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Microbial and Chemical Changes during Fermentation of Coconut Water Kefir Beverage

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Abstract: This study investigated changes in the microbial growth and chemical characteristics of coconut water kefir (CWK) during fermentation. The carbohydrate profile, in terms of glucose and sucrose consumption, production of carboxylic acids, and changes in amino acid profile, was determined during CWK fermentation over a period of 96 h. The results showed that the kefir grains were able to utilise both glucose and sucrose and produce significant quantities of carboxylic acids after 96 h of fermentation. The total titratable acidity significantly increased throughout 96 h of incubation at 30 °C, which correlated to a significant drop in pH to 2.8 for CWK supplemented with 12 g/L of sucrose. In addition, this was accompanied by a significant increase in lactic acid, acetic acid, and pyruvic acid. During fermentation, a total of eighteen amino acids were generated, with a notable decline observed across all amino acids. Among them, glutamic acid exhibited a higher concentration compared to the other amino acids. The Scanning Electron Microscope (SEM) results confirmed a higher density of lactic acid bacteria (LAB) and acetic acid bacteria (AAB), with fewer yeast cells through morphological identification. Overall, the findings support the notion that coconut water fermented with kefir could be used as a potential functional starter to produce other fermented food products or a refreshing beverage.

Keywords: coconut water kefir; amino acids; carboxylic acid; D-/L-lactic acid; kefir; fermentation

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1. Introduction

Milk kefir and water kefir are traditionally made using different gelatinous particles that contain probiotics. These particles are called "milk kefir grains" and "water kefir grains", respectively [1]. Both types of kefirs can influence the composition and activity of gut microbiota [2]. However, people who are vegan, severely lactose intolerant, or allergic to dairy products cannot consume milk kefir. Hence, water kefir is fast becoming a cheaper and more popular alternative to developing milk kefir beverages. Water kefir can be made with fruit juices, coconut water, organic sugar water, or filtered water. Some of the widely predominant microorganisms in kefir are *Lactobacillus kefiri*, *L. kefiranofaciens*, *L. paracasei*, *L. acidophilus*, *L. delbrueckii*, and *L. plantarum*; Acetobacter is also an integral component of kefir grains [3–7]. A detailed list of all of the species identified in kefir and beverages fermented with kefir has been reported by [8]. Egea et al. [9] provided a comprehensive review of non-dairy kefir products that described their characteristics and potential health benefits.

Kefir grains contain a complex mixture of lactic acid bacteria (LAB), acetic acid bacteria (AAB), and various yeasts embedded in a polysaccharide matrix [10–12]. The main polysaccharide speific to milk kefir grain is known as "kefran", while water kefir grains contain a dextran matrix [1]. Milk kefir grains and water kefir grains have different microbial composition. It was reported that water kefir grains do not grow in milk, mainly because *L. hilgardii* does not assimilate or produce polysaccharides from lactose [13]. Milk kefir grains, on the other hand, can grow in sugar solutions and juices [14]. Kefir grains can grow on different substrates, such as sucrose, molasses, honey, fruit and vegetable juice, and water-soluble plant-based extracts, acting as a starter culture [15]. Kefir made from coconut water has shown a promising sensory profile in green coconut water kefir and sourdough bread [16]. Coconut water has grown rapidly in the functional beverages category as it is low in calories, rich in potassium, fat and cholesterol free, and has a good hydration quality [17]. Recently, a fermented beverage was developed using water kefir in a water-soluble coconut extract (WSCE) with inulin [18]. The WSCE obtained from the pulp (endosperm) of the coconut was an interesting option as it has a white colour like milk. The authors reported that the growth of the kefir grains was greater when the concentrations of inulin were close to 2.8 and 3.0% (*w/v*) and up to 0.26% (*w/v*) xanthan gum.

Monitoring the chemical changes during the kefir fermentation process is important to improve the characteristics of kefir products. Sabokba and Khodaiya [19] evaluated a mixture of pomegranate juice and whey as potential substrates to produce a novel probiotic beverage using kefir grains. They found that fermentation temperature and kefir grains inoculum influenced pH, acidity, lactose consumption, and organic acid formation over 32 h of fermentation. The growth of microorganisms in kefir results in enzymatic reactions involving invertase that can increase the content of reducing sugars, such as glucose and fructose [20]. Glycolytic enzymes can further act on these sugars to produce metabolites, such as ethanol, CO_2, and organic acids. Lactic, citric, malic, and succinic acids are also found in kefir beverages [20,21]. The production of these acids depends on both the type of microorganisms in kefir and substrate used in the fermentation. For example, the use of glucose as a substrate, compared to lactose and sucrose, was found to increase LAB counts and, consequently, the production of lactic acid in soymilk [22]. In general, the decrease in pH and resulting increase in acidity coincides with the fermentation progress; while, the microbial growth increases and soluble solids decrease [20].

There is little information available on the changes in free amino acids content in the growth medium after the cultivation of kefir starter cultures. Only Simova et al. [12] reported an increase in most free amino acids after fermentation with a kefir starter culture (*Lactococcus lactis*, *Lactobacillus helveticus*, *Streptococcus thermophilus*, and *Lactobacillus bulgaricus*) cultivated in pasteurized cow's milk from 2 to 168 h after inoculation. In another study, the major amino acids in a tomato seed protein isolate that included glutamic acid, aspartic acid, arginine, lysine, leucine, and phenylalanine increased after 24 h of fermentation using a water kefir mixture culture (Genesis Laboratories, USA) that comprised LAB (*Lactococcus lactis* sp. *lactis*, *L. lactis* sp. *lactis biovar diacetylactis*, *L. lactis* sp. *cremoris*, *Leuconostoc mesenteroides* sp. *cremoris*, and *Lactobacillus kefyr*), *Candida kefyr*, and *Saccharomyces unisporus* spp. [23]. The amino acids released by the bacteria will influence the nutritional properties and biological value of kefir-fermented products.

This study showcases the challenges in the development of fermented coconut water with kefir as a potential functional starter. Kefir is a fermented milk drink that contains a variety of bacteria and yeasts, which can potentially contribute to gut health and immune system function. It is important to note that, according to the ISSAP definition, kefir does not meet the criteria to be classified as a probiotic. The microbiota of kefir can readily adapt to different food substrates, which can be utilized to produce new beverages with a similar probiotic function. Water kefir production is influenced by the amount of inoculum, fermentation time, substrates, and temperature. Coconut water was selected as a substrate for kefir fermentation in this study due to its novelty and popularity as a natural and refreshing drink. Coconut water is also known to be rich in nutrients, including vitamins,

minerals, and antioxidants, making it a beneficial substrate for fermentation [17]. Hence, in this study, the effects of varying substrates (glucose and sucrose) using coconut water kefir product on chemical changes over 96 h of fermentation were investigated. The change in microbial content of kefir during fermentation was also determined to understand the changes in the growth of microorganisms with fermentation time.

2. Materials and Methods

2.1. Microbial Cultures

Commercial kefir starter (Body Ecology™, Auckland, New Zealand), which contained the following strains: *Lactococcus lactis*, *Lactococcus cremoris*, *Lactococcus diacetylactis*, *Leuconostoc cremoris*, *Lactobacillus plantarum*, *Lactobacillus casei*, and *Saccharomyces boulardii*, was used to ferment coconut water with and without the addition of different sugars (glucose and sucrose) in a LabServ incubator (Thermo Fisher Scientific, Auckland, New Zealand). The fermentation was monitored from 0 to 96 h of incubation at 30 °C.

