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Influence of Substrate on the Fermentation Characteristics and Culture-Dependent Microbial Composition of Water Kefir

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Abstract: Water kefir is a sparkling fermented beverage produced by fermenting water kefir grains in a sucrose solution containing dried fruits or fruit extracts. The objective of this study was to investigate the influence of substrate composition on the fermentation kinetics and culture-dependent microbial composition of water kefir. First, the impact of different fruit substrates and nitrogen limitation was examined. Fermentation of different fruit-based media with a single water kefir culture demonstrated that the substrate mainly influenced the type and ratio of the organic acids produced. These organic acid profiles could be linked to the culture-dependent microbial composition. In addition, the microbial composition and the associated dominant microorganisms observed were influenced by the water kefir fermentation conditions. Investigation of the effect of nitrogen limitation on the fermentation kinetics of several water kefir cultures showed that under such conditions, the fermentative capacity of the cultures declined. However, this decline was not immediate, and specific water kefir microorganisms may have enabled some cultures to maintain a higher fermentative capacity for longer. Thus, the water kefir fermentation kinetics and characteristics could be linked to the substrate composition, microorganisms present, and the process conditions under which the fermentations were performed.

Keywords: water kefir; organic acid; fermentation resilience; nitrogen depletion

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1. Introduction

Water kefir is a sparkling fermented beverage prepared with water kefir grains. The slightly acidic drink is produced by fermenting a solution of sucrose, to which dried fruits have been added, with water kefir grains; most often, dried figs are used, sometimes with added lemon slices [1]. These gelatinous grains are a symbiotic culture of bacteria and yeast embedded in a polysaccharide matrix, typically a glucan homoexopolysaccharide [2,3]. It is hypothesised that these grains may have more than one geographical origin [4–6]. The water kefir grain microbiota consists of a range of lactic acid bacteria (LAB), acetic acid bacteria (AAB) and yeast species. Recent studies have highlighted the critical interactions and mutualistic relationship between LAB and yeast within the grain community [7,8]. Beside acid production, LAB species produce the homoexopolysaccharide matrix from which the kefir grain is formed [3], while yeast, through their proteolytic activity, assists the bacteria by providing nitrogen in a form that can be assimilated (di-, tri-peptides and amino acids) [7–9]. In addition, AAB's presence depends on the oxygen level [10]. LAB are represented primarily by the former *Lactobacillus* genus, AAB by the genus *Acetobacter*, and yeast by the genera *Saccharomyces*, *Zygosaccharomyces* and *Brettanomyces*. However, which species dominate and the prevalence of species identified in studies depends on several factors, including the fermentation substrate and conditions. In addition, the origin

of the grains and the breadth and diversity of samples (complex cultures/grains) examined may be reflected in those species that have been commonly isolated [1,10]. *Lacticaseibacillus casei* (*Lactobacillus casei*/*paracasei*, *Lentilactobacillus hilgardii* (*Lactobacillus hilgardii*), and *Liquorilactobacillus nagelii* (*Lactobacillus nagelii*) have each been found regularly in studies on water kefir fermentations. The presence of AAB is dependent on the fermentation conditions (level of oxygen), with *Acetobacter fabarum* being a commonly isolated species. Yeast species frequently detected in water kefir grains include *Zygotorulaspora florentina*, *Saccharomyces cerevisiae* and *Brettanomyces bruxellensis* [1,5,7,10–14]. The substrate and its composition play an essential role in fermentation characteristics and, ultimately, the final (beverage) product qualities. Both carbon and nitrogen sources are key to the metabolism and growth of the water kefir grain microbial consortia and the fermentative capacity of the grains as a whole. Sucrose is typically used as a carbon source, while fresh or dried fruits are generally used as a source of nitrogen. The most common fruit applied (in the scientific literature) is fig, either as a whole, dried fruit [1] or extract [13]. While figs are the most common, a variety of fruits or even vegetables could be added [15]. Fig's popularity may be linked to the empirical observation of vigorous and successful fermentations with this substrate. The exact reason for the superiority of figs in these fermentations is not fully understood but may relate to their relatively high calcium content. Calcium content and the buffering capacity of the medium or substrate (water/fruits) are key factors for good grain growth, lowering pH, and overall fermentative vigour [16,17]. The main metabolites of the water kefir fermentation are ethanol, lactic and acetic acid. Their relative concentration is strictly dependent on (i) the composition of the microbial consortium characterising the water kefir grains, (ii) the fermentation substrate, and (iii) the growth conditions applied during the fermentation step [6]. These metabolites along with other flavour products, such as acetaldehyde and acetoin conferring to the fermented beverage a refreshing exotic sour and slightly alcoholic flavour. Calcium may be provided by the water source (amount dependent on the mineral content or hardness), with a contribution from the fruit added. Laureys et al. [16] found that the buffering capacity and calcium content impacted the water kefir fermentation characteristics, particularly grain growth. Higher buffering capacity and calcium concentrations of the water used for fermentation promoted increased growth. Conversely, when the buffering capacity and/or calcium content were below a certain level, the pH values were significantly lower at the start of the fermentation and grain growth decreased throughout a back-slopping of the water kefir during the fermentation process [16]. This study aimed to investigate the influence of substrate composition on the fermentation kinetics and culture-dependent microbial composition of water kefir. Firstly, the impact of different fruit substrates, namely apple, fig and raisin extracts, was examined. This was performed using a single water kefir culture to ferment the different substrates. Secondly, the effect of nitrogen limitation on the kinetics and microbial composition of water kefir was examined. Here, a number of different water kefir cultures were used to ferment a nitrogen-rich substrate, followed by subsequent fermentations in nitrogen-poor media. Thus, the effect of this change in nitrogen availability on the fermentative capacity of the different water kefir cultures was examined. Finally, sequential fermentations were performed to account for the possibility of the grains adapting to the media over time. Thus, changes in the fermentation characteristics became apparent because of this adaption. After the initial fermentation, the grains were removed, washed, and placed into fresh media for one or more subsequent fermentations.

2. Materials and Methods

2.1. Preparation of Water Kefir Grain Cultures

Six commercial brands of water kefir grains were purchased and designated the following codes: HKUK, CFUK, CLUK, FFUK, KGIE and KOUK. All cultures were sourced from the United Kingdom (Amazon, London, UK) except for the KGIE culture purchased from an Irish online retailer. Upon reception, the water kefir grains were revived and/or rehydrated in a solution of 10% of sucrose (Sigma-Aldrich, St. Louis, MO, USA), 0.3% of

yeast extract (Fermaid O; Lallemand, Vienna, Austria) and 0.3% of peptone (from meat; Merck, Darmstadt, Germany). Fermentation was performed in glass jars covered with a muslin cloth at 26 °C for four days. The grains were then removed and placed into fresh medium and fermented for an additional four days prior to use in subsequent experiments.

2.2. Fruit-Based Water Kefir Fermentations

Apples, raisins and dried figs were purchased at a local supermarket. The fruits were chopped and puréed in a Kenwood kitchen blender. Following this, 0.39 kg, 0.5 kg and 2 kg of raisin, fig and apple purée were added to 1 L of water and extracted at 80 °C for 1 h in a water bath. Extracts were allowed to cool for 30 min before filtering through a cheesecloth to separate the liquid and solids. Extract (*w*/*w*) analysis was performed on the liquid via density meter DMA 4500M with an Alcolyzer Beer ME (Anton-Paar GmbH, Graz, Austria) at 20 °C. The liquid fruit extracts were diluted appropriately in water to achieve a final extract concentration of 7.5% (*w*/*w*), as measured by Anton Paar density meter DMA 4500M. As controls, 7.5 % sucrose solution was prepared. Each fruit substrate was fermented with 30 g of HKUK water kefir grains (Figure 1A) statically at 26 °C for four days in glass jars covered with a muslin cloth. The grains were then placed in fresh fruit substrate (grain refreshment) for an additional four days; thus, fermentations were performed for eight days. Fermentations were performed in duplicate.

