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Fermentation dynamics of water kefir in coconut and coconut–pineapple substrates: an evaluation of carbon source effects

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ABSTRACT

Water kefir is a promising non-dairy probiotic beverage, yet its microbial dynamics in coconut water and coconut–pineapple substrates remain insufficiently explored. This single-run study evaluated microbial growth using three carbon sources (glucose, sucrose, and commercial sugar). Fermentation was carried out with 5% (w/v) water kefir grains and 5% sugar at 25–28 °C for 72 h, with sampling every 24 h. Optical density (OD600) ranged from 0.13 to 0.83, generally increasing during the first 48 h and declining thereafter, while pH remained acidic (3.65–3.93). Plate counts on MRS agar were too numerous to count during the first 48 h, followed by enumerable counts at 72 h. Coconut–pineapple substrates showed higher culturable lactic acid bacteria counts (~10^8–10^9 CFU/mL), whereas coconut-only substrates were lower (~10^7 CFU/mL or below detection). Pineapple-containing and sucrose-based treatments were associated with higher activity. Because the study was a single run without replication, results are presented descriptively as observed trends and should be regarded as preliminary. Fermentation up to 48 h supported microbial growth, whereas extended fermentation reduced culturability under MRS-based enumeration. These observations highlight the roles of fermentation time and substrate composition, providing a basis for future replicated studies.

Keywords: oconut water, fermentation, lactic acid bacteria, water kefir, carbon source

PLAIN LANGUAGE SUMMARY

Non-dairy fermented drinks are increasingly popular among people who avoid milk. We studied water kefir, a lightly fizzy fermented beverage, prepared in coconut water and in a coconut–pineapple blend, using three sugars: glucose, sucrose, and table sugar. Over three days we tracked turbidity, acidity, and the number of culturable bacteria. Microbial growth was strongest during the first 48 hours and then declined. Coconut–pineapple blends and sucrose generally supported more growth than coconut alone or glucose. Our findings suggest that fermenting for about 48 hours and adding pineapple may help in developing non-dairy probiotic beverages, and warrant confirmation in replicated studies.

Introduction

Fermentation is a long-established biochemical process in which microorganisms convert complex organic substrates into simpler compounds such as organic acids, alcohols, and gases. Beyond its traditional role in food preservation, fermented products are increasingly associated with functional properties and potential health benefits, including improved gut health and metabolic regulation [1–3]. In recent years, interest in non-dairy probiotic beverages has increased significantly due to the growing demand for plant-based diets and the prevalence of lactose intolerance [4,5]. Within this context, water kefir has emerged as a promising alternative probiotic product with diverse microbial composition and functional potential [5,6].

Water kefir is produced by a complex microbial consortium consisting of lactic acid bacteria, acetic acid bacteria, and yeasts embedded in kefir grains [7,8]. During fermentation, these microorganisms interact synergistically to produce metabolites such as lactic acid, ethanol, carbon dioxide, and various bioactive compounds that contribute to both functionality and sensory properties [9,10]. Previous studies have reported that water kefir fermentation can reach microbial populations in the range of 10^7–10^9 CFU/mL, depending on substrate composition and fermentation conditions [5]. These viable microorganisms are associated with functional benefits, including modulation of gut microbiota and antimicrobial activity [3,11].

Despite these advantages, water kefir fermentation remains highly dynamic and is strongly influenced by substrate composition, environmental conditions, and microbial interactions [7,12]. Among these factors, the type of substrate and carbon source

plays a critical role in determining microbial growth kinetics and metabolite production [7,13]. Sugars serve as the primary energy source, and differences in sugar type, such as monosaccharides and disaccharides, can influence microbial utilization rates and fermentation efficiency [7,13,14].

Previous studies have primarily investigated water kefir fermentation using single substrates or focused on isolated variables such as sugar concentration or starter culture [12,15]. In these systems, microbial growth typically shows a rapid increase within 24–48 h, followed by stabilization or decline due to substrate depletion and metabolite accumulation [7,16–18]. However, studies rarely examine the combined effects of multiple substrates and carbon sources within a single system. Consequently, the interaction between substrate composition and carbon source, particularly in relation to microbial biomass, viability, and acidification, remains insufficiently characterized in mixed-substrate fermentation systems [7,17].