2.2. Coconut Water

UFC coconut water (CW), manufactured by Universal Food Public Company Limited, Thailand and purchased at Countdown, Auckland, New Zealand, was used in this study. The coconut water container was opened in a laminar air hood to obtain the coconut water under aseptic conditions.

2.3. Carbohydrates

Glucose (Food-grade, Thermo Fisher Auckland, New Zealand) and sucrose (Chelsea Sugar Limited, Auckland, New Zealand) were used as carbohydrate sources in this study. Cevik and Aydogdu et al. [24] have reported that the addition of sugars serves as a carbon source in fruit juices and can increase kefir biomass. Autoclavable, lidded, plastic containers with 500 mL capacities (Thermo Fisher, Auckland, New Zealand) were used for carrying out the fermentation. The sugars were filter-sterilised by preparing a concentrated solution with autoclaved distilled water. The concentrated stock of sugar solution was sterilised using a sterile syringe and a sterile filter membrane with a 0.2 µM pore size (Thermo Fisher NZ). Membrane filtration was performed aseptically in a laminar air-flow hood. The sterile sugar solution was collected in a sterile bottle.

2.4. CWK Preparation

CWK was formulated using coconut water (300 mL) and kefir grains (1.5 g/L). The influence of sugars on CWK fermentation was studied by employing a full factorial design [25]. Sucrose and glucose were added at varying concentrations (0, 6 and 12 g/L) to determine their effects on fermentation, as shown in Table 1. The sucrose level selected in this study was predetermined from a separate pilot study that was carried out where low sugar concentration (1.5 g/L) did not result in significant microbial growth, while high sugar concentrations (13.5 g/L) could inhibit the growth of the kefir biomass. The different formulations were incubated aerobically at 30 °C for a period of 96 h in a LabServ incubator (Thermo Fisher Scientific, New Zealand). Samples were taken aseptically every 24 h for monitoring pH and the cell counts (CFU/mL) for LAB and yeasts, as well as determining the residual sugars, carboxylic acids, and amino acids. This study selected glucose and sucrose to be used in the development of a CWK beverage as both sugars are suitable for fermentation and have positive impacts in terms of sensory quality, economic viability, and clean labelling.

2.5. Total Viable Counts

The method described by Boczek, Rice, and Johnson [26] was used to determine the total viable counts, and the results were expressed as colony-forming units per mL (CFU/mL) for each sample. Samples were taken from each of the nine experimental sets at a uniform time interval of 24 h over 96 h (0, 24, 48, 72, and 96 h). LAB, yeasts, and

AAB were grown on De Man, Rogosa, and Sharpe (MRS); Malt Extract (ME); and acetic acid bacteria (AAB) agar medium, respectively. All the media were supplied by DIFCO (Fort Richard, New Zealand, Auckland) and prepared according to the manufacturer's instructions. The media were autoclaved at 121 °C for 15 min at 15 psi.

Table 1. The composition of coconut water kefir media used in this study.

Experimental Sets	Coconut Water	Kefir Grains (g/L)	Glucose (g/L)	Sucrose (g/L)
Set 1	300	1.5	0	0
Set 2	300	1.5	0	6
Set 3	300	1.5	0	12
Set 4	300	1.5	6	0
Set 5	300	1.5	6	6
Set 6	300	1.5	6	12
Set 7	300	1.5	12	0
Set 8	300	1.5	12	6
Set 9	300	1.5	12	12

A spread plate technique was used for spreading the culture uniformly across the circular agar surface with a sterile glass spreader (Sanders, 2012). Ten-fold dilutions in peptone water were prepared for all samples from each set. A 100 µL aliquot of sample from each dilution was spread on the different agar media. All the MRS, ME, and AAB agar spread plates were incubated at 30 °C in a 5% CO_2 incubator (LabServ incubator, Thermo Fisher Scientific, New Zealand). The colonies were counted, after which, the colony forming units (CFU/mL) were calculated where CFU/mL = (no. of colonies × dilution factor)/volume of sample plated.

2.6. pH, Total Titratable Acidity (TTA) and D-/L-Lactic Acid

The pH was determined using a pH meter (Eutech pH 700 m, Thermo Fisher Scientific Inc., New Zealand) with a glass electrode (Electrode ECFC7252101B, Thermo Fisher Inc., New Zealand). The pH meter electrodes were calibrated against known buffer solutions. The readings were taken when the pH meter was stabilised. Total titratable acidity was determined using the AACC International Method 02-31.01 (AACC, 1999), where 10 mL of CWK sample was titrated against 0.1 M NaOH (pH 8.5). The concentrations of D- and L-lactic acids were determined using the Megazyme Lactic Acid Kit K-DLATE01/14 (Food Tech Solutions Limited, Auckland, New Zealand) following the manufacturer's instructions.

2.7. Residual Sugars

The concentration of total reducing sugars was determined using the 3,5-dinitrosalicylic acid method [27]. The concentration of sucrose was estimated using a modified Anthrone method [28], which is a colorimetric method involving the ethanol-based extraction of sucrose. Reducing sugar (glucose) concentration in the experimental sets of CWK during fermentation, between 0 and 96 h, was determined using the 3,5-di-nitro salicylic acid method (DNS); whereas, the non-reducing sugar (sucrose) concentration was determined using a modified anthrone method. The CWK sets were centrifuged for 5 min at 3000× *g*. A volume of 3 mL of supernatant was added to the reagents to carry out the titration for reducing sugar estimation. In total, 2 mL of the supernatant was added to the reagents to estimate sucrose content. Coconut water was used as a blank for estimating the glucose and sucrose concentrations. All the experiments were completed in triplicates. Error bars for the graphs represent the standard deviations in the data. Absorbance was determined at 570 nm using a spectrophotometer (Ultrospec 2100 pro UV/visible spectrophotometer). A calibration curve was created using standard solutions of glucose and fructose.

2.8. Scanning Electron Microscopy of CWK Granules

Kefir granules extracted after 72 h of incubation in coconut water at 30 °C were filtered using a biological membrane with a pore size of 0.20 µm and thickness of 150 µm. The granules were then freeze-dried at −80 °C using a freeze drier (ALPHA 2-4 LDplus, Martin Christ, Osterode am Harz, Germany). The freeze-dried CWK samples were cut transversely into sizes of about 1 × 1 × 0.5 cm (length × breadth × height) using a sharp razor blade. This section of the sample was mounted onto a stainless-steel metal using a double-sided carbon-conductive adhesive tape. A platinum coating was applied under vacuum conditions using the Hitachi E-1045 ion sputter coater for better resolution and to increase ion conductivity of the LAB and yeast due to their small sizes. This helped increase electron emissions, which helped capture a stable micrograph of the granules. A field emission scanning electron micrograph apparatus (SU-70 Schottky, Hitachi, Tokyo, Japan) at an accelerating voltage of 5 kV SEM was used to view the microscopic structure of CWK granules.