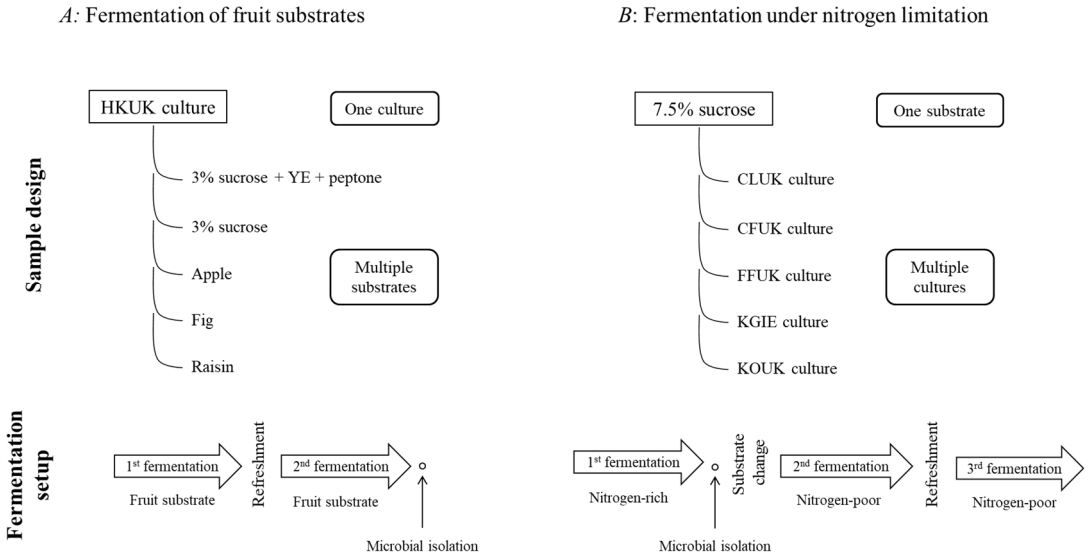

Figure 1. Experimental design. (**A**) Fermentation of fruit substrates. (**B**) Fermentation under nitrogen limitation.

2.3. Nitrogen-Limited Water Kefir Fermentations

CFUK, CLUK, FFUK, KGIE and KOUK water kefir grain cultures were used in the nitrogen-limitation fermentations (Figure 1B). Initially, a solution containing 7.5% sucrose, 0.3% Fermaid O yeast extract and 0.3% peptone (nitrogen-rich substrate) was fermented with 30 g of grains for four days. Following this, the grains were transferred to a 7.5% sucrose solution (nitrogen-poor substrate) and fermented for four days, after which the grains were placed in a fresh 7.5% sucrose solution and fermented for a further four days. All fermentations were performed statically at 26 °C in glass jars covered with a muslin cloth.

2.4. Analysis of Water Kefirs

pH was measured using a digital pH meter (Mettler Toledo LLC, Columbus, OH, USA). Total titratable acidity (TTA) was determined via titration of 10 mL sample against 0.1 M NaOH until pH 7 using an EasyPlus Titrator Easy pH (Mettler Toledo, Columbus, OH, USA). Ethanol (% *v*/*v*) and extract (% *w*/*v*) were analysed via density meter DMA 4500M with Alcolyzer Beer ME (Anton-Paar GmbH, Graz, Austria) at 20 °C. Sugars were

determined by high performance liquid chromatography (HPLC) Agilent 1260 Infinity (Agilent Technologies, Santa Clara, CA, USA) equipped a refractive index detector (RID) and a Sugar-Pak I 10 mm, 6.5 mm 300 mm column (Waters, Milford, MA, USA) with 0.1 mM of Ca-EDTA as the mobile phase and a flow rate of 0.5 mL/min at 80 °C. Organic acids were analysed using a Thermo Scientific Dionex UltiMate 3000 UPLC equipped with a diode array detector (DAD) set to 210 nm at 85 °C.

2.5. Microbial Isolation

Microbial isolation was performed from both the liquid (fermentate) and the grains of the water kefir fermentations. De Man Rogosa and Sharpe (MRS) media was used to isolate LAB. Glucose yeast extract carbonate (GYC), acetic acid ethanol (AE) [18], yeast extract peptone mannitol (YPM), malt yeast extract (MYA) [19], deoxycholate mannitol sorbitol (DMS) and modified DMS (mDMS) media were used for the growth and isolation of AAB [20–23]. Yeasts were isolated on yeast extract peptone dextrose (YPD) and YPM media. Cycloheximide (50 µg/mL) and chloramphenicol (30 µg/mL) were added to agars for the selective isolation of bacteria and yeast, respectively. Plates were incubated at 26 °C for three days under aerobic (yeast and AAB) or anaerobic (LAB) conditions. Grown cultures were examined for differences in colony morphology and evidence of acid production. Based on these indicators, different isolates were chosen for subculture until single, pure isolates were obtained. Isolates were stocked in the Culture Collection of the School of Food and Nutritional Sciences, UCC, in a final concentration of 40% glycerol for later identification via DNA sequencing.

2.6. Species Identification

DNA was extracted from isolates using a High Pure PCR Template Preparation Kit (Roche, West Sussex, UK) according to manufacturer's guidelines. For bacterial isolates, PCR of the 16S rRNA coding region was performed with primers fD1 and rP2 according to Weisburg et al. [24]. The primer pair ITS1F-ITS2 was used for yeast identification [25]. Amplified products were purified with a GenElute PCR Clean-Up kit (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. DNA sequencing was performed by Eurofins Genomics (Ebersberg, Germany). Isolate identification was performed using nBLAST of the forward and reverse strand contig [26] against the GenBank database. For bacteria, confirmation of species identity was performed by nBLAST against the 16S sequence of the type strain, available on the list of prokaryotic names with standing in nomenclature [27].

2.7. Statistical Analysis

Statistical analysis was performed with R version 3.6.3. Analysis of Variance (ANOVA) was followed by pairwise comparisons by Tukey's Honestly Significant Difference (HSD) test using the agricolae package v. 1.3-2 [28]. All statistical tests were evaluated at a significance level of $p = 0.05$.

3. Results and Discussion

This study aimed to investigate the influence of substrate composition on the fermentation kinetics and culture-dependent microbial composition of water kefir. The study was performed two parts, A and B (Figure 1). In part A the influence of different fruit substrates, namely extracts of apple, fig and raisin was examined. This was performed using a single water kefir culture, HKUK, to ferment the different substrates. Two sequential fermentations were performed to account for the possibility of the grains adapting to the fruit media over time. Thus, changes in the fermentation characteristics became apparent because of this adaption. Thereby, after the initial 4-day fermentation the grains were removed, washed, and placed into fresh fruit media for another 4-day fermentation (Figure 1A). Fermentation characteristics were measured at the start (0 h) and at the end of fermentation (96 h) for both fermentation periods. Part B investigated the effect of nitrogen

limitation on water kefir fermentation kinetics and culture-dependent microbial composition. Here, several different water kefir cultures were used to ferment a nitrogen-rich substrate (sucrose, yeast extract and peptone), followed by two subsequent fermentations in nitrogen-poor media (sucrose alone) (Figure 1B). The effect of this change in nitrogen availability on the fermentative capacity of the different water kefir cultures was examined.

