kefirs mainly did not result in significant changes in organic acid content of the beverages (Table 6). While some statistically significant difference in individual organic acid content was found between the recipes, the levels of malic acid, ascorbic acid, and lactic acid remained low and close to the detection threshold in all three studied recipes.

The observed concentration of lactic acid was found to be lower compared to previous water kefir studies (Laureys & De Vuyst, 2014; Laureys & De Vuyst, 2017; Patel et al., 2022) both for sucrose-based water kefir and water kefirs with other sweeteners. There are various possibilities potentially explaining this difference, one being a smaller mass of water kefir grains (4% w/v) used for the fermentation compared to the studies in question: 9–25% (w/v) in the study of Laureys & De Vuyst (2017), 10.7% (w/v) in the study of Patel et al. (2022). As Laureys & De Vuyst (2017) concluded in their study, the fermentation medium can affect the microbial metabolism. However, in their study, the water kefir with lower initial nutrient content contained 1.32 g/L of lactic acid on average,

while the highest value among ready products in this study was 0.151 g/L. Another possible reason is a different microbial composition of the water kefir grains used in the current work. Lynch et al. (2021) in their review demonstrated that the microbial content of water kefir highly varies (see Table 1 for the microbial species detected in different studies), and Laureys & De Vuyst (2017) in their study concluded that the starter culture affects the fermentation process and its products. As the microbial composition was not in the focus in the current work, it is possible that the starter culture had a lower ratio of lactic acid bacteria to yeasts, or that the LAB strains in the used water kefir grains are less metabolically active.

The change in the citric acid concentration by the end of the cold storage period in water kefir WK1 cannot be readily explained. Citric acid is reported to be synthesised by fungi, yeasts, and some bacteria, but its more active production is linked with high sucrose concentrations and aerobic conditions (Grewal & Kalra, 1995). During cold storage, the conditions were close to anaerobic, and the amount of glucose, fructose, and sucrose in water kefir WK1 was the lowest after cold storage. Another iteration of the study would be needed to confirm the obtained results.

The predominant lack of change in the organic acid content did not provide an explanation for the pH decrease in all recipes during production and cold storage. The pH changes may, however, result from the acetic acid, carbon dioxide, or other metabolites that were not quantified or identified in this work. As mentioned in the study of Patel et al. (2022), acetic acid is one of the major components in water kefir reaching 11.77 g/L at the end of fermentation, but it was not quantified in the current work. Although the citric acid content increased in water kefir WK1 towards the end of the cold storage period, its pH value did not significantly differ from other recipes. This allows to assume that the observed change in the concentration of citric acid did not factor in the pH decrease in the beverage during storage or that the observed citric acid concentration was caused by a measurement error.

4.2 Dynamics of the carbohydrate content in water kefirs

The carbohydrate content of water kefirs produced with three different recipes reduced expectedly during production but remained at a sufficient level at the end of the second fermentation (Table 6). Sucrose-based water kefir WK0 contained the most sucrose, glucose, and fructose at all measurement timepoints, and water kefir WK1 containing

other sweeteners exhibited the lowest sucrose concentration, reaching less than 1% (w/v) by the end of the second fermentation and production. However, water kefir WK1 exhibited a subsequent decrease in glucose, fructose, and sucrose concentrations during cold storage. This demonstrates that even the recipe with the lowest amount of added sucrose contained it in sufficient amounts for residual fermentation when the product was stored.

Water kefirs made with all three recipes demonstrated a slower reduction in glucose and fructose levels compared to sucrose. Particularly large amounts of glucose and fructose were observed in water kefir WK0 after 20 days of cold storage. This might be explained by the metabolic interactions in water kefir microbiota, in which invertase that converts sucrose into glucose and fructose might be more active compared to the enzymes of their subsequent metabolism. As Laureys & De Vuyst (2017) stated in their work, glucose might be a more preferred substrate than fructose, as fructose demonstrated a bigger lag between the initial increase in its concentration and the subsequent decrease in their study as well. In the current work, however, both glucose and fructose exhibited a similar initial increase in their concentration in water kefirs with reduced sucrose content. In sucrose-based water kefir WK0, the concentration of glucose and fructose continuously increased until the end of cold storage.