Fruit-based substrates are widely used due to their natural sugar content, vitamins, and minerals that support microbial growth [4,16,19]. Coconut water contains simple sugars (approximately 2–5%), electrolytes, and minerals that facilitate microbial metabolism, while pineapple juice provides additional fermentable sugars, organic acids, and bioactive compounds that may enhance fermentation performance [16,18,20]. Although both substrates have been individually applied, experimental evidence on their combined use remains limited. Their combination may create a more complex nutritional environment that supports microbial growth and metabolic activity. Such compositional differences may also influence microbial interactions within the kefir consortium, including sugar utilization and metabolite exchange between bacteria and yeasts [7,8,17].

In addition to substrate composition, the type of carbon source is a critical variable influencing fermentation dynamics. Glucose can be directly utilized by microorganisms, whereas sucrose requires hydrolysis into glucose and fructose prior to metabolism, which may affect fermentation kinetics and biomass accumulation in complex microbial systems [7,13,14].

To evaluate fermentation dynamics, parameters such as optical density (OD), pH, and total plate count (TPC) are commonly used. OD reflects biomass accumulation, TPC represents viable microbial populations, and pH indicates metabolic activity through acid production [15,17,21]. The integration of these parameters enables a comprehensive understanding of microbial growth behavior and fermentation kinetics.

Based on these considerations, this study evaluates how mixed substrates (coconut water and coconut–pineapple) and different carbon sources influence microbial biomass (OD), viability (TPC), and acidification (pH) during water kefir fermentation. By systematically examining the combined effects of substrate composition and carbon source, this study addresses the limitations of previous single-substrate approaches and provides a more integrated understanding of fermentation dynamics in complex, multi-substrate systems.

Methods

Experimental design

This study was conducted as a single-run exploratory experiment without biological or technical replication. Consequently, no statistical analysis was performed, and all results are reported descriptively as observed trends under the tested conditions. The design comprised two factors: substrate type (coconut water and coconut–pineapple, 1:1 v/v) and carbon source (glucose, sucrose, and commercial sugar), yielding six treatment combinations: coconut–glucose, coconut–sucrose, coconut–commercial sugar, coconut–pineapple–glucose, coconut–pineapple–sucrose, and coconut–pineapple–commercial sugar.

Fermentation experiment

Substrate preparation and treatment design. Two substrate systems were used: coconut water alone and a coconut–pineapple mixture (1:1, v/v), representing single- and mixed-substrate conditions. Fresh young coconut water and freshly extracted pineapple juice served as the main substrates. A pineapple-only treatment was not included; therefore, substrate effects are limited to the tested systems. All glassware, including 250 mL Schott

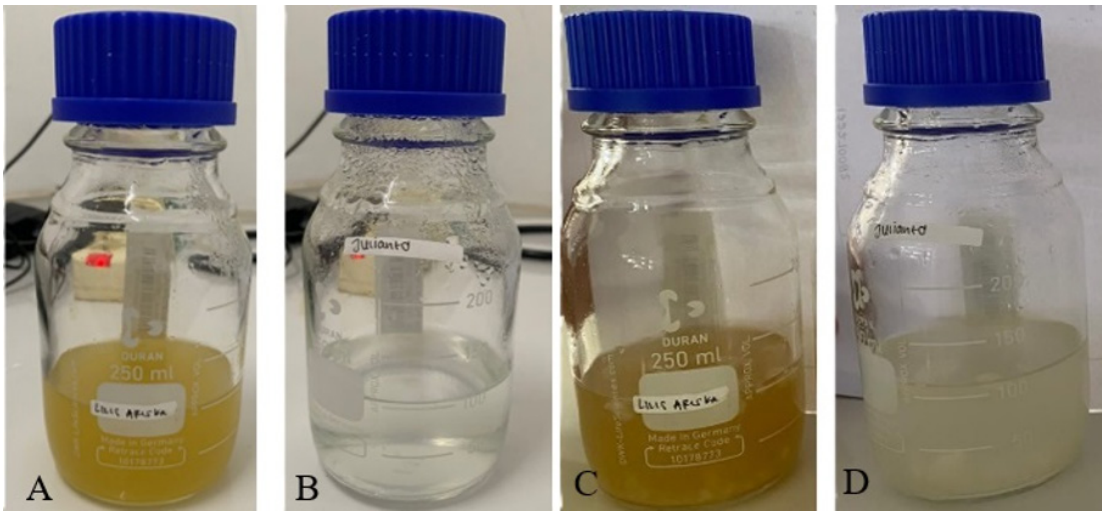

Figure 1. Visual appearance of the fermentation substrates before and after inoculation with water kefir grains. (A) Coconut–pineapple mixture before fermentation (without grains); (B) coconut substrate before fermentation (without grains); (C) coconut–pineapple mixture after 24 h of fermentation with grains; and (D) coconut substrate after 24 h of fermentation with grains. The 24 h time point is shown as representative because no distinct visual differences were observed between 24 h and 48 h.

bottles, pipettes, and Petri dishes, was sterilized by autoclaving (GEA Medical YX-18LDJ) before use, and aseptic technique was maintained throughout substrate handling.