3.1. Part A: Influence of Fruit Substrate on the Water Kefir Fermentation

The HKUK grains fermented each fruit water kefir media, with little difference in the fermentation characteristics apparent between the first and second 4-day fermentation, particularly for fig and raisin (Table 1). This suggests that the grains needed little adaption to these substrates. In the apple medium, the end TTA of the second fermentation period was higher than that for the first fermentation indicating higher acid production and suggesting some adaption of the grains to this medium. Figure 2 shows the organic acid levels produced in the fruit water kefir fermentations. Examination of Figure 2C shows that in the apple water kefir fermentations there were higher levels of acetic acid present on days 3 and 4 of the second fermentation period compared to the same days of the first fermentation period. Apart from these higher acetic acid levels, the buffering capacity of acetic acid could have contributed to the higher TTA observed at the end of the second fermentation. While the final TTA of the raisin and fig fermentations did not differ significantly between the first and subsequent fermentation, there were changes in the organic acid profile and the relative proportions of the different acids present, as shown in Figure 2D,E. A similar change in the types of acids presents was also apparent in the apple water kefir and likely was reflective of chances in the microbial composition during the fermentations. As already stated, acetic acid levels increased at the end of the second apple water kefir fermentation period; the ratio of acetic acid to lactic acid increased from near 1:1 on day 4 of the first fermentation to 2:1 by the end of the second fermentation (Figure 2C). Lactic acid levels remained constant in the apple fermentation. This is in contrast to the raisin water kefir fermentation. Although acetic acid levels were relatively constant between the fermentations in this substrate, gluconic acid levels increased in the second fermentation period and lactic acid levels decreased; the latter was not detectable at the end of the second raisin water kefir fermentation period (Figure 2D). Such changes may reflect shifts in the dominant microorganisms due to substrate adaption, such as a decrease in LAB numbers or their metabolic capacity and increase in AAB, discussed further below. Fermentation of the fig resulted in the most diverse organic acid profile; all organic acids assayed were found, including citric acid and succinic acid. Figure 2E shows that in the first 4-day fermentation period the levels of these acids were constant and present at relatively lower levels compared to the concentrations of these individual acids found for the other fruits during this first fermentation period. In both the apple and fig fermentations gluconic acid was dominant initially in the early days of the second fermentation, but decreased thereafter, with acetic acid becoming dominant (Figure 2C,E).

This shift in organic acid profile within the fermentation suggests a change in the dominant microorganisms, their metabolism or in their patterns of cross-feeding between the microorganism in the fermentation community. For example, Xu et al. [8] found that *Liquorilactobacillus hordei* (*L. hordei*), a species commonly associated with water kefir, can consume gluconate; such gluconate could arise from the metabolism of members of the AAB such as species of *Gluconobacter*.

Table 1. Characteristics of fruit water kefir fermentations.

Ferm. Substrates	Ferm. n°	Time (h)	Parameters						
			pH	TTA (meq/L)	Alcohol (% *v*/*v*)	Extract (% *w*/*w*)	Sucrose (g/L)	Glucose (g/L)	Fructose (g/L)
Sucrose, yeast extract, peptone	1st	0	6.3 ± 0.0 ^{c}	4.3 ± 0.5 ^{i}	9.7 ± 0.1 ^{abc}	0.1 ± 0.0 ^{d}	87.7 ± 2.4 ^{a}	0.0 ± 0.0 ^{c}	0.0 ± 0.0 ^{e}
		96	3.3 ± 0.1 ^{i}	44.5 ± 0.4 ^{e}	3.3 ± 2.1 ^{e}	4.4 ± 1.2 ^{b}	0.5 ± 0.2 ^{f}	6.2 ± 8.1 ^{c}	13.6 ± 13.6 ^{cde}
	2nd	0	6.5 ±0.0 ^{b}	6.2 ± 0.0 ^{i}	10.4 ± 0.0 ^{a}	0.1 ± 0.0 ^{d}	89.9 ± 0.0 ^{a}	0.0 ± 0.0 ^{c}	0.0 ± 0.0 ^{e}
		96	3.3 ± 0.0 ^{hi}	66.3 ± 0.5 ^{c}	1.2 ± 0.0 ^{e}	5.7 ± 0.1 ^{a}	0.7 ± 0.1 ^{f}	0.0 ± 0.0 ^{c}	0.1 ± 0.1 ^{e}
Sucrose alone	1st	0	7.0 ± 0.3 ^{a}	0.0 ± 0.0 ^{i}	7.3 ± 0.1 ^{cd}	0.0 ± 0.0 ^{d}	58.8 ± 5.3 ^{c}	0.4 ± 0.6 ^{c}	0.1 ± 0.2 ^{e}
		96	2.5 ± 0.0 ^{k}	58.2 ± 0.1 ^{cd}	7.0 ± 0.4 ^{d}	0.3 ± 0.1 ^{d}	1.4 ± 0.1 ^{f}	25.7 ± 0.4 ^{ab}	35.5 ± 0.4 ^{ab}
	2nd	0	7.0 ± 0.0 ^{a}	0.2 ± 0.0 ^{i}	7.4 ± 0.0 ^{bcd}	0.0 ± 0.0 ^{d}	76.0 ± 0.8 ^{b}	0.0 ± 0.0 ^{c}	0.0 ± 0.0 ^{e}
		96	2.9 ± 0.1 ^{j}	21.1 ± 0.3 ^{gh}	6.7 ± 1.3 ^{d}	0.1 ± 0.1 ^{d}	0.2 ± 0.3 ^{f}	0.6 ± 0.1 ^{c}	6.9 ± 0.3 ^{de}
Apple	1st	0	3.5 ± 0.0 ^{fg}	47.9 ± 0.6 ^{de}	7.2 ± 0.0 ^{d}	0.0 ± 0.0 ^{d}	8.1 ± 0.1 ^{e}	17.7 ± 0.1 ^{b}	35.7 ± 0.2 ^{ab}
		96	3.6 ± 0.0 ^{f}	69.3 ± 0.0 ^{c}	1.2 ± 0.0 ^{e}	3.1 ± 0.0 ^{c}	0.0 ± 0.0 ^{f}	0.0 ± 0.0 ^{c}	0.0 ± 0.0 ^{e}
	2nd	0	3.5 ± 0.0 ^{fg}	42.1 ± 0.3 ^{ef}	7.7 ± 0.0 b^{cd}	0.1 ± 0.1 ^{d}	2.5 ± 1.8 ^{ef}	3.8 ± 4.7 ^{c}	16.1 ± 22.1 ^{bcde}
		96	3.5 ± 0.0 ^{fg}	93.7 ± 0.5 ^{b}	1.3 ± 0.0 ^{e}	3.3 ± 0.0 ^{bc}	0.0 ± 0.1 ^{f}	0.0 ± 0.0 ^{c}	0.1 ± 0.0 ^{e}
Fig	1st	0	4.2 ± 0.0 ^{d}	14.2 ± 0.0 ^{ghi}	8.5 ± 0.0 ^{abcd}	0.0 ± 0.0 ^{d}	1.6 ± 0.0 ^{f}	35.4 ± 0.1 ^{a}	32.8 ± 0.1 ^{abc}
		96	3.4 ± 0.0 ^{gh}	102.5 ± 0.2 ^{b}	2.4 ± 0.0 ^{e}	3.3 ± 0.0 ^{bc}	0.0 ± 0.0 ^{f}	0.0 ± 0.0 ^{c}	0.3 ± 0.0 ^{e}
	2nd	0	4.3 ± 0.0 ^{d}	12.5 ± 0.1 ^{hi}	7.9 ± 0.0 ^{bcd}	0.0 ± 0.0 ^{d}	1.8 ± 0.4 ^{f}	2.9 ± 2.4 ^{c}	12.6 ± 4.8 ^{cde}
		96	3.4 ± 0.0 ^{fg}	92.6 ± 0.6 ^{b}	2.1 ± 0.6 ^{e}	3.1 ± 0.0 ^{c}	16.5 ± 2.6 ^{d}	28.3 ± 1.5 ^{a}	30.0 ± 1.0 ^{abcd}
Raisin	1st	0	3.9 ± 0.0 ^{e}	26.4 ± 0.8 ^{gh}	8.2 ± 0.0 ^{abcd}	0.0 ± 0.0 ^{d}	0.3 ± 0.0 ^{f}	34.7 ± 0.0 ^{a}	36.3 ± 0.0 ^{ab}
		96	3.2 ± 0.0 ^{i}	138.2 ± 0.3 ^{a}	1.2 ± 0.0 ^{e}	3.0 ± 0.2 ^{c}	0.3 ± 0.0 ^{f}	0.0 ± 0.0 ^{c}	1.0 ± 0.1 ^{e}
	2nd	0	4.0 ± 0.0 ^{e}	27.7 ± 0.3 ^{fg}	10.1 ± 0.1 ^{ab}	0.1 ± 0.0 ^{d}	0.0 ± 0.0 ^{f}	32.8 ± 0.6 ^{a}	44.7 ± 0.6 ^{a}
		96	3.2 ± 0.0 ^{i}	146.3 ± 0.5 ^{a}	2.3 ± 0.0 ^{e}	3.9 ± 0.1 ^{bc}	0.0 ± 0.0 ^{f}	0.0 ± 0.0 ^{c}	0.1 ± 0.3 ^{e}