2.9. Determination of Carboxylic Acids Liquid Chromatography-Mass Spectrometry (LC-MS)

One millilitre aliquot of the CWK sample was collected from each treatment, as shown in Table 1, at 0, 24, 48, 72, and 96 h, and added to ice-cold 50% MeOH containing 10 mg/L 4-chlorobenzoic acid (CBA) as an internal standard. The mixture was centrifuged at 10,000× *g* for 5 min at 4 °C (Z216MK, HERMLE Labortechnik GmbH, Wehingen, Germany). The 2-hydrazinoquinoline (HQ) derivatization reaction was conducted by mixing 5 µL of the sample with 100 µL of acetonitrile solution containing 1 mM 2,2′-dipyridyl dipyridyl disulfide (DPDS), 1 mM triphenylphosphine (TPP), and 1 mM HQ. The reaction mixture was incubated at 60 °C for 60 min. The HQ derivatives in the reaction mixture were stored in a −80 °C freezer (ULT freezer, Haier Biomedical ULT freezers, Auckland, New Zealand) until further analysis using LC-MS. The carboxylic acids standards used for this assay were lactic acid, succinic acid, acetic acid, glutamic acid, pyruvic acid, and malic acid prepared with 50% methanol and 20 mM sodium hydroxide.

The LC-MS was an Agilent 1260 Series liquid chromatograph that comprised a G1311B quaternary pump, G1329B thermostatted autosampler, and a G1330B thermostatted column compartment (Agilent Technologies, Santa Clara, CA, USA). Mobile phase A involved 0.1% formic acid in ultrapure water and mobile phase B involved 0.1% acetic acid in acetonitrile. The column was a Phenomenex Kinetex EVO C18 column, measuring 2.1 × 150 mm with a 1.7 µm diameter of packing material, and was maintained at 25 °C. The chromatographic gradient was held for 0.1 min at 3% B and then ramped to 6 min at 15% B; it was then increased to 9 min at 90% B before decreasing to 3% B at 11 min. The flow rate was 200 µL min^{-1}, total run time was 16 min, and injection volume was 5 µL. For detection, an Agilent 6420 triple quadrupole mass spectrometer fitted with an Agilent Multimode Ionisation source was operated in positive electrospray mode. The peak areas of derivatized carboxylic acids were normalized and quantified using a series of diluted external standards. The external standards, which included succinic acid, lactic acid, acetic acid, malic acid, and pyruvic acid, were prepared for this purpose. Data were collected and processed using Agilent Mass Hunter Version B06.00 software.

2.10. Determination of Amino Acids Using LC-MS

An aliquot of 50% methanol containing 10 mg/L of 2,3,3,3-d4-alanine [d4A] (1.4 mL) was added to 1 mL of the CWK sample in a microcentrifuge tube. The tubes were then vortexed to obtain a homogenous mixture. This was followed by centrifugation at 10,000× *g* for 5 min at 4 °C (Z216MK, HERMLE Labortechnik GmbH, Germany). A 10 µL volume of extract was used to perform pre-column derivatisation with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, following a method adapted from Salazar et al. (2012).

The LC-MS was an Agilent 1260 Series liquid chromatograph comprising a G1311B quaternary pump, G1329B thermostatted autosampler, and a G1330B thermostatted column compartment (Agilent Technologies, Santa Clara, CA, USA). The mobile phase A consisted

of 0.6% formic acid in ultrapure water, while the mobile phase B consisted of 0.1% formic acid in acetonitrile. The column used was a Phenomenex Kinetex EVO C18 column, measuring 2.1 × 150 mm with 1.7 µm diameter of packing material that was maintained at 25 °C. The chromatographic gradient was held for 1 min at 1.5% B and was then ramped to 13% B at 8 min, 17% B at 15 min, and 80% B at 16 min before returning to 1.5% B at 17.5 min. The flow rate was 300 µL min^{-1}, total run time was 28 min, and injection volume was 5 µL. For detection, an Agilent 6420 triple quadrupole mass spectrometer fitted with an Agilent Multimode Ionisation source was operated in positive electrospray mode. Optimum Multiple Reaction Monitoring [MRM] transitions were established using the Agilent Mass Hunter Optimiser Version B06.00 software. The peak areas of derivatized amino acids were normalized to the recovery of d4A and quantified using a dilution series of external standards. These standards were prepared from a commercially available amino acid mix (Sigma product A9906, Sigma-Aldrich Pty. Ltd., Sydney, Australia). Data were collected and processed using the Agilent Mass Hunter Version B06.00 software.

2.11. Statistical Analysis

At least five individual replicates of each experimental treatment shown in Table 1 were analysed. All the results were expressed as means of values and the ± standard deviation for five replicates per sample. One-way and two-way analyses of the variance (ANOVA) were applied to determine the level of significance; when the ANOVA was significant. The means were separated by pairwise comparison using the Tukey's (HSD) test at a 5% significance level ($p < 0.05$). All statistical analyses were performed using XLSTAT-MX version 2012.4.02 (Addinsoft, New York, NY, USA). For analysing the amino acid data for the CWK, web-based MetaboAnalyst version 3.0 was used (Chong et al., 2018). All the data were normalized by log-transforming and mean centering.

Principal Components Analysis (PCA) was used to identify the effect of time on the changes in the amino acid profile. ANOVA Simultaneous Component Analysis (ASCA) was performed to identify major patterns in terms of glucose and sucrose concentrations and their interaction [29]. Due to the vast number of data generated from the 9 experimental sets with 5 replicates each, the interpretation of data would have been exceedingly difficult. Therefore, to visualise trends in the amino acids data, PCA was used to view the dominant patterns in a data set.

3. Results and Discussion

3.1. Total Viable Counts (TVC)

3.1.1. LAB

The total viable counts (TVC) of LAB during fermentation are shown in Table S1. The LAB viable count increased over 96 h of incubation. The F-values obtained for the variables affecting LAB showed that the length of incubation time had the most significant effect (357.99, $p < 0.01$). This is a known fact as with any cell growth there would be an increase in growth as time increased until the stationary phase is reached. The TVC of LAB increased from 3.34 log CFU/mL to above 5.1 log CFU/mL for all CW samples. However, the TVC of LAB produced in all experimental sets (approximately 5 log CFU/mL) are low compared to LAB fermentation using other substrates, such as fructose and lactose, as reported in other studies [30–32]. LAB are known to be fastidious microorganisms which require growth factors, such as vitamins and amino acids, and can also be affected by nitrogen concentration [33–37]. A recent study further showed that sugary water substrates produced less LAB compared to milk-based substrates [14].

In general, the sucrose and glucose addition increased the TVC of LAB after 96 h of incubation. Coconut water without any sugar additions showed the least TVC of LAB (5.02 log CFU/mL, $p < 0.05$). This contrasts with the CWK sets containing glucose and sucrose, which increased the growth of LAB. The CWK supplemented with sucrose at 12 g/L (Set 3) produced the highest LAB TVC, 5.72 ± 0.04 log CFU/mL ($p < 0.05$) after 96 h. The second-highest LAB was obtained from Set 9 (5.64 ± 0.02 log CFU/mL, $p < 0.05$),

which contained CW with added glucose (12 g/L) and sucrose (12 g/L) in the ratio of 1:1. The difference in TVC between Sets 3 and 9 was less than one order of magnitude, despite the additional 12 g/L glucose in the Set 9 sample. This suggests that there might be some sugar (substrate) saturation of the enzymes involved in the metabolism of sugar [31,38]. In addition to the substrate saturation effect, the results also suggest that there might be catabolite repression occurring between the glucose and sucrose. The acidic products of fermentation could also inhibit the growth of LAB [39], as supported by a 2D score plot that showed an increase in organic acids during CWK fermentation with increasing time.