Fermentation conditions. Carbon sources—glucose, sucrose, or commercial sugar—were added at 5% (w/v), and water kefir grains (Kefiree, Grade A) were inoculated at 5% (w/v) as the starter culture under aseptic conditions. Fermentation was carried out at 25–28 °C for 72 h under static conditions.

Sampling procedure. Samples (5 mL) were aseptically collected from the same fermentation batch at 0, 24, 48, and 72 h for physicochemical and microbiological analyses.

Analytical procedures

pH measurement. The pH of the fermentation broth was measured directly on the collected samples at each time point using a calibrated digital pH meter (Ohaus) to assess acidification during fermentation.

Optical density (OD_{600}) analysis. Biomass accumulation was estimated spectrophotometrically at 600 nm (Thermo Scientific). Samples were diluted as necessary (typically 10-fold) with sterile distilled water prior to measurement to ensure readings within the linear range. OD_{600} values were not corrected against a substrate blank and therefore represent relative turbidity rather than absolute cell concentration.

Microbial enumeration. Viable lactic acid bacteria (LAB) were enumerated by the spread-plate method on De Man, Rogosa, and Sharpe (MRS) agar (Merck) [25]. Serial dilutions were prepared in sterile distilled water, plated, and incubated (Memmert) at 30 °C for 24–48 h, after which colonies were counted and expressed as CFU/mL [18]. Because MRS agar selectively supports LAB growth, the reported counts represent culturable LAB only and do not capture yeasts, acetic acid bacteria, or non-culturable members of the consortium; yeast enumeration was not performed in this study.

Results

Substrate characteristics and fermentation observation

Before fermentation, the coconut–pineapple substrate (1:1) was homogeneous with a pale-yellow color, whereas coconut water alone was clear and colorless. After fermentation, the coconut–pineapple system became turbid and yellowish-white, while the coconut-only system appeared turbid and milky-white, indicating the presence of suspended biomass and fermentation-derived particulates (Figure 1). Following pasteurization (65 °C for 30 min), no noticeable change in color or aroma was observed. Visible signs of microbial activity, including gas