abcdefghiMean values ± standard deviations. Values in the same row followed by the same superscript letters are not significantly different ($p > 0.05$).

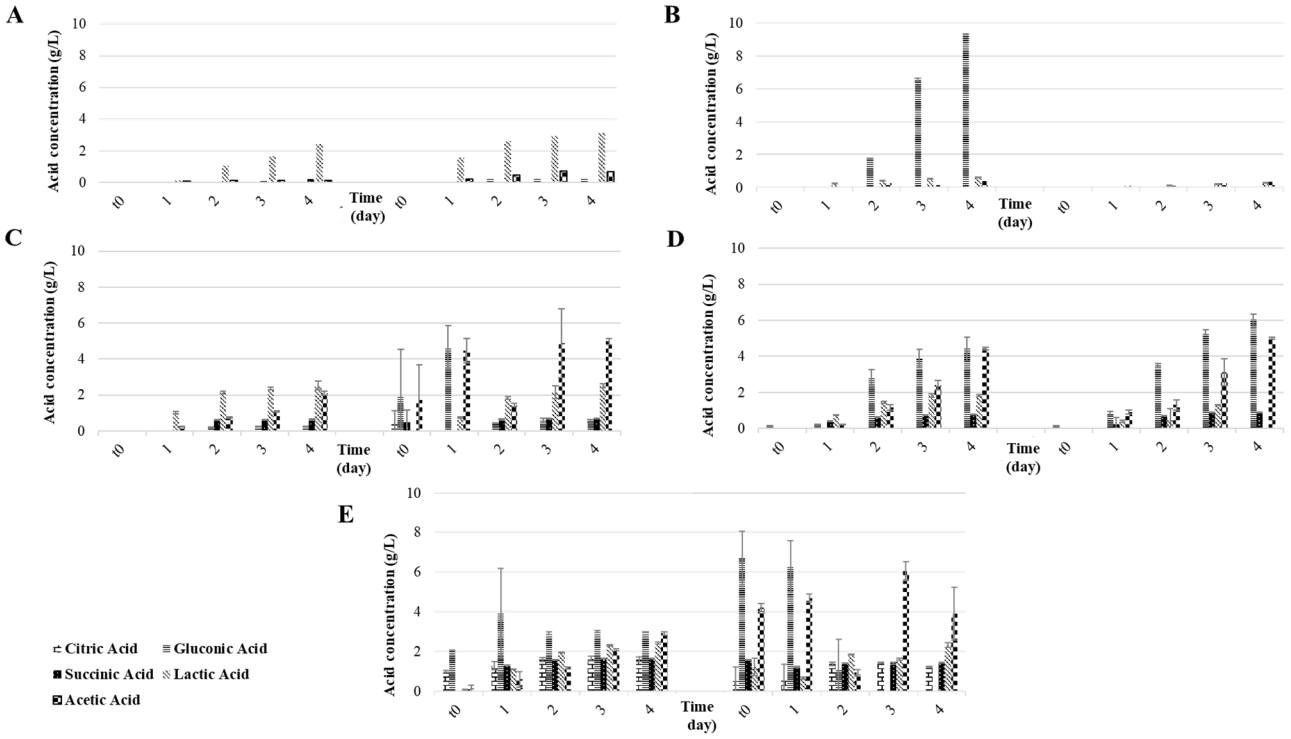

Figure 2. Organic acid development in fruit water kefirs. (**A**) Sucrose, yeast extract and peptone, (**B**) sucrose, (**C**) apple, (**D**) raisin, (**E**) fig.

Microbial isolation from the grains and fermentate of the fruit water kefirs provided some insight into the observed organic acid profiles (Table 2). Acetic acid bacteria of the genera *Acetobacter* and *Gluconobacter* were dominant isolates from all fruit water kefirs. Two *Acetobacter* species were found in the apple and raisin water kefirs, and three in the fig water kefir; *A. fabarum* was common to all, a species commonly found in other studies of

water kefir [1,10,13]. In contrast, only a single species of LAB, *Liquorilactobacillus satsumensis* (*L. satsumensis*) was found two of the three fruit water kefirs, with *L. hilgardii* also present in the fig water kefir. Both species have previously been identified in water kefir fermentations, in particular *L. hilgardii* [5,12,13,29,30]. The apparent dominance of *Acetobacter* may have been reason for the observed high levels of acetic acid. In addition, some lactobacilli species, those that are heterofermentative, can produce acetic acid when utilising fructose as an electron acceptor; however, *L. satsumensis* is unable to perform this reaction given its homofermentative metabolism. This species can, however, produce acid from gluconate which may be significant given the presence of gluconic acid in the water kefirs [31]. *L. hilgardii* was also found in the fig water kefir, as it has in many studies of water kefir, most of which have used fig as a substrate [29,32]. This species is heterofermentative and may have been responsible for some of the acetic acid produced in this water kefir through the use of fructose as an electron acceptor [33]. Some heterofermentative LAB can also use oxygen as an electron acceptor if they possess NADH oxidase activity [34]. This would be relevant for this study given that oxygen was not excluded, however, *L. hilgardii* has been shown not to contain any NADH oxidase activity [35].

Table 2. Bacteria and yeast species isolated from fruit water kefirs fermented with HKUK grains.