3.1.2. Yeast

The changes in concentration of yeast during fermentation are summarised in Table S2. Fermentation time had the most significant effect ($F = 6728.24$, $p < 0.001$) on the TVC of yeast similar to LAB growth. The growth of yeast was, however, much less, with around a 1 log increase after 96 h of incubation, compared to the 2 log increase in LAB during fermentation. Sucrose concentration also showed a significant impact ($F = 961$, $p < 0.001$) on the growth of yeast cells. Sets 3 and 9, which both contained 12 g of sucrose, showed the highest yeast TVC similar to LAB cell growth. Laureys and De Vuyst [40] also reported the growth of yeast cells in water kefir (fermented sugar solutions) supplemented with sucrose.

3.1.3. Acetic Acid Bacteria

The growth of AAB was observed only after incubation for 48 h on Acetic Acid Bacteria Agar (Table S3). Acetic acid bacteria are known to grow better in the presence of lactic acid produced during fermentation [13,41]. The highest growth of AAB was observed in Set 3 (supplemented with sucrose at 12 g/L) and Set 9 (supplemented with both glucose and sucrose at 12 g/L each). There was a significant increase in the population of AAB during the later stages of fermentation, particularly in the presence of sucrose.

3.2. Determination of Residual Glucose and Sucrose, pH and Total Titratable Acidity

As shown in Figure 1A,B, the concentrations of residual glucose and sucrose were determined at regular time intervals during fermentation. A significant increase in glucose concentration ($p < 0.05$) was observed after 24 h for all experimental sets (Figure 1A). This could be due to the metabolism of sucrose by yeast, yielding glucose and fructose.

In the absence of sucrose addition, glucose utilisation is maximized. Set 7 (12 g/L of glucose) and Set 4 (6 g/L of glucose) demonstrated high glucose utilisation values of 70% and 74%, respectively. Set 2 (supplemented with 6 g/L of sucrose) and Set 8 (supplemented with 12 g/L of glucose and 6 g/L of sucrose) showed high sucrose utilisation values of 79% and 81%, respectively. In general, sucrose was utilised more than glucose. The utilisation of sugars is reflected by an increase in the TVC of LAB, yeast, and AAB (Table S1, Table S2 and Table S3, respectively). The utilisation of sucrose by water kefir microorganisms has been reported by Laureys and De Vuyst [40]. Sucrose can be hydrolysed to D-glucose and D-fructose by dextransucrase (invertase) secreted by microorganisms, such as LAB and yeast. D-fructose is utilized as an energy and carbon source, whereas D-glucose is used for the biosynthesis of dextran via polymerization [42,43].

During fermentation, glucose and sucrose were metabolised to produce acids, which was supported by a drop in pH (Figure 1C and Table S6), and an increase in the overall acidity of the medium. This total titratable acidity (TTA) (Figure 1D and Table S7) increased significantly over time ($p < 0.05$). Set 3 (supplemented with 12 g/L of sucrose) showed the highest amount of TTA (13.65 ± 0.04, $p < 0.05$) at 96 h compared to the other sets. A significant increase in TTA (up to 1% at the end of fermentation) was previously reported for milk kefir grains by Chen et al. [44]. This was accompanied by a decrease in pH and an increase in lactic acid and other organic acids. The transformation of sugars to lactic acid and other organic acids by LAB is an important step during fermentation that influences the pH and acidity of fermented products, such as kimchi [45,46]. Studies have also been

carried out on milk kefir with or without supplementing soy [47]. pH in this study was found to steadily decrease during fermentation for up to 21 days. In a separate study on milk kefir by Chen et al. [44], fresh milk was provided daily for 28 cycles and the pH was observed to gradually decrease to 3.4 during fermentation.