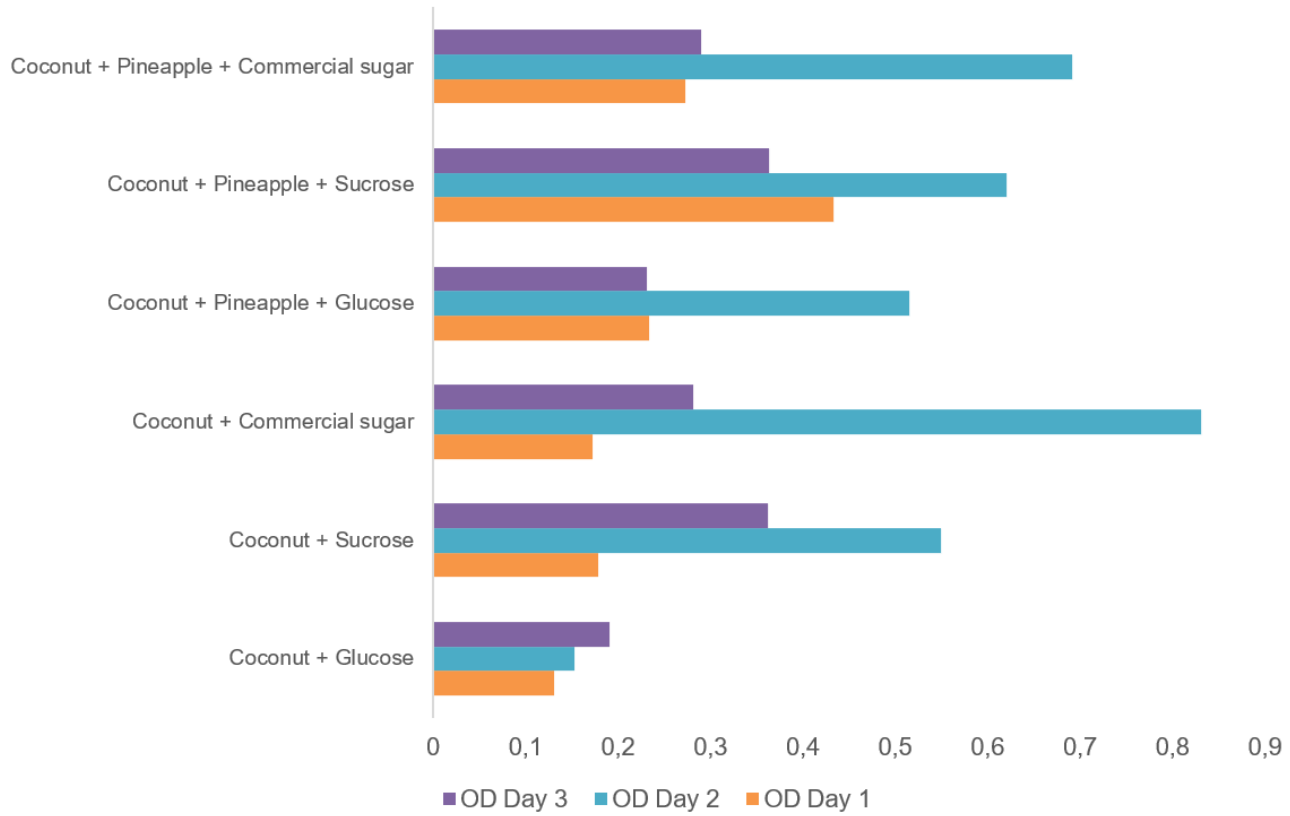

Figure 2. Changes in optical density (OD_{600}) during fermentation. Values were recorded over three days (Day 1–Day 3) in coconut water and coconut–pineapple substrates supplemented with glucose, sucrose, or commercial sugar. OD_{600} was measured at 600 nm and reflects relative turbidity rather than absolute cell concentration

formation and increased turbidity, were apparent after 24 h, with no distinct visual differences between 24 h and 48 h under the tested conditions.

Changes in optical density (OD_{600})

OD_{600} values ranged from 0.131 to 0.831 across all treatments (Figure 2). Most treatments increased from Day 1 to Day 2 and then decreased on Day 3, a pattern observed in both coconut-only and coconut–pineapple substrates supplemented with sucrose or commercial sugar. In contrast, the coconut + glucose treatment increased gradually throughout the observation period without a distinct peak. The highest OD_{600} values occurred in coconut–pineapple systems supplemented with sucrose and commercial sugar, whereas the lowest values were generally observed in coconut-only treatments.

Changes in pH

The pH values ranged from 3.65 to 3.93 across all treatments (Figure 3). Variation among treatments was limited: some treatments showed slight decreases over time, while others exhibited minor fluctuations or small increases at specific time points. All treatments remained within an acidic range throughout fermentation. Because baseline (0 h) measurements were not available, the extent of acidification could not be quantified and no absolute change in pH could be calculated; results are therefore interpreted as relative changes from Day 1 to Day 3. These observations indicate that pH behavior during fermentation was dynamic and influenced by carbon source and substrate composition rather than following a single consistent trend across all conditions.

Bacterial growth analysis during fermentation

Bacterial growth was assessed by the plate-count method. On Day 1 and Day 2, all treatments produced dense, confluent colonies that prevented reliable enumeration; these plates were classified as too numerous to count (TNTC) and are presented qualitatively (Figure 4). Because the dilution range applied on Days 1 and 2 was insufficient to yield