Carbon Source	Liquid (L) or Grain (G) Isolation	Isolated Species		
		Lactic Acid Bacteria	**Acetic Acid Bacteria**	**Yeast**
Sucrose	L	n.d.	*Gluconobacter cerinus*	n.d.
	G	*Liquorilactobacillus satsumensis*	*Acetobacter syzgii, Gluconobacter cerinus*	n.d.
Apple	L	*Liquorilactobacillus satsumensis*	*Acetobacter fabarum, Acetobacter suratthaniensis*	n.d.
	G	n.d.	*Acetobacter fabarum*	n.d.
Fig	L	n.d.	*Acetobacter persici*	*Pichia kudriavzevii*
	G	*Lentilactobacillus hilgardii, Liquorilactobacillus satsumensis*	*Gluconobacter cerinus, Acetobacter fabarum, Acetobacter syzgii, Komagataeibacter saccharivorans*	n.d.
Raisin	L	n.d.	*Acetobacter orientalis, Acetobacter syzygii, Gluconobacter cerinus*	n.d.
	G	*Liquorilactobacillus satsumensis, Liquorilactobacillus oeni*	*Acetobacter fabarum, Gluconobacter cerinus, Gluconobacter oxydans*	*Pichia kudriavzevii*

In addition to acetic acid, gluconic acid levels were dominant in the raisin water kefir fermentations, and lactic acid levels were very low throughout the second fermentation and absent by day 4 of this period. While a single species of *Gluconobacter*, *G. cerinus*, was present in all fruit water kefirs, an additional species, *G. oxydans*, was found in the raisin water kefir. Few previous studies of water kefir have found *Gluconobacter* species [10,30] with one identifying *G. roseus/oxydans* [10]. The *Gluconobacter* species identified in this study are more commonly associated with kombucha fermentations, that are intentionally aerobic, and in which AAB of the genera *Acetobacter* and *Gluconobacter* play the dominant role [36]. Laureys et al. [37] found that in anaerobic water kefir fermentations, *G. roseus/oxydans* and *A. indonesiensis* were more abundant, whereas *A. fabarum* was most abundant under aerobic fermentation conditions [10]. In the water kefir fermentations of our current study *A. fabarum* was the AAB species most frequently isolated; this suggests that the partial aerobic nature under which the water kefir fermentations were performed influenced the dominance of AAB, leading to the production of significant levels of acetic acid in the apple and fig water kefirs and gluconic acid in the raisin water kefir. Only in raisin and fig water kefier, yeast strains belonging to the species *Pichia kudriavzevii* (synonym *Issatchenkia*

orientalis) were detected as part of the kefir microbiota. Such species are commonly part of the flora of commercial and traditional kefir drinks [38,39].

Regarding the influence of the individual fruit substrates on the microbial composition and ultimate fermentation characteristics, all extracts were pasteurised prior to use which would suggest little microbial contribution directly from the fruit material. As mentioned, beside differences in the profile of TTA development between the fruits, the evolution of the pH, extract consumption and alcohol production were similar between the fruits. Only the fructose consumption was delayed in the second fermentation of raisin water kefir compared to apple and Figure This delayed fructose consumption accompanied the very low lactic acid levels observed at the same time. However, on day 4 the fructose was fully consumed (Table 1). The absence of lactic acid and near doubling of the acetic acid levels between days 3 and 4 suggests that heterofermentative LAB could have been involved in utilisation of fructose as an electron acceptor with the concomitant production of acetic acid. While no heterofermentative species were isolated from the raisin water kefir, the presence of unculturable LAB species could not be discounted, as discussed further below, some of which may have performed this reaction.

An examination of the general chemical composition of fig, apple and raisin did clearly indicate the suitability of one fruit over the another. Analysis of compositional data on USDA FoodData Central [37]—Adjusting for the quantity of fruit material used in the preparation of the fruit extracts—Suggests that, the apple extract was potentially lower in protein, and certain minerals and micronutrients such as calcium, iron, potassium, sodium, phosphorous, copper and selenium. This could explain the lower TTA achieved in the apple water kefir fermentations. On the other hand, the apple extract was predicted to be higher in vitamins such as vitamin C, riboflavin and folate. It is relevant to point out that the proximate composition of the target fruit samples has not been determined, and the metabolic performance of the commercial water kefir grains has been discussed by taking into consideration the average nutritional profile of the fruit samples resulting from the public database (USDA-FoodData). This represents a limitation of this study.

The fruit extracts were predicted to be similar in terms of level of carbohydrates present. As mentioned in the Introduction section, the calcium content and the buffering capacity of the medium have been identified as key factors for good grain growth, lowering of the pH and overall fermentative vigour [16,17]. Calcium and substances with buffering capacity may be provided by the water used but also the fruit. Reiß [40] found that figs produced the most optimum fermentation when compared to other fruits. Omission of figs significantly slowed the consumption of glucose and thus the fermentation rate. Substitution with other dried fruit, namely raisins, dates and plums, modified the fermentation and rate of production of lactic acid and acetic acid [40]. In the current study, based on the TTA and total acid levels during the fermentations, raisin appeared to be the substrate eliciting the highest fermentative capacity from the grains. Similarly, Laureys et al. [10] showed that dried raisins produced a stable water kefir fermentation that had the lowest pH (compared to dried fig, dried apricot or fresh fig) but also the lowest grain growth [10].

Our results suggested that AAB became dominant in the fruit water kefir fermentations. This was likely a consequence of factors such as composition of the substrate medium and the incubation conditions, as discussed. However, it is noteworthy that the culture-dependent methodology utilised may have missed unculturable species. Zanirati et al. showed that many LAB species detected in water kefir by culture-independent methods were not identified by the culture-dependent methods, including *L. hilgardii* and *L. nagelii* [5].

The influence of substrate and in particular buffering capacity was evident when considering the fermentation characteristics of the control media (sucrose, peptone and yeast extract, and sucrose alone). The pH values of these control media where much higher at the beginning of the fermentations (pH 6–7) compared to the fruit media (pH 3.5–4) (Table 1). In addition, the pH of the control media dropped rapidly within the first 24 h, suggesting an absence of buffering capacity. In contrast, the pH of fruit water kefirs gradually dropped over the 4-day fermentation period. Despite the minimal

overall decrease in the pH of the fruit water kefirs compared to the controls, the change and increase in TTA was significantly higher in the former compared to the latter fermentations. Thus, the rapid decrease in pH accompanied by low overall acid production reinforced the conclusion that buffering capacity in the control media was effectively absent. The organic acid profiles of the control media (Figure 2A,B) also highlight the effect of nutrient composition. In the medium containing sucrose, peptone and yeast extract (nitrogen-rich) the dominant acid was lactic acid, with minimal levels of acetic acid (Figure 2A). Indeed, the lactic acid levels showed a similar profile to the levels found in the apple and fig water kefirs. However, unlike the fruit water kefirs, lactic acid was the main acid found. It is probable that the nitrogen-rich nature of this medium favoured a dominance of LAB at the expense of the AAB. The presence of this nitrogen in an easy to assimilate form (in peptone and yeast extract) may have contrasted with the nitrogen present in the fruit water kefirs. The naturally less assimilable nature of the nitrogen sources in the latter may have created an advantage for the AAB. In addition, the partially aerobic nature of the fermentations coupled with the ability of these bacteria to derive energy via oxidative fermentation would have created an advantage over the LAB [41]. Thus, the acid profiles of the fruit water kefirs reflected this dominance in the form of high acetic acid and gluconic acid levels (Figure 2C–E). Furthermore, in the absence of an added nitrogen source (i.e., in media sucrose alone) gluconic acid was the only acid produced (Figure 2B). Here, the absence of a nitrogen source likely significantly reduced LAB growth and fermentative capacity, allowing AAB to dominant (for reasons discussed above) in particular *Gluconobacter; G. cerinus* was a dominant isolate from this water kefir fermentation. Therefore, the importance of substrate and nitrogen source in determining the microbial composition and, ultimately, the fermentation characteristics are evident [42], as will be discussed further in part B.

3.2. Part B: Effect of Nitrogen Limitation on the Water Kefir Fermentation

To further examine the effect of nitrogen limitation on water kefir fermentation, several other brands of water kefir culture were investigated for their fermentation and microbial characteristics under such conditions. The cultures were used to ferment, initially, a nitrogen-rich substrate (7.5% sucrose, 0.3% yeast extract and 0.3% peptone), followed by two subsequent fermentations in nitrogen-poor media (sucrose alone) (Figure 1B). The effect of nitrogen availability on the fermentative capacity of the different water kefir cultures was examined.