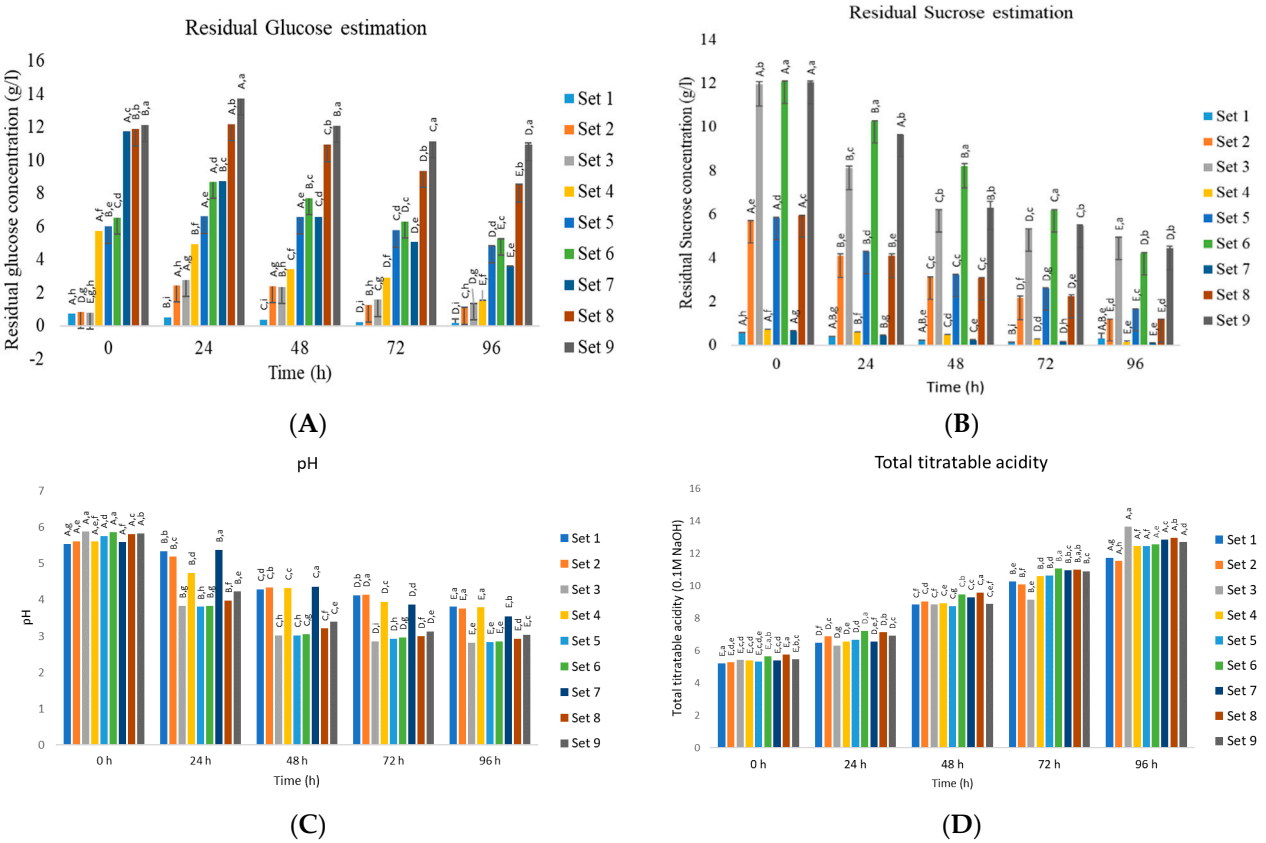


Figure 1. The estimation of residual glucose (**A**), residual sucrose (**B**), pH (**C**), and TTA (**D**) for the different treatments of CWK fermented at 30 °C with 5% CO_2 concentration. All of the values for each experimental set represent the mean of triplicate readings. A, B, C, D, E = Different letters within the same row (different fermentation times—0 h, 24 h, 48 h, 72 h and 96 h for the same set treatment) differ significantly using Fisher's least significant difference ($p < 0.05$). a, b, c, d, e, f, g, h—Different letters within the same column (different treatments for the same time interval) differ significantly using Fisher's least significant difference ($p < 0.05$).

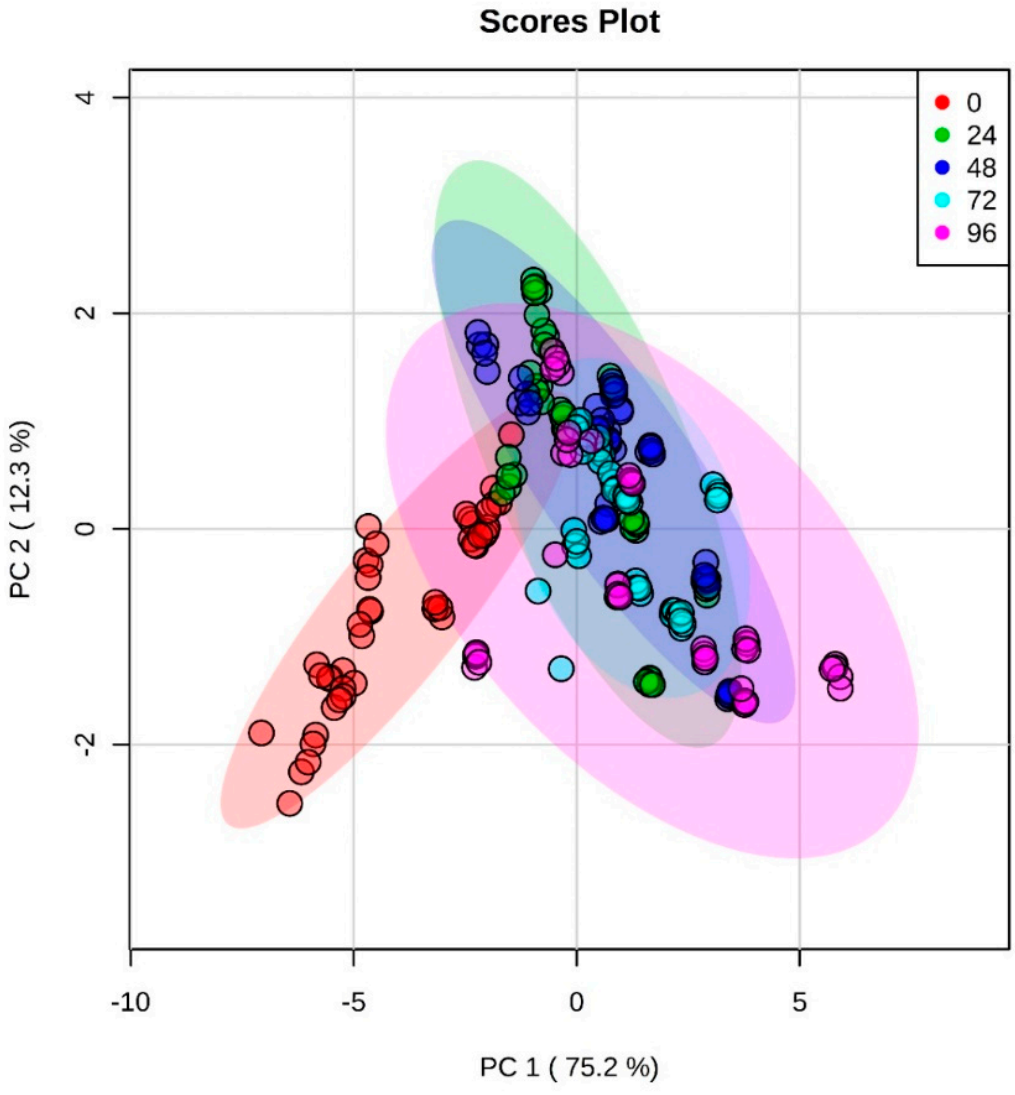
In the present study using CWK grains, sucrose was utilized more efficiently compared to glucose during fermentation. To date, no studies have compared the efficiency of sucrose and glucose utilisation during CWK fermentation. Reid and Abratt [48] reported that the efficiency of sucrose uptake by LAB and yeast depends on the genetic architecture of microorganisms. The changes in pH values in this study are similar to fermentation studies carried out on milk, where a steady drop in pH was reported after 96 h without any replenishment of the medium during fermentation. According to Odet [49], the pH of milk kefir made using cow's milk with 5% kefir grains (fermented for 24 h between 18–24 °C) varied between 4.2 and 4.6. This was attributed to the production of acid and metabolites by the kefir microorganisms, which supports the results obtained in this study. Lower pH values were also observed by Garrote et al. [50], who attributed this to the metabolic activity of kefir grains.

3.3. Carboxylic Acids

Organic acid analysis was carried out for kefir-fermented coconut water to determine changes in the concentration of malic acid, lactic acid, acetic acid, pyruvic acid, and succinic