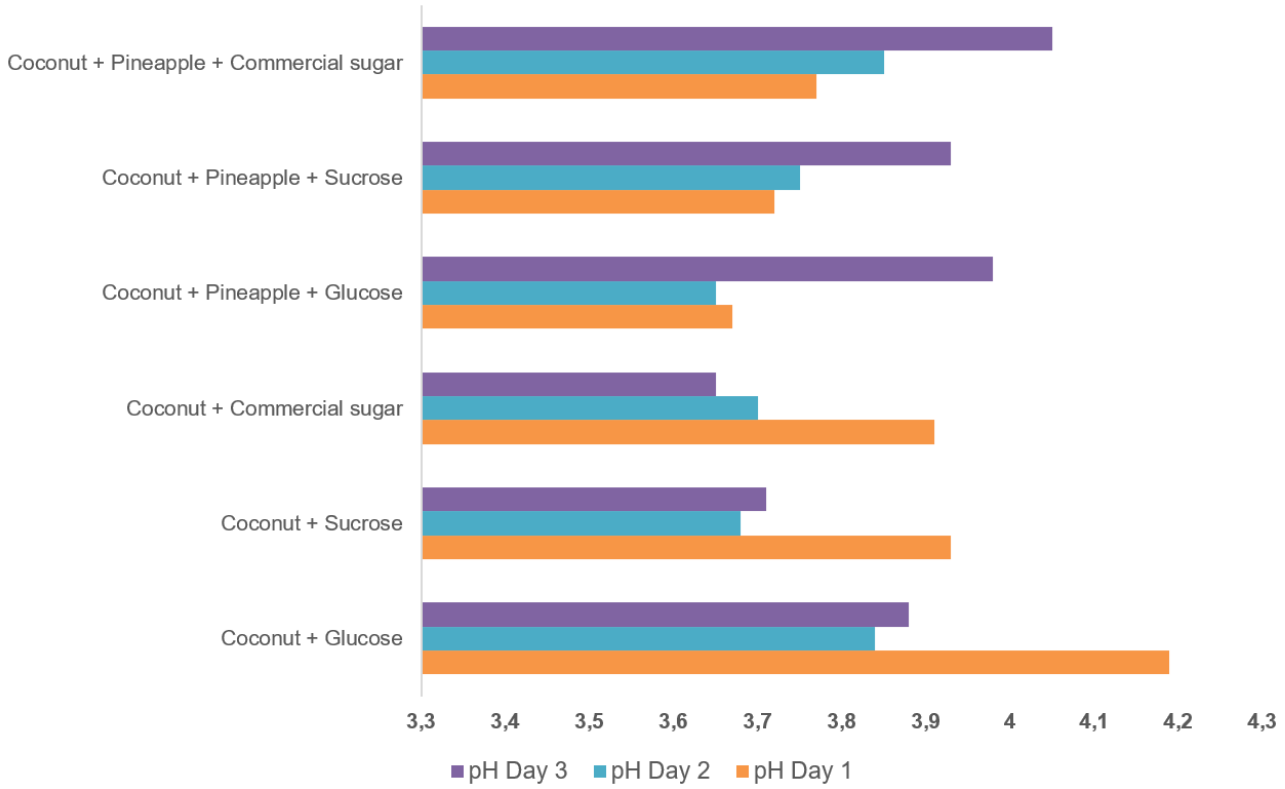

Figure 3. Changes in pH during fermentation. Values were recorded over three days (Day 1–Day 3) in coconut water and coconut–pineapple substrates supplemented with glucose, sucrose, or commercial sugar. Baseline (0 h) values were not recorded; therefore, only relative changes from Day 1 to Day 3 are presented