The data in Table 3 and Figure 3, reporting the profiles of TTA evolution, extract consumption alcohol production and acids profiles, show a marked change in the fermentation characteristics over the three fermentation periods. This mainly occurred during the switch from the nitrogen-rich to nitrogen-poor media. Secondly, there were differences in the fermentation characteristics among the tested water kefir cultures. The most significant difference in the kinetics between the cultures was apparent in the initial nitrogen-rich fermentation period. Particularly noteworthy was the difference between the cultures CLUK and FFUK. The CLUK fermentation was characterised by the lowest drop in pH and low acid production, as evidenced from the TTA and organic acid production levels (Table 3). In addition, CLUK fermentation exhibited the highest extract consumption and the highest alcohol production. This combination of fermentation characteristics could suggest that the dominant metabolism was that of yeast.

Table 3. Characteristics of water kefir fermentations performed under nitrogen limitation.

Water Kefir Culture	Fermentation Period	Time (h)	pH	TTA (meq/L)	Extract (% *w*/*w*)	Alcohol (% *v*/*v*)	Sucrose (g/L)	Glucose (g/L)	Fructose (g/L)
CLUK	1st	0	6.4 ± 0.0 ^{b}	1.0 ± 0.0 ^{h}	10.5 ± 0.0 ^{a}	0.1 ± 0.0 ^{d}	110.5 ± 0.0 ^{a}	0.0 ± 0.0 ^{g}	0.0 ± 0.0 ^{h}
		96	3.8 ± 0.1 ^{c}	15.2 ± 0.4 ^{fgh}	0.9 ± 0.0 ^{c}	5.8 ± 0.1 ^{a}	1.1 ± 1.6 ^{f}	0.0 ± 0.0 ^{g}	0.0 ± 0.0 ^{h}
	2nd	0	3.7 ± 0.0 ^{c}	0.0 ± 0.0 ^{h}	7.5 ± 0.0 ^{b}	0.0 ± 0.0 ^{d}	80.6 ± 0.0 ^{b}	0.0 ± 0.0 ^{g}	0.0 ± 0.0 ^{h}
		96	2.5 ± 0.0 ^{i}	46.2 ± 0.6 ^{de}	6.9 ± 0.5 ^{b}	0.5 ± 0.3 ^{cd}	6.7 ± 6.0 ^{f}	32.2 ± 1.7 ^{b}	32.2 ± 1.7 ^{ab}
	3rd	0	7.2 ± 0.0 ^{a}	0.1 ± 0.0 ^{h}	7.5 ± 0.0 ^{b}	0.0 ± 0.0 ^{d}	83.6 ± 0.0 ^{b}	0.0 ± 0.0 ^{g}	0.0 ± 0.0 ^{h}
		96	3.0 ± 0.1 ^{fgh}	10.3 ± 0.2 ^{gh}	7.5 ± 0.4 ^{b}	0.0 ± 0.0 ^{d}	36.1 ± 5.9 ^{d}	20.8 ± 3.9 ^{bc}	20.8 ± 3.9 ^{cde}
CFUK	1st	0	6.4 ± 0.0 ^{b}	1.0 ± 0.0 ^{h}	10.5 ± 0.0 ^{a}	0.1 ± 0.0 ^{d}	110.5 ± 0.0 ^{a}	0.0 ± 0.0 ^{g}	0.0 ± 0.0 ^{h}
		96	3.2 ± 0.0 ^{de}	123.3 ± 0.8 ^{a}	2.2 ± 0.4 ^{c}	5.0 ± 0.1 ^{a}	2.9 ± 0.0 ^{f}	0.3 ± 0.4 ^{fg}	4.5 ± 2.4 ^{gh}
	2nd	0	7.2 ± 0.0 ^{a}	0.0 ± 0.0 ^{h}	7.7 ± 0.0 ^{b}	0.0 ± 0.0 ^{d}	81.0 ± 0.0 ^{b}	0.0 ± 0.0 ^{g}	0.0 ± 0.0 ^{h}
		96	2.7 ± 0.0 ^{hi}	88.3 ± 0.2 ^{b}	7.5 ± 0.1 ^{b}	0.1 ± 0.0 ^{d}	2.8 ± 0.1 ^{f}	33.4 ± 0.0 ^{a}	37.1 ± 0.0 ^{a}
	3rd	0	7.2 ± 0.0 ^{a}	0.3 ± 0.0 ^{h}	7.5 ± 0.0 ^{b}	0.0 ± 0.0 ^{d}	84.4 ± 0.0 ^{b}	0.0 ± 0.0 ^{g}	0.0 ± 0.0 ^{h}
		96	3.1 ± 0.0 ^{defg}	13.5 ± 0.2 ^{fgh}	7.7 ± 0.01 ^{b}	0.0 ± 0.0 ^{d}	34.8 ± 0.8 ^{de}	21.7 ± 0.3 ^{b}	21.9 ± 0.2 ^{cde}
KGIE	1st	0	6.4 ± 0.0 ^{b}	1.0 ± 0.0 ^{h}	10.5± 0.0 ^{a}	0.1 ± 0.0 ^{d}	110.5 ± 0.0 ^{a}	0.0 ± 0.0 ^{g}	0.0 ± 0.0 ^{h}
		96	3.5 ± 0.0 ^{cd}	67.9 ± 0.1 ^{bc}	1.9 ± 0.1 ^{c}	4.9 ± 0.1 ^{a}	3.0 ± 0.0 ^{f}	0.0 ± 0.0 ^{g}	0.0 ± 0.0 ^{h}
	2nd	0	7.2 ± 0.1 ^{a}	0.0 ± 0.0 ^{h}	7.6 ± 0.8 ^{b}	0.0 ± 0.0 ^{d}	80.8 ± 0.3 ^{b}	0.0 ± 0.0 ^{g}	0.0± 0.0 ^{h}
		96	3.0 ± 0.4 ^{efgh}	63.3 ± 1.1 ^{cd}	2.3 ± 0.5 ^{c}	2.9 ± 0.1 ^{b}	2.5 ± 0.0 ^{f}	0.0 ± 0.0 ^{g}	10.7 ± 3.6 ^{fg}
	3rd	0	7.2 ± 0.0 ^{a}	0.2 ± 0.0 ^{h}	7.5 ± 0.0 ^{b}	0.0 ± 0.0 ^{d}	84.0 ± 0.6 ^{b}	0.0 ± 0.0 ^{g}	0.0 ± 0.0 ^{h}
		96	3.1 ± 0.0 ^{defg}	24.9 ± 0.2 ^{efg}	6.2 ± 1.1 ^{b}	0.7 ± 0.5 ^{cd}	11.7 ± 7.1 ^{f}	20.4 ± 4.5 ^{b}	26.87 ± 0.7 ^{bcd}
FFUK	1st	0	6.4 ± 0.0 ^{b}	1.0 ± 0.0 ^{gh}	10.5 ± 0.0 ^{a}	0.1 ± 0.0 ^{cd}	110.5 ± 0.0 ^{a}	0.0 ± 0.0 ^{g}	0.0 ± 0.0 ^{h}
		96	3.3 ±0.0 ^{def}	119.5 ± 2.0 ^{a}	10.0 ± 0.2 ^{a}	0.5 ± 0.4 ^{d}	24.1 ± 1.2 ^{e}	19.1 ± 2.5 ^{bcd}	29.4 ± 4.4 ^{abc}
	2nd	0	7.3 ± 0.0 ^{a}	0.0 ± 0.0 ^{h}	7.5 ± 0.0 ^{b}	0.0 ± 0.0 ^{d}	80.6 ± 0.0 ^{b}	0.0 ± 0.0 ^{g}	0.0 ± 0.0 ^{h}
		96	2.9 ± 0.1 ^{ghi}	72.9 ± 0.5 ^{bc}	6.0 ± 1.1 ^{b}	1.0 ± 0.6 ^{cd}	25.3 ± 6.1 ^{de}	10.9 ± 9.4 ^{de}	18.8 ± 8.5 ^{def}
	3rd	0	7.2 ± 0.0 ^{a}	0.1 ± 0.0 ^{h}	7.5 ± 0.0 ^{b}	0.0 ± 0.0 ^{d}	83.6 ± 0.0 ^{b}	0.0 ± 0.0 ^{g}	0.0 ± 0.0 ^{h}
		96	3.1 ± 0.2 ^{defg}	13.0 ± 0.3 ^{fgh}	6.2 ± 1.6 ^{b}	0.0 ± 0.4 ^{d}	53.7 ± 2.21 ^{c}	10.9 ± 1.0 ^{cde}	11.4 ± 0.9 ^{fg}
KOUK	1st	0	6.4 ± 0.0 ^{b}	1.0 ± 1.0 ^{h}	10.5 ± 0.0 ^{a}	0.1 ± 0.0 ^{d}	100.5 ± 0.0 ^{a}	0.0 ± 0.0 ^{g}	0.0 ± 0.0 ^{h}
		96	3.3 ± 0.0 ^{def}	120.8 ± 1.1 ^{a}	6.1 ± 1.5 ^{b}	2.8 ± 0.8 ^{b}	3.6 ± 0.9 ^{f}	9.7 ± 5.2 ^{def}	23.7 ± 4.9 ^{bcde}
	2nd	0	7.2 ± 0.0 ^{a}	0.0 ± 0.0 ^{h}	7.7 ± 0.0 ^{b}	0.0 ± 0.0 ^{d}	81.0 ± 0.0 ^{b}	0.0 ± 0.0 ^{g}	0.0 ± 0.0 ^{h}
		96	3.0 ± 0.1 ^{fgh}	31.4 ± 0.2 ^{ef}	5.9 ± 0.5 ^{b}	1.2 ± 0.3 ^{c}	26.8 ± 5.0 ^{de}	9.4 ± 0.1 ^{efg}	17.4 ± 0.1 ^{ef}
	3rd	0	7.2 ± 0.0 ^{a}	0.3 ± 0.0 ^{h}	7.5 ± 0.0 ^{b}	0.0 ± 0.0 ^{d}	84.4 ± 0.0 ^{b}	0.0 ± 0.0 ^{g}	0.0 ± 0.0 ^{h}
		96	3.1 ± 0.0 ^{defg}	12.1 ± 0.3 ^{fgh}	7.1 ± 0.7 ^{b}	0.1 ± 0.1 ^{d}	52.8 ± 6.5 ^{c}	8.3 ± 0.0 ^{efg}	9.5 ± 0.3 ^{fg}