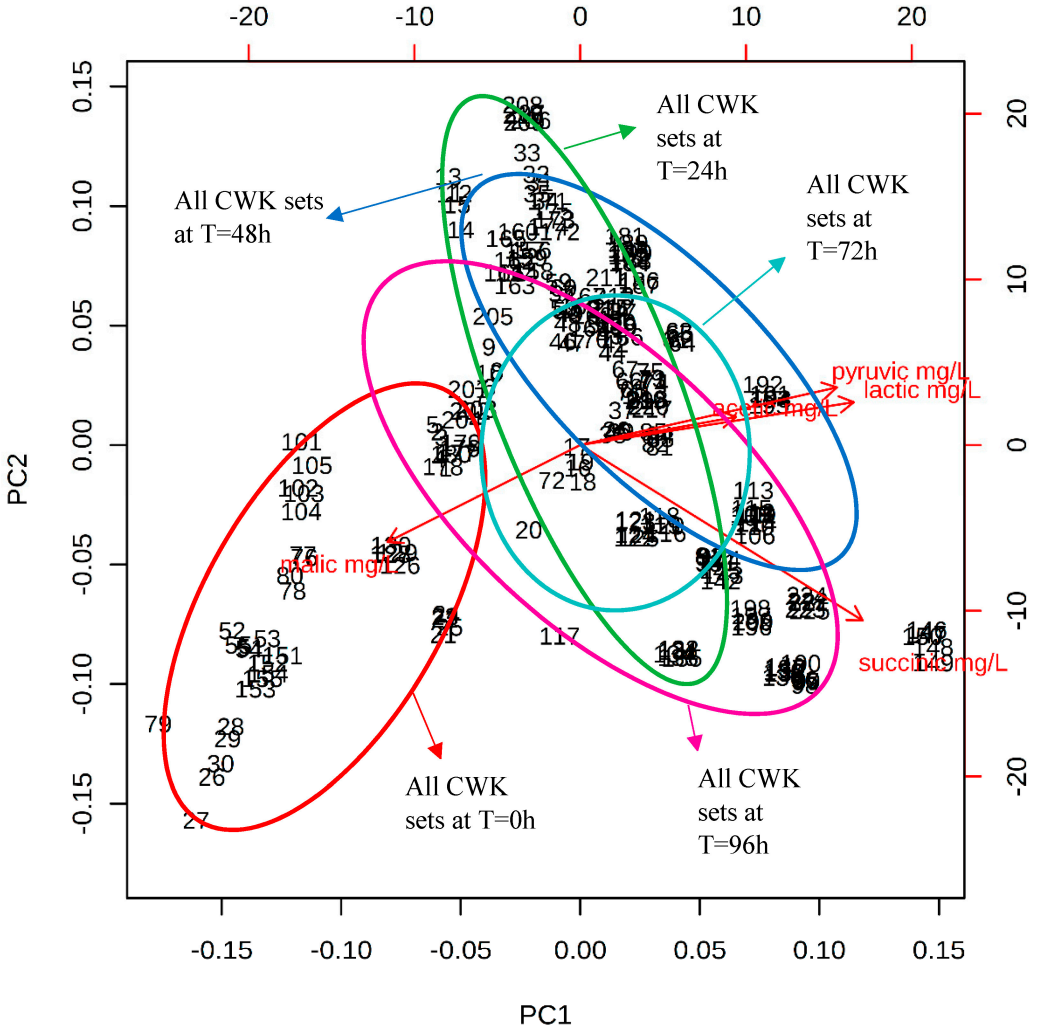
acid. Principal component analysis (PCA) was carried out to assess the variation in these acids for all experimental sets containing different concentrations of glucose and sucrose during the fermentation that took place over 96 h. Samples were taken at 0, 24, 48, 72, and 96 h.

The PCA- 2D score plots (Figure 2A) distinguished the CWK sets after longer fermentation times (24 h to 96 h) that were associated with higher concentrations of carboxylic acids from the non-fermented sample (0 h). PC1 and PC2 accounted for 75.2% and 12.3% of the total variation, respectively. The scores for the different kefir fermented coconut water were clearly separated according to fermentation time (T = 0, 24, 48, 72 and 96 h) indicating distinct changes in acids during fermentation. Samples at time T = 0 had negative scores along PC1. With increasing fermentation time (T = 24, 48, 72, and 96 h), sample scores became increasingly positive.



(**A**)

Figure 2. *Cont.*



(**B**)

Figure 2. (**A**) PCA score plots of PC1 and PC2 for the carboxylic acids present in the different sets of CWK with different fermentation times (T = 0, 24, 48, 72 and 96 h). The samples are colour-coded as below: Legends-●: T= 0 h; ●: T= 24 h; ●: T = 48 h; ●: T = 72 h; ●: T= 96 h. (**B**) Principal components analysis (PCA) biplot showing PC scores for the experimental sets fermented at different times (T = 0 in red, 24 in green, 48 in blue, 72 in cyan, and 96 h in purple) that are presented as numbers and loadings of carboxylic acids in the CWK sets. The product loadings (1–150) are representative of samples and replicates. PC1 and PC2 explained 75.2% and 12.3% variances in the data, respectively.

The PCA biplot shown in Figure 2B shows the scores for all the CWK sets fermented at 0, 24, 48, 72, and 96 h. The control, (T = 0 h) had high negative scores that corresponded to high negative loadings for malic acid. This finding was supported by ANOVA results shown in Supplementary Table S1-E. The malic acid concentration was significantly high for Set 3, while Set 9 had significantly low levels of malic acid ($p < 0.05$). The fact that malic acid was only found at the start time of fermentation (T = 0 h) indicated that malolactic fermentation occurred during CWK fermentation. Malic acid can be converted to lactic acid [51,52] during fermentation. With increasing fermentation time, most of the sets fermented for 24, 48, and 72 h had significantly high positive scores, which corresponded to the high positive loadings of lactic acid, pyruvic acid, and succinic acid. This finding was supported by the ANOVA results shown in Supplementary Table S1-A,C,D, which showed significantly higher concentrations of lactic acid, pyruvic acid, and succinic acid in fermented samples with increasing fermentation time. The successful conversion of malic acid to lactic acid

using *L. casei* and *Lactococcus oenos*, known as malolactic fermentation, has been reported in wine [53,54]. Lactic acid has also been produced in fermented food and beverages, such as kimchi and wine, which involve both lactic acid bacteria and yeast [55–57]. Lactic acid was identified as the primary non-volatile organic acid that increased gradually throughout fermentation. It has been observed that kefir microorganisms were able to increase the lactic acid concentration along with other acids present in the fermentation medium ($p < 0.05$).

Pyruvic acid was generated in significant amounts due to the presence of various strains of lactic acid bacteria during CWK fermentation. Pyruvate is a critical intermediate in the homo- and hetero-fermentative pathways and can be reduced to lactic acid. Different strains of lactic acid bacteria break down pyruvate differently. *L. brevis* degrades pyruvate to lactate and acetate (thus increasing both lactic acid and acetic acid concentrations in the medium [58]. Along PC1, CWK samples fermented for 96 h had the highest positive scores that corresponded to the high positive loading of succinic acid (Figure 2B). This observation was supported by the ANOVA results, where succinic acid was significantly the highest in 96 h fermented samples ($p < 0.05$) (Supplementary Table S1-B). Weckx et al. [59] reported a correlation between the presence of *L. plantarum* in the fermentation medium with succinic acid production.