4.3 Volatile profile of water kefirs with sucrose and non-fermentable sweeteners

The addition of non-fermentable sweeteners did cause differences in the range of detected and identified volatile compounds in water kefirs with different recipes. Water kefirs produced with sucrose and other sweeteners contained the same volatile compounds without an immediate difference in their concentration based on the peak intensity of GC-MS chromatograms. To draw conclusions about the relative content of individual volatile compounds, quantification or semi-quantification of the identified molecules needs to be conducted, which was left out of scope in the current work.

The majority of the volatile compounds that became more prominent in the water kefir after cold storage belong to esters (Figure 8). These compounds are associated with yeast metabolism (Lynch et al., 2021; Patel et al., 2022), which points indirectly at the yeast activity being a major actor in the development of a volatile profile in water kefirs during cold storage. While Patel et al. (2022) reported that the most abundant categories of volatile compounds in ready water kefirs included higher alcohols, higher aldehydes, and

acetic acid, the predominant volatile compounds in this work are different based on their peak intensity. The most abundant compounds detected in water kefirs, except for isoamyl alcohol, belong to the class of esters: isobutyl acetate, isoamyl acetate, methyl octanoate, ethyl decanoate were compounds with the highest peak intensity. The volatile compound profile of water kefir in the current work mostly follows the results of Laureys & De Vuyst (2014), in which isoamyl alcohol, ethyl acetate, and 2-methyl-1-propanol were discovered in the largest quantities and a range of esters including isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl butanoate, and ethyl 2-methylbutanoate were discovered in smaller amounts, yet higher than the threshold concentration of their perception. The difference in the most abundant volatile compounds between the current work and previous studies cannot be attributed to the substitution of sucrose with other sweeteners. In this study, sucrose-based water kefir WK0 exhibited a similar volatile compound profile compared to the water kefirs with non-fermentable sweeteners. Instead, the difference can be explained by the water kefir grains composition, nutrient content of the fermentation medium, and other fermentation conditions, as Laureys & De Vuyst previously reported that the starter culture (Laureys & De Vuyst, 2017) as well as presence of nutrients and oxygen (Laureys & De Vuyst, 2018) determine the metabolite content of water kefir.

A range of volatile compounds that were identified in the final product might be linked to the used ingredients and not fermentation. For example, α-ionol and β-ionone that were present in water kefirs after the addition of the raspberry extract and sweeteners (Figure 7) have been previously detected among volatile organic compounds of raspberries (Aprea et al., 2015). While an introduction of additional compounds with the raspberry extract could be expected, its volatile profile analysis would be required to confirm that some volatiles present in the flavoured water kefirs in this work are derived from the added raspberry extract.

4.4 Sensory qualities of water kefirs with sucrose and other sweeteners

Substitution of sucrose with non-fermentable sweeteners caused a significant difference in perceived sweetness and fizziness between different water kefir recipes (Table 8). Creating water kefir recipes was not in the objectives of the current work, and the difference in sweetness can be explained by the difference in the used sweeteners and

their concentrations (see section 1.4.3 for the detailed description of xylitol, erythritol, and steviol glycosides).

The observed difference in the perceived fizziness of water kefirs WK0 and WK7 before and after cold storage can be attributed to the continuing fermentation and production of carbon dioxide when the product was stored. This finding goes in line with the assumed increase in carbonation levels in sucrose-containing water kefirs during cold storage, which was discussed in section 1.4.1. The results demonstrate that the change in carbonation is perceivable during tasting as a change in the corresponding mouthfeel.