1st fermentation period: (nitrogen-rich); 2nd and 3rd fermentation period: (nitrogen-poor). Mean values ± standard deviations. Values in the same row followed by the same superscript letters are not significantly different ($p > 0.05$).

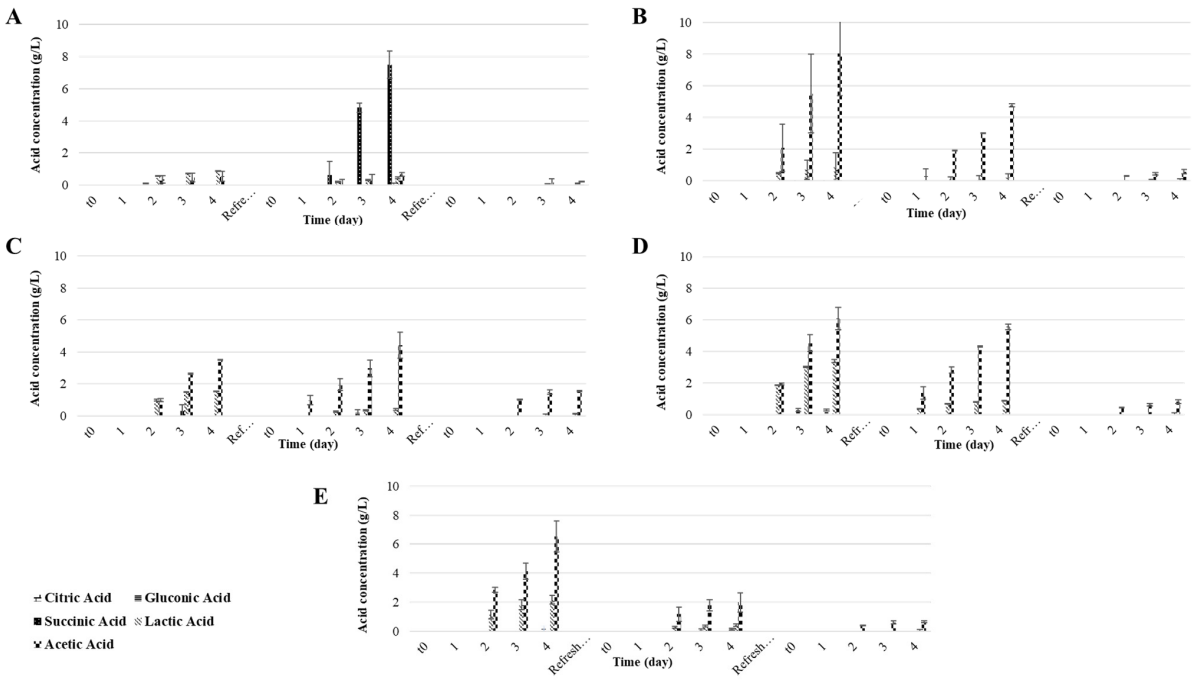

Figure 3. Organic acid development in water kefirs under nitrogen limitation. (**A**) CLUK, (**B**) FFUK, (**C**) KGIE, (**D**) CFUK, (**E**) KOUK.

In contrast, the FFUK grains showed a significant increase in TTA over the nitrogen-rich fermentation period, and a similar drop in pH to other grains, but with minimal extract consumption and minimal alcohol production. This fermentation pattern suggests a dominance of AAB metabolism and is reinforced by the high acetic acid levels (Figure 3B) and low alcohol levels found. Therefore, it could be postulated that the AAB were utilising alcohol for energy production and growth, with minimal carbohydrate (extract) consumption [41].

During the fermentation under a nitrogen-poor environment, the fermentative capacity of the cultures decreased, as exemplified by the minimal consumption of extract and alcohol production compared to the first fermentation period (Table 3) as were the levels of organic acids produced (Figure 3). However, most cultures retained some fermentative capacity in the second fermentation period, with evidence of continued acid production relative to the minimal production observed in the final fermentation period (Table 3). In particular, KGIE displayed a higher extract consumption and alcohol production than the other cultures.

The reason for the maintenance of some fermentative capacity in the second fermentation period, even though the grains were in a nitrogen-poor environment, may have been due to the carry-over within the grain matrix/environment itself of a certain level of residual nitrogenous substances. This probably supported a low level of fermentative activity of the grains into the second fermentation period.