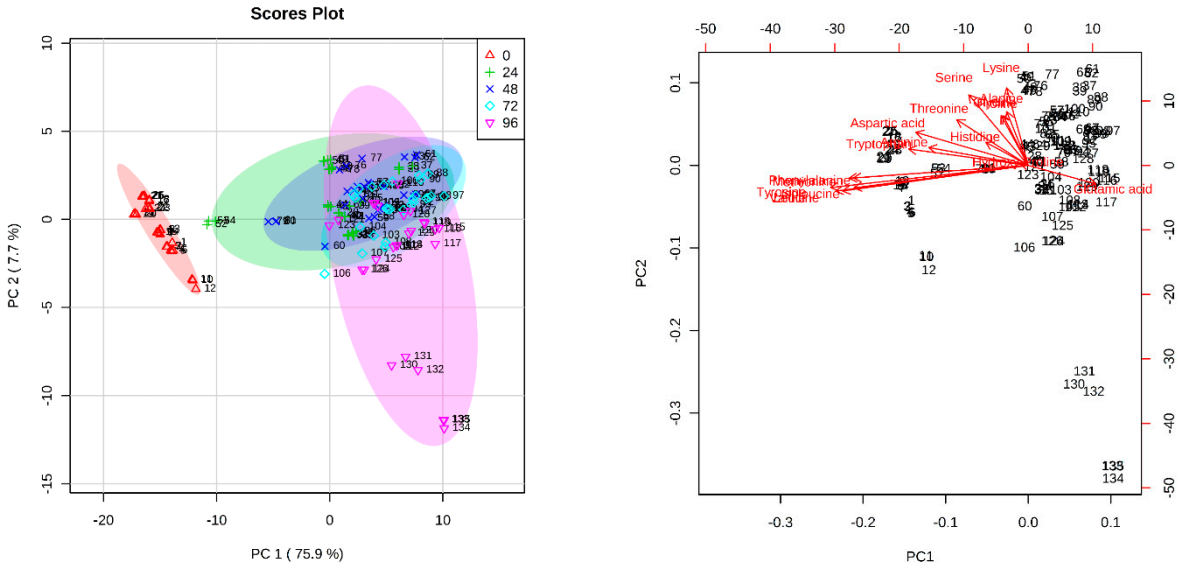
Fermented CWK Sets 4, 5, 6, 8, and 9 that contained added sucrose had high lactic acid concentrations. This was consistent with their rapid growth when consuming sucrose during the early stage of fermentation. Sucrose was reported to be the preferred substrate for lactic acid bacteria during sauerkraut fermentation in studies conducted by Cho et al. [60] and Xiong et al. [61], with better utilization observed compared to fructose or glucose. Siragusa et al. [62] also found that sucrose was gradually degraded into glucose and fructose for lactic acid production.

3.4. Amino Acid Composition

Amino acid analysis was carried out using LCMS to determine changes in amino acid composition in the kefir-fermented coconut water. All the nine experimental sets of CWK (Table 1) were analysed at 0, 24, 48, 72, and 96 h. The PCA-2D score plot shown in Figure 3A described 75.9% and 7.7% of the total variances for PC1 and PC2, respectively. The scores for the different kefir-fermented coconut waters were clearly separated according to fermentation time (T = 0, 24, 48, 72, and 96 h). Samples at time T = 0 had negative scores along PC1. With increasing fermentation time (T= 24, 48, 72 and 96 h), the sample scores became increasingly positive. The PCA biplot in Figure 3B, generated for all the 18 identified amino acids showed both the scores and the loadings of CWK fermented at 0, 24, 48, 72, and 96 h. The 0 h (control) samples had high negative scores that corresponded to high negative loadings for tyrosine, valine, leucine, isoleucine, methionine, and phenylalanine. This finding was supported by the ANOVA results shown in Tables S7 and S8, which showed significantly higher values for tyrosine, valine, leucine, isoleucine, methionine, and phenylalanine in 0 h experimental sets.

With increasing fermentation time, samples fermented for 24, 48, and 72 h had high positive scores, which corresponded to the high positive loadings of glutamic acid. All the samples fermented for 24, 48, 72, and 96 h had positive scores, which corresponded to high positive loadings of glutamic acid. These observations are supported by results summarised in the ANOVA table shown in Tables S7 and S8, which showed significantly higher values for glutamic acid, particularly in samples fermented for 24, 48, and 72 h. The presence of glutamic acid in these fermentations is desirable in the current study as this amino acid could potentially enhance the flavour profile of CWK beverage. The 95% confidence ellipse can be seen in Figure 3A. The ellipses clearly define statistically significant class separation. The ellipsoid for 96 h samples was wide vertically, with a variance that can be explained along PC2. The ellipses for samples fermented for T = 48 and 72 h overlapped tightly and corresponded to high positive glutamic acid loading along PC1, as seen in the PCA-biplot in Figure 3B. Glutamate dehydrogenase activity in LAB, which leads to the production of

glutamic acid, is a very important criterion for selecting the strains utilised for producing fermented foods, such as cheese. The production of glutamate from α-ketoglutarate by LAB produces flavour compounds which are associated with the formation of desirable aromas in fermented foods [63]. Glutamate, the anionic form of glutamic acid, can be further converted into glutamine or gamma-aminobutyric acid (GABA). GABA possesses several well-known physiological functions, such as anti-hypertension [64] and anti-diabetic [65]. Studies carried out on sourdough show that glutamine deamidation by LAB during wheat sourdough fermentation could account for the increase in glutamate levels that could positively impact the flavour of bread [66]. An increased production of glutamic acid with incubation time has also been reported for the fermentation of kefir grains in cow and buffalo milk [47].



(**A**) (**B**)

Figure 3. PCA plots as shown in figures (**A**,**B**). (**A**): The PCA 2D score plot derived for 18 amino acids present in kefir fermented coconut water sets at times, T = 0, 24, 48, 72, and 96 h. The score plot shows a clear differentiation between samples with fermentation time. (**B**): PCA biplot of amino acids for all the kefir coconut water sets fermented for 0, 24, 48, 72, and 96 h. The correlation loadings refer to the amino acids present in the samples. PC1 and PC2 explained 75.2% and 12.3% variances in the data, respectively. Experimental sets numbered refer to different experimental sets: Numbers 1–15: refer to Set 1, numbers 16–30: refer to Set 2, numbers 31–45: refer to Set 3, numbers 46–60: refer to Set 4, numbers 61–75: refer to Set 5, numbers 76–90: refer to Set 6, numbers 91–105: refer to Set 7, numbers 106–120: refer to Set 8, and numbers 121–135: refer to Set 9.

Changes in the amino acid profile can be related to the differences in the number of microorganisms that thrive in the different sugar mediums during CWK fermentation. A similar study was carried out on the water kefir microflora by Neve and Heller in 2002 [67], who reported that yeasts in kefir are responsible for the breakdown of sucrose into glucose and fructose units. Gronnevik et al. [39] further reported that the presence of amino acids in milk kefir may be due to the combined effects of the release of amino acids during fermentation, assimilation of peptides, and proteolytic activity.

3.5. Visualisation of CWK Grains Using Scanning Electron Microscopy

Samples of CWK was frozen at −80 °C and then freeze-dried using Alpha 2-4 LD plus. These freeze-dried samples were then coated with platinum using the Hitachi E-1045 ion sputter coater and observed using the Hitachi TM3030 plus SEM, 15 KV, secondary electrons (SE), and back-scatter detector (BSE). Figure 4 shows the SEM images of CWK.

The frozen CWK samples and dehydrated CWK showed a smooth surface. A thin cross-section obtained from both of the samples appeared to have a rough surface when observed under SEM. A magnification of up to 45× produced a good image of the kefir grain surface (Figure 4C). Under higher magnification, coccus-shaped and bacillus-shaped bacteria were visible (Figure 4A,B). These were most likely the LAB. The SEM results could not distinguish between the lactobacilli and the acetic acid bacteria. In the present study, the CWK cross-section showed a relatively higher density of bacterial cells than those of yeast cells. Previous studies carried out on the structure of milk kefir supported the SEM results observed in this study; that is, a greater proportion of lactic acid bacteria were found to be present compared to yeast [10,68,69]. A study carried out by Rea et al. [69] and Neve and Heller [67] reported that lactococci were the dominant bacteria on the kefir surface. They also reported that cocci were dominantly present in the water kefir samples, along with some short and long rods [67,69].