However, higher fizziness of water kefir WK7 compared to water kefir WK0 before cold storage does not have a ready explanation as water kefir WK7 contains a lower amount of added sucrose. The attribute "fizziness" used in the current study was defined as a mouthfeel sensation similar to the sensation of a carbonated beverage: tickling feeling on the tongue related to bubbles in the beverage. As Pelchat et al. (2014) state, carbonation provokes a complex sensation involving the trigeminal nerve in a mechanism that is not yet fully understood. The authors refer to a previously conducted study that demonstrates that the experience of carbonation is greatly affected by the temperature. The temperature of the served samples could fluctuate once they were removed from cold storage during sensory evaluation sessions, which could affect the scoring of the attribute "fizziness". In addition, the perceived fizziness could be affected by the bottles, in which water kefirs underwent the second fermentation and were stored after the production. It was noticed that some of the bottles could not be closed in a completely airtight manner, which evidently led to the loss of CO_2 pressure.

Dry mouthfeel can be associated with tannins and other polyphenolic compounds, some organic and inorganic acids (such as malic or hydrochloric acid), some dehydrating agents (for example, ethanol), and multivalent salts, and proteins (Pires et al., 2020; Paissoni et al., 2023). Since this sensory attribute can be associated with various compounds, and not all of them were analysed in the current work (for example, polyphenolic compounds), the observed difference in the dry mouthfeel between water kefir WK7 and water kefirs WK0 and WK1 (Figure 14) cannot be readily explained. In addition, Pearson's Chi-square has been reported not to be robust for small-sized samples (McHugh, 2013), therefore the results need to be taken into consideration with caution.

The lack of significant difference in other taste, odour, and mouthfeel attributes of water kefirs (see Figure 12, Figure 13, Table 8) can be linked to the similar organic acid and volatile compound content for all studied water kefirs. The set of identified volatile compounds was the same for all water kefirs with different sweeteners and the volatile compounds did not exhibit a visible difference in their intensity in GC-MS chromatograms. As the absolute concentration of these metabolites was not quantified, it is possible that their concentrations did not differ sufficiently, or the concentrations of some metabolites were below the perception threshold. As volatile compounds contribute to the odour and flavour of water kefir (Table 2), the similarity in their profiles can explain the lack of perceived differences in the odour of water kefir made with different recipes.

The obtained results of the sensory evaluation can also be linked to the performance of sensory study participants (see section 3.4.1). The results of panel performance demonstrated that the study participants might have understood a part of attributes differently or used a scale in a different way when evaluating those attributes. Consequently, this could lead to a large variance in the answers of the sensory panellists and mask possible significant differences due to an increased noise in the data.

4.5 Methodological considerations

This study is important in the study of water kefirs as if it examines the possible effects of non-fermentable sweeteners on fermentation and the sensory qualities of the beverages. This work combined several methods and examined the metabolite content of water kefirs with different sweeteners together with their perceived sensory qualities. This allowed to evaluate the sensory qualities of water kefirs within the context of their metabolite content.

The scope of the metabolite analysis in this work, however, did not include the quantification of carbon dioxide nor volatile compounds. This did not allow to determine whether the absence of difference in most sensory attributes related to odour and taste can be linked to the volatile compound profile of different water kefir recipes. While the volatile profile compound profile of different water kefirs exhibited the same metabolites, quantifying these metabolites would allow to determine whether a statistically significant change in their concentration exists. In addition, their quantitative analysis would allow to determine, which volatile compounds are present in the concentration above their perception threshold.

To assess whether the observed difference in fizziness during sensory evaluation is associated with a difference in carbon dioxide content, the level of carbon dioxide in water kefirs can be measured directly, for example with the help of a pressure gauge or an analysis of dissolved CO_2 content. Future work involving water kefirs can include using containers that ensure no gas exchange with the environment, such as bottles equipped with airlocks or bottles sealed with a capping machine. This would help minimise the effect of gas losses on the perceived fizziness during sensory evaluation.

Some limitations of the sensory evaluation must be acknowledged as well. In particular, more consistent panel performance could be achieved with more training sessions. Due to the time constraints of the Master's thesis work, the number of trainings was limited to three. The limited number of training sessions did not allow for active work with each panellist nor for the determination of all points that were causing confusion. The process of panel training as a routine part of product development can consist of significantly bigger amount of training time, which allows to prepare the panellists for consistent scale use, helps them become familiar with the evaluated samples and attributes, and increases their ability to discriminate samples (Chambers & Chambers, 2020). Besides that, the evaluation of all sensory attributes should remain in the form of linear scales. If participants express wish to assess them in the categories of either being present or absent, like it happened in the current work, such attributes might need to be removed from the evaluation. Including some attributes in the form of categorical questions does not allow for the assessment of the panel performance related to these attributes and does not allow to draw strong conclusions from the obtained results.