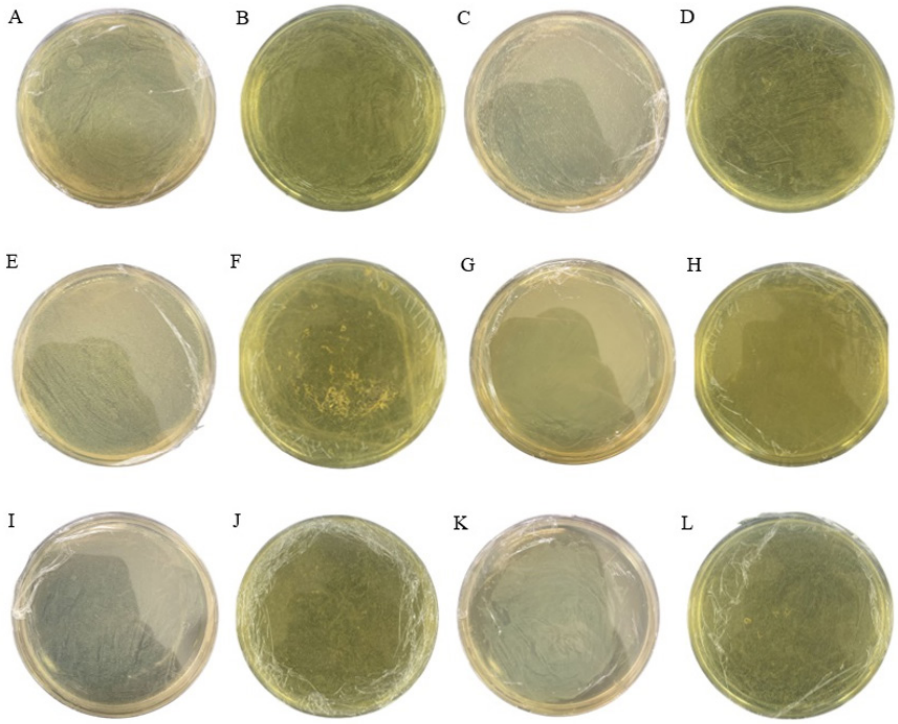

Figure 4. Bacterial growth on MRS agar after 1 and 2 days of fermentation. Plates correspond to coconut–pineapple and coconut water substrates supplemented with 5% sucrose, glucose, or commercial sugar: (A, C, E, G, I, K) Day 1 and (B, D, F, H, J, L) Day 2; (A–F) coconut–pineapple and (G–L) coconut water; (A–B, G–H) sucrose, (C–D, I–J) glucose, and (E–F, K–L) commercial sugar. All plates were too numerous to count (TNTC)

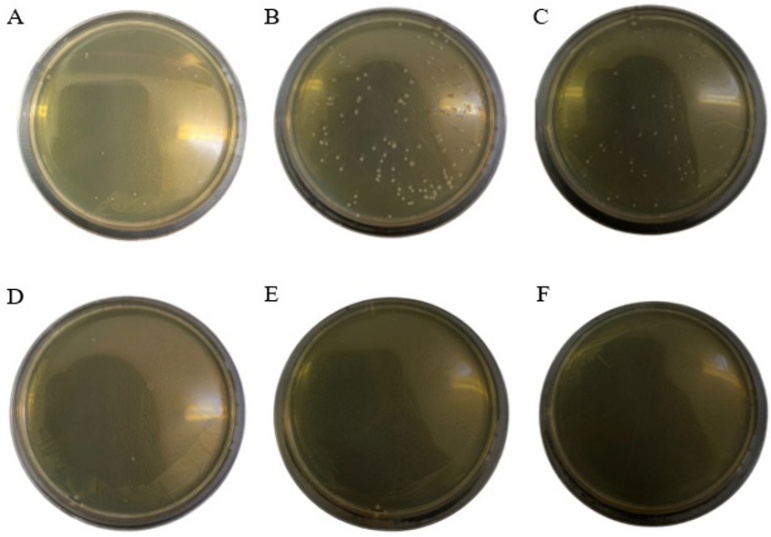

Figure 5. Bacterial growth on MRS agar after 3 days of fermentation. Panels A–C represent coconut–pineapple treatments and panels D–F represent coconut water treatments. Sucrose, glucose, and commercial sugar treatments correspond to panels A/D, B/E, and C/F, respectively. Colonies were counted from serial dilutions up to 10^{-6} and expressed as CFU/mL

isolated colonies, growth at these time points is reported qualitatively rather than as quantitative CFU/mL. This pattern was consistent across all treatments.

On Day 3, enumeration was performed using serial dilution up to 10^{-6}. In coconut–pineapple substrates, colony counts at the selected dilution were 11, 120, and 49 for the sucrose, glucose, and commercial sugar treatments, corresponding to approximately 1.1×10^8, 1.2×10^9, and 4.9×10^8 CFU/mL, respectively. In coconut-only substrates, counts were lower: 2 colonies (2.0×10^7 CFU/mL) in the sucrose treatment, while no colonies were detected in the glucose and commercial sugar treatments at the same dilution, indicating values below the detection limit on the selective MRS medium. Overall, higher culturable LAB counts were observed in coconut–pineapple substrates than in coconut-only systems at Day 3 (Figure 5).

Discussion

The integrated analysis of OD_{600}, pH, and bacterial growth revealed a dynamic, non-linear pattern of microbial activity during fermentation. The increase in OD_{600} from Day 1 to Day 2 is generally associated with enhanced light scattering due to biomass formation. In complex fermentation matrices, however, OD_{600} should be interpreted with caution, as it may also be affected by cell aggregation, extracellular polymeric substances, and substrate-derived particulates rather than reflecting cell concentration alone. Nevertheless, the concurrent too-numerous-to-count (TNTC) plate counts on Days 1 and 2 are consistent with a high microbial load during the early fermentation phase.