By the third fermentation period any residual nitrogen sources would have been depleted and the cultures starved of nitrogen, as indicated by the plateaued kinetics, suggestive of minimal fermentation activity. The KGIE culture survived the nitrogen limitation best (Figure 3C), as discussed further below. The observation of a rapid fall in pH (within 24 h) of all cultures during the nitrogen-poor fermentation periods suggested minimal buffering capacity in the fermentations, which would be expected in a medium consisting solely of sucrose. In addition, further examination of the types of organic acid produced (Figure 3) showed that acetic acid, followed by lactic acid were produced in the highest concentrations; the amounts formed were lower with each subsequent fermentation, in line with the decreasing fermentative capacity. However, for all cultures except CLUK, the main acid formed was acetic acid, followed by lactic acid and in similar ratios. Species of *Acetobacter,* and lactobacilli were isolated from all culture fermentations; however, *Acetobacter* dominated these isolates, which may account for the high acetic acid levels; among the different water kefir cultures, eight different species were isolated (Table 4).

Two species for CFUK (L) one for (G) and five species for KOUK (L) were isolated. The reason for the dominance of *Acetobacter* in the cultures likely relates to the aerobic nature under which these fermentations were performed, as discussed in Part A for the fruit water kefirs. As the main difference from the other cultures, the dominant organic acid in the CLUK fermentation was gluconic acid (Figure 3A), with high levels observed after the switch from nitrogen-rich to nitrogen-poor media. A similar organic acid profile was observed in the sucrose-containing media fermented with the HKUK grains (Figure 2B) and where the dominant isolate was *G. cerinus*. In the CLUK fermentations *G. oxydans* was isolated from both the grains and liquid fermentate. In all other water kefir culture fermentations, the dominant acid was acetic acid, followed by lactic acid.

Of the complex cultures used in this study, the KGIE culture maintained fermentative capacity for the longest when fermenting the nitrogen-poor media; this culture had the highest TTA and alcohol production in the third fermentation period (Table 3). Additionally, noteworthy is that this culture contained the highest lactic acid levels of all the cultures, suggesting that the LAB present may have dominated the fermentation longer than in the other cultures. Furthermore, *L. nagelii*, a typical water kefir bacteria, was isolated from the liquid fermentate and the grains of this fermentation. In addition, two yeast species were isolated, including *Zygotorulaspora florentina*, a common yeast isolate, while only a single yeast species was isolated from other cultures. The observation of these common water kefir microorganism and their metabolic resilience under the investigated adverse fermentation may have been the reason for the maintenance of the fermentative capacity of

the KGIE culture for longer. Furthermore, a mutualistic relationship existed between these microorganisms [7]. Moreover, the KGIE culture contained several bacteria not previously isolated from water kefir, such as *Curtobacterium flaccumfaciens*. However, the role of such bacteria in water kefir fermentation remains unknown.

Table 4. Microorganisms isolated from water kefir cultures used in the nitrogen limitation study.

Water Kefir Culture	Liquid (L) or Grain (G) Isolation	Isolated Species			
		LAB	AAB	Yeast	Other
CLUK	L	*Liquorilactobacillus nagelii*	*Acetobacter indonesiensis*, *Acetobacter orientalis*, *Acetobacter syzygii*, *Gluconobacter oxydans*	*Pichia membranifaciens*, *Saccharomyces cerevisiae*	n.d.
	G	n.d.	*Gluconobacter oxydans*	*Pichia membranifaciens*	n.d.
CFUK	L	*Liquorilactobacillus nagelii*	*Acetobacter persici*, *Acetobacter syzygii*	*Zygotorulaspora florentina*	n.d.
	G	*Liquorilactobacillus nagelii*	*Acetobacter persici*	*Zygotorulaspora florentina*	n.d.
FFUK	L	*Lacticaseibacillus casei*, *Lacticaseibacillus paracasei*	*Acetobacter cerevisiae*, *Acetobacter fabarum*, *Acetobacter indonesiensis*, *Acetobacter orientalis*, *Gluconobacter oxydans*	n.d.	n.d.
	G	*Lacticaseibacillus paracasei*	n.d.	n.d.	Uncultured bacterium
KGIE	L	*Liquorilactobacillus nagelii*	*Acetobacter fabarum*, *Acetobacter indonesiensis*, *Acetobacter orientalis*, *Acetobacter tropicalis*	*Pichia membranifaciens*, *Zygotorulaspora florentina*	Uncultured bacterium, uncultured fungus, *Aureobasidium pullulans*, *Curtobacterium flaccumfaciens*, *Deinococcus xingiangensis*, *Paenibacillus humicus*
	G	*Liquorilactobacillus nagelii*	n.d.	n.d.	-
KOUK	L	*Liquorilactobacillus nagelii*	*Acetobacter cerevisiae*, *Acetobacter fabarum*, *Acetobacter papayae*, *Acetobacter persici*, *Acetobacter suratthaniensis*	*Issatchenkia orientalis*	Uncultured bacterium
	G	n.d.	n.d.	n.d.	n.d.

The results highlight the importance of a sufficient nitrogen source to support the fermentative capacity of the water kefir grain community. Additionally, different aspects can significantly influence the "sufficient nitrogen source" required for microbial growth, specifically: (i) the type of microorganism targeted LAB vs. AAB, (ii) the quality (bioavailability) of the nitrogen sources available in the growth medium (i.e., different kinds of fruit) and (iii) the type of microbial community in which the microorganism is expressing its metabolic performance (presence of mutualistic/symbiotic phenomena within the microbial community of the kefir grains).

Furthermore, while some cultures produced a different organic acid profile, fermentation of the same substrate led to similar organic acid production. The dominance of AAB in the isolates may have been related to the partially aerobic nature of the fermentations. Finally, some cultures maintained fermentative capacity for longer, which could have been associated with the presence of specific water kefir microorganisms.

4. Conclusions

This study aimed to investigate the influence of substrate composition on the fermentation kinetics and culture-dependent microbial composition of water kefir. The water kefir culture used was able to ferment each fruit medium; however, differences in the organic acid profiles and the relative proportions of the acids were observed. In particular, there were differences in the levels of acetic acid and gluconic acid produced between and within the fruit water kefir fermentations. Such changes were reflected in the dominant microorganisms that were isolated and likely reflected adaption of the grain microbiota to the fermentation substrate or conditions. Acetic acid bacteria of the genera *Acetobacter* and *Gluconobacter* were dominant isolates from all water kefirs in this study which correlated with the finding that acetic acid was the predominant organic acid in many of the fermentations. The aerobic nature under which the fermentations were performed likely influenced the dominance of AAB and associated acetic acid and gluconic acid levels. The influence of substrate composition and buffering capacity were evident when considering the fermentation characteristics and differences in the nitrogen-rich and nitrogen-poor media. It was observed that a switch to nitrogen-poor media appeared to favour AAB's dominance at the LAB's expense. While each fruit substrate supported the water kefir fermentation, differences in the ratio of the organic acid produced were observed. However, aside from the choice of fruit as raw material, the nitrogen content and fermentation environment clearly influenced the fermentation performance.

Further examination of the effect of nitrogen limitation on the water kefir fermentation using different water kefir cultures highlighted the marked impact of nitrogen limitation on the fermentations. Differences in the fermentation kinetics were evident after the switch from the nitrogen-rich to nitrogen-poor media, when the fermentative capacity of the cultures declined. However, the water kefir cultures maintained fermentative power for some time, even in a nitrogen-poor medium; this may have been due to the presence of a certain level of residual nitrogenous substances remaining within the grains, which supported continued growth and metabolism of the microorganisms. The maintenance of a relatively higher fermentative capacity for longer in the nitrogen-poor media may have also been linked to the presence and maintenance of common water kefir microorganisms within the grain environment and the fermentation. In conclusion, this study highlighted the potential for using different fruits as substrates for water kefir fermentation. The interrelationship between medium composition, fermentation conditions, microbial composition and resulting water kefir fermentation kinetics and products was demonstrated. In addition, the importance of a sufficient nitrogen supply in maintaining the water kefir fermentation was highlighted.

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