5 Conclusions

In this work, seven water kefir recipes with xylitol, erythritol, and steviol glycosides added as individual sweeteners and in blends were created after several iterations of recipe development. Monitoring the production of five pre-selected water kefir recipes demonstrated they followed the same trend of gradual pH and °Bx value reduction, indicating similar fermentation activity in these products.

While the water kefirs with sucrose and non-fermentable exhibited a different carbohydrate content, their organic acid concentrations mostly remained at a similarly low level. The volatile profile of the water kefirs produced with non-fermentable sweeteners remained predominantly similar to the volatile profile of the sucrose-based water kefir. This indicates that substitution of a part of sucrose with non-fermentable sweeteners introduced little difference to the metabolism of water kefir microbiota.

The lack of significant difference in organic acid and volatile compound content of water kefirs with different sweeteners goes in line with the similar sensory evaluation results for the products. In the result of the descriptive sensory analysis, the difference was mostly observed for the attributes directly related to the concentration of sucrose and other sweeteners in the beverages but not for the attributes that could be associated with the volatile compounds present in the water kefir. However, panel performance analysis revealed that the lack of significant difference for some attributes could be explained by the performance of the sensory evaluation participants. The panellists might have had a lack of consensus regarding the attribute definition or scale use or might have been unable to discriminate different intensities of the attributes in question.

Overall, the study provides insights into the low effect of erythritol, xylitol, and steviol glycosides on the fermentation process and sensory qualities of water kefir. The current work demonstrates the potential for commercial production of water kefir sweetened with non-fermentable sweeteners. It also highlights areas for future research, such the effect of non-sucrose sweeteners on the microbial composition and carbon dioxide content of water kefirs.

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Appendix I. List of all volatile compounds detected and identified in water kefirs

Compound	Retention index according to NIST 14 library
Acetaldehyde	702
Ethyl acetate	888
2-Butanone	907
Methyl isobutyrate	921
Ethanol	932
Propyl acetate	973
3-Methylbutanal	918
Diacetyl	979
2-Pentanone	982
Isobutyl acetate	1012
Ethyl butanoate	1036
Butyl acetate	1074
Dodecane	1200
Isobutyl alcohol	1092
2-Methyl-2-pentanol	1099
Isoamyl acetate	1123
1-Butanol	1142
4-Methyl-2-heptanone	1206
Isoamyl alcohol	1209
Ethyl hexanoate	1233
Eucalyptol	1212
Acetoin	1285
Methyl octanoate	1385
2-Nonanone	1390
2-Methyl-2-octanol	1397
Nonanal	1391
m-Di-tert-benzene	1427
Ethyl octanoate	1435
Acetic acid	1449
Decanal	1498
Linalool	1547
n-octyl formate	1553
Isobutyric acid	1570
Bornyl acetate	1580
Methyl decanoate	1593
Terpinen-4-ol	1602
Methyl benzoate	1612
2-Methylbenzaldehyde	1632
Ethyl decanoate	1639
α-Terpinyl acetate	1693
Ethyl 9-decenoate	1694
α-Terpineol	1697
endo-Borneol	1702
Methyl hexadecanoate	1804

β-Phenethyl acetate	1813
Hexanoic acid	1846
α-Ionone	1840
Ethyl dodecanoate	1843
α-Ionol	1895
Phenylethyl alcohol	1907
β-Ionone	1967
Cinnamaldehyde	2018
4-Ethylguaiacol	2032
Octanoic acid	2060
Eugenol	2169
Nonanoic acid	2170
Aceteugenol	2263
n-Decanoic acid	2276
9-Decenoic acid	2345
2,4-Di-ter-butylphenol	2321
Dodecanoic acid	2496