In water kefir systems, early-stage fermentation is typically characterized by rapid sugar utilization by lactic acid bacteria (LAB) and yeasts, leading to biomass accumulation and increased turbidity [8,16]. The acidic pH observed across all treatments is consistent with active conversion of carbohydrates into organic acids, particularly lactic acid, a hallmark of LAB-driven acidogenesis [8]. The alignment between low pH, rising OD_{600}, and TNTC-level growth suggests an active growth phase during the first 48 h; however, because these indicators were not measured against a common baseline, they are best interpreted as complementary rather than directly proportional measures of microbial abundance.

By Day 3, microbial dynamics appeared to shift. The recovery of countable colonies after serial dilution suggests a reduction in culturable population density relative to the earlier TNTC condition. It should be emphasized that enumeration reflects only culturable LAB recovered on MRS agar

and not the full consortium. The counts obtained (~10^8–10^9 CFU/mL in coconut–pineapple substrates and lower or undetectable levels in coconut-only substrates) may indicate differential survival and adaptation depending on substrate composition.

The stabilization or slight increase in pH observed in some treatments at later stages may reflect reduced net acid production or metabolic shifts within the community, such as partial utilization of organic acids or reduced glycolytic activity under substrate limitation. Comparable pH dynamics have been reported in mixed-culture water kefir fermentations, where shifts in the dominant microorganisms and their metabolism alter the organic-acid profile over time [16,26]. In the absence of direct metabolite profiling, however, this interpretation remains tentative.

Substrate composition appeared to be an important determinant of microbial performance. Treatments supplemented with sucrose and commercial sugar generally showed higher OD_{600} and culturable LAB counts, which may relate to the enzymatic hydrolysis of sucrose into glucose and fructose, increasing substrate accessibility for multiple microbial groups [8,17]. Glucose-based systems, in contrast, may support more direct but metabolically constrained growth. The enhanced activity observed in coconut–pineapple systems is consistent with a contribution of additional fermentable sugars and micronutrients from pineapple, which together may create a more favorable environment for LAB proliferation [21,22,26]. Such substrate- and nutrient-dependent effects on microbial community structure and metabolism have also been reported elsewhere in water kefir [27].

Several limitations should be considered when interpreting these findings. First, the study was a single run without biological or technical replication, precluding statistical analysis; the reported values therefore describe observed trends rather than statistically validated effects. Second, microbial enumeration was restricted to culturable LAB on MRS agar and did not quantify yeasts or acetic acid bacteria, so the results do not represent the full consortium. Third, OD_{600} in heterogeneous substrates may be influenced by particulates, cell aggregation, and extracellular polymers rather than cell number alone. Fourth, the absence of a 0 h pH baseline and of a pineapple-only treatment limits the quantification of acidification and the full deconvolution of substrate effects. These constraints define the present work as exploratory and hypothesis-generating.

Taken together, the combined dataset suggests that fermentation progressed from rapid initial growth toward metabolic stabilization within 48–72 h. Within the stated limitations, fermentation beyond 48 h still supported microbial activity, but its efficiency appeared increasingly constrained by nutrient depletion and metabolite accumulation, particularly in the less nutritionally complex coconut-only substrate.

Conclusion

In this study, water kefir fermentation in coconut water and coconut–pineapple substrates with 5% sugar supported active microbial growth during the early stages. Integrated OD_{600}, pH, and plate-count observations indicated peak culturable activity within 48 h—reflected by high turbidity, acidic conditions (pH 3.65–3.93), and TNTC growth—followed by lower culturable counts at 72 h. Coconut–pineapple and sucrose-based treatments tended to show higher culturable LAB counts than coconut-only and glucose-based systems. Overall, 48 h appeared to be the most effective fermentation period under the tested conditions. These trends require confirmation in future replicated studies with statistical analysis and broader microbial enumeration, including yeasts and acetic acid bacteria.

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Declaration of interest

Competing interests: The authors declare no competing interests.

Author contributions

Conceptualization: FKF. Methodology: FKF. Investigation: LA, POS, MC, J, PEIBS, YFMH.

Data curation: FKF, LA. Formal analysis: FKF, LA. Supervision: FKF. Project administration: FKF. Funding acquisition: FKF Writing – original draft: FKF.

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