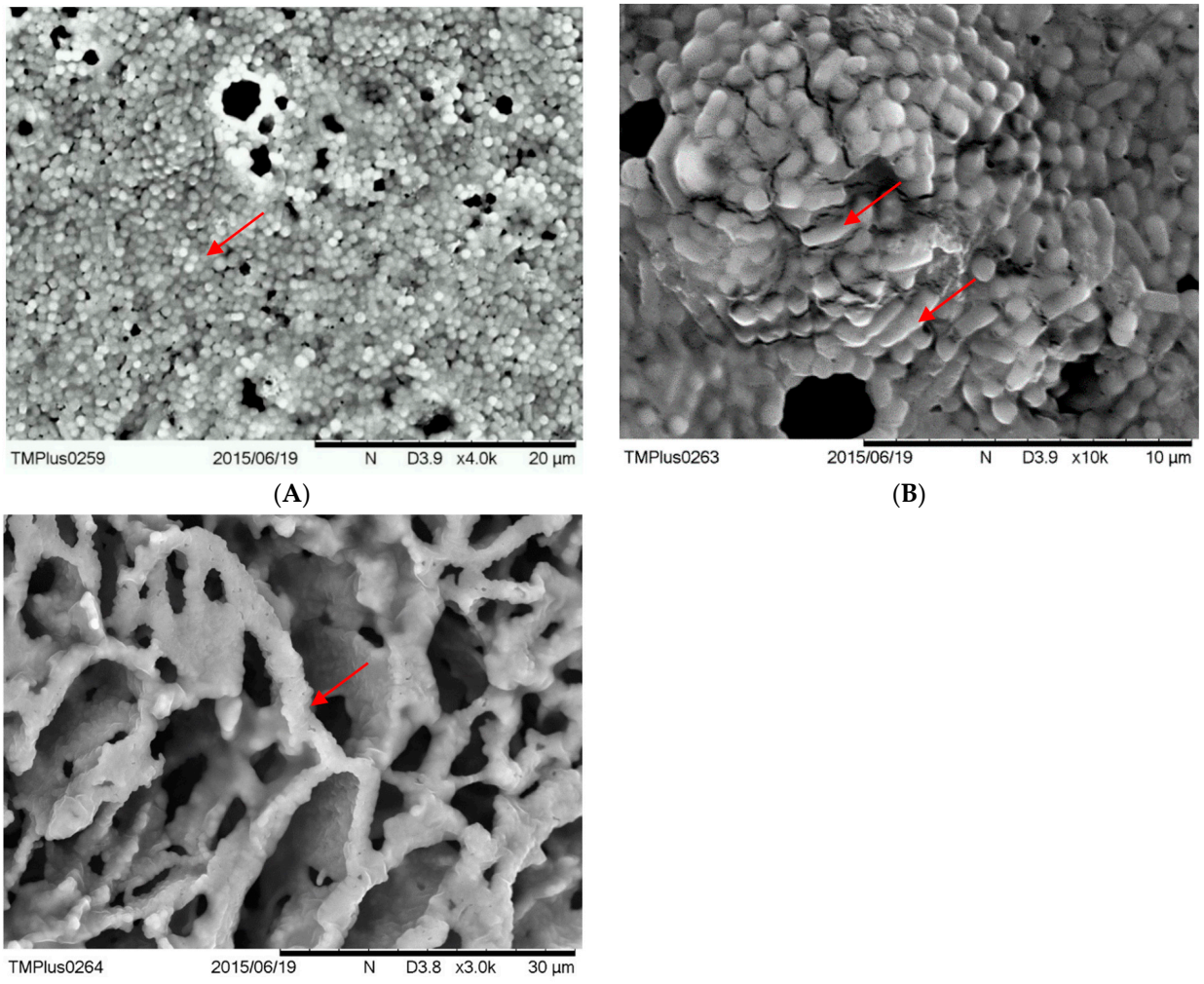


Figure 4. The scanning electron micrographs of CWK grains. (**A**): The surface appearance of CWK microflora (magnification of up to ×4000. The red arrow indicates the presence of coccus-shaped lactic acid bacteria, as shown in (**B**): The surface appearance of the cross-section of the CWK microflora (magnification of up to ×10,000). The red arrows indicate the presence of rod-shaped lactic acid bacteria; (**C**): SEM of kefir network, in CWK, on the polysaccharide scaffold (magnification of up to ×3000). The red arrow indicates a polysaccharide matrix with symbiotic yeast and lactic acid bacteria (kefiran complex).

4. Conclusions

This paper contributes to the understanding of desirable microorganisms in kefir grains and processing conditions, which can be used to improve the quality of coconut water kefir beverages. Results showed that the different sugar types and concentrations influenced the microbial and chemical characteristics of CWK. In fermented CWK, sucrose was identified as the preferred substrate for LAB growth, surpassing glucose. Samples fermented for 48 h using only sucrose exhibited desirable pH levels and the highest production of glutamic acid during fermentation. Scanning electron microscopy of the CWK granules revealed that bacillus- and coccus-shaped microorganisms (most probably LAB) were present at higher densities than yeast. To develop value-added non-dairy fruit-based coconut kefir beverages with desirable functional properties, it is recommended that further research be conducted to determine the bioactive precursor, gamma-aminobutyric acid, in fermented CWK. Additionally, further microbial studies are needed to explore the interactions between microorganisms (LAB-yeast, LAB-AAB, and AAB-yeast) to provide insight into metabolic cross-feeding. However, caution should be exercised when characterizing kefir as a probiotic, as the specific probiotic properties of its numerous microorganisms have not been fully determined in the present study. Additional research is required to investigate the specific strains and their probiotic potential, and to reassess the classification of kefir as a probiotic. Furthermore, optimization of the fermentation conditions, including evaluation of oxygen levels and nutrient composition with a focus on nitrogen sources to assess nutrient accessibility for fermentation is necessary to ensure robust bacterial growth. Future studies should also evaluate the potential of non-thermal technologies [70,71], bioavailability and overall health impact of the CWK fermented drink in a healthy population and conduct sensory [72] and consumer testing [73] to understand its market potential.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13127257/s1, Table S1: The cell concentration of LAB (log CFU/mL) isolated from CWK fermented at 30 °C with 5% CO_2 on MRS agar. Table S2: The cell concentration of yeast (log CFU/mL) isolated from CWK fermented at 27 ± 3 °C on ME agar. Table S3: The cell concentration of AAB (log CFU/mL) isolated from CWK fermented at 30 °C on AAA agar. Table S4: The estimation of residual glucose concentration for the different treatments of coconut water kefir fermented at 30 °C with 5% CO_2 concentration. Table S5: The estimation of residual sucrose concentration for the different treatments of coconut water kefir fermented at 30 °C with 5% CO_2 concentration. Table S6: The estimation of pH for the different treatments of coconut water kefir fermented at 30 °C with 5% CO_2 concentration. Table S7: The estimation of TTA for the different treatments of coconut water kefir fermented at 30 °C with 5% CO_2 concentration. Table S8: Amino acid results for all nine experimental sets sampled over time, expressed as mean and standard deviation.

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