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Impact of fermentation on biological activities and bioactive compounds of coconut milk kefir

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Abstract: Coconut milk serves as an excellent plant-based substitute for animal milk in fermentation with kefir grains, producing a vegetarian-friendly and allergy-safe fermented beverage. This study examined how fermenting coconut milk with milk kefir grains affects its antioxidant capacity, tyrosinase inhibition, probiotic growth, total phenolic content, and protein profile. Results showed significant improvements in cellular antioxidant and anti-tyrosinase effects, along with enhanced growth of the probiotic *Lactobacillus acidophilus* during fermentation, especially at 120 h. Conversely, fermentation decreased the overall phenolic content and led to the breakdown of most proteins in the coconut milk. Nonetheless, the process promoted the formation of smaller proteins and peptides around 10 kDa, possibly contributing to the increased antioxidant, anti-tyrosinase, and probiotic growth-promoting effects. These findings offer valuable insights into how fermentation with kefir alters the biological activities and bioactive compounds of coconut milk, helping to identify the optimal fermentation duration for producing a health-enhancing, high-quality fermented beverage.

Keywords: antioxidant; kefir-fermented coconut milk; phenolics; probiotic growth; tyrosinase inhibition

Kefir is a traditional drink that originated in the Caucasus Mountains, Tibet, and Mongolia. It is made by fermenting animal milk with kefir grains, which harbour a diverse community of microorganisms including lactic acid bacteria (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Streptococcus*), yeasts (*Candida*, *Kluyveromyces*, *Saccharomyces*, *Torulopsis*, and *Zygosaccharomyces*), and sometimes acetic acid bacteria (*Acetobacter*) (Arslan 2015). During fermentation, these microorganisms metabolise the milk, giving kefir its characteristic yeasty flavour and a fizzy mouthfeel that many find appealing. This process also enriches kefir with various nutrients and bioactive compounds such as vitamins, minerals, essential amino acids, phenolics, exopolysaccharides, bioactive peptides, organic acids, and antibiotics. Studies have shown that consuming kefir regularly can aid digestion, enhance lactose digestion, and offer health benefits such as antibacterial, antioxidant, anti-cancer, anti-allergenic, anti-hypertensive, and healing properties. As a result, the global demand for kefir-based products has grown significantly in recent years (Vieira et al. 2021).

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Plant-based milks have recently gained significant popularity as alternatives to dairy products due to their health benefits and lower environmental impact. They are particularly suitable for individuals who are lactose intolerant, follow vegetarian diets, or seek to reduce the consumption of animal-derived products for ethical or health reasons (Ramsing et al. 2023). Among these, coconut milk – which is widely used in Asian cuisine – stands out as a versatile option, containing water, lipids, carbohydrates, proteins, vitamins, and minerals that make it an excellent substrate for fermentation, including kefir production. Previous studies have reported that coconut milk kefir exhibits antimicrobial activity against *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, and *Saccharomyces cerevisiae*, as well as cytotoxic effects on breast cancer (MCF-7) cells and notable *in vitro* free radical-scavenging activity (Lakshmi et al. 2017; Abadl et al. 2022). However, its potential roles in scavenging intracellular free radicals, inhibiting cellular tyrosinase activity, and promoting probiotic growth remain largely unexplored and need further investigation.

Coconut milk kefir is typically produced by fermenting coconut milk with kefir grains for 12 to 24 h, and most studies evaluating its biological and physicochemical properties have been limited to this fermentation period (Lakshmi et al. 2017; Abadl et al. 2022, 2023). However, as fermentation progresses, microbial growth enhances metabolic activity, leading to increased production of bioactive compounds and consequently improved biological properties (Sarıtaş et al. 2024). This trend has also been observed in fermented fireweed (*Chamerion angustifolium*) leaves, where antioxidant activity increased over time, with the highest activity recorded after 72 h of fermentation (Lasinskas et al. 2023). Based on this premise, the present study aimed to investigate the effects of extended fermentation on cellular antioxidant activity, cellular tyrosinase inhibitory activity, and probiotic growth-promoting capacity, as well as on total phenolic content and protein profile of coconut milk kefir. This work seeks to provide deeper insight into prolonged fermentation and support the development of coconut milk kefir products with enhanced health benefits.

MATERIAL AND METHODS

Material. Milk kefir grains were obtained from a Yogourmet® kefir starter (Lallemand, France). Coconut milk (15% fat, 1% protein, 2% carbohydrate) was supplied by Asian Coconut Processing JSC (Vietnam). *Lactobacillus acidophilus* ATCC 4356, human dermal fibroblast (HDF), and mouse B16F10 melanoma (CRL-6475) were sourced from ATCC. Chemicals were purchased from Sigma-Aldrich (Germany), and culture media from Himedia (India) and Oxoid (UK).

Preparation of coconut milk kefir. Kefir grains were activated in pasteurised cow's milk for a period of 12 to 24 h. Following activation, the grains were separated and transferred to a fresh batch of milk, a process repeated 2 to 3 times. Once activation was complete, 10 g of kefir grains, thoroughly washed with sterile deionised water to remove any residual milk, were added to 200 mL of coconut milk. The mixture was fermented for 120 h at 28 °C and the coconut milk kefir was then collected every 24 h over a total period of 120 h, designated as CMF0, CMF24, CMF48, CM72, CM96, and CMF120. Subsequently, all samples were heat-inactivated at 90 °C for 1 h, centrifuged at 10 000 rpm for 10 min, adjusted to pH 7.0, and filtered through a 0.22 μm membrane to remove all microorganisms. The resulting supernatants were then used for further analysis.

Measurements of pH, total soluble solid content, and microbial growth. pH was measured using a pH meter and total soluble solids (°Brix) with a refractometer. Microbial growth was determined by culturing lactic acid bacteria (LAB) on de Man-Rogosa-Sharpe (MRS) agar with 400 $mg \cdot L^{-1}$ cycloheximide, acetic acid bacteria (AAB) on glucose-yeast extract-calcium carbonate (GYC) agar with 400 $mg \cdot L^{-1}$ cycloheximide, and yeasts on potato dextrose (PD) agar. The microbial counts were expressed as colony-forming units per millilitre of the samples ($CFU \cdot mL^{-1}$).

Determination of cellular antioxidant activity. Fibroblast cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were detached by trypsin prior to subsequent analyses.

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Fibroblasts were seeded in 96-well plates (1×10^5 $cells \cdot mL^{-1}$) and incubated for 48 h, followed by treatment with different sample concentrations (3.125, 6.25, 12.5, 25, and 50 $mg \cdot mL^{-1}$). Water served as the negative control. After the addition of MTT solution (5 $mg \cdot mL^{-1}$) and incubation for 2 h, the resulting formazan crystals were dissolved in dimethyl sulfoxide (DMSO), and absorbance was measured

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at 570 nm. Cell inhibition was calculated using the following formula:

$$Cell\ viability\ (\%) = 100 \times (A_s - A_{b1}) / (A_c - A_{b2}) \quad (1)$$

where: A_c – absorbance of the negative control; A_s – absorbance of the sample; A_{b1} – absorbance of the blank of sample; A_{b2} – absorbance of the blank of DMSO.

Cellular antioxidant activity in fibroblasts was evaluated using the 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay. Fibroblasts (1×10^5 cells·mL^{-1}) were seeded in 24-well plates. To stimulate intracellular ROS production, the cells were treated with 3 μM H_2O_2 in culture medium and incubated at 37 °C in a 5% CO_2 for 30 min. The H_2O_2-containing medium was then replaced with samples at a concentration of 12.5 mg·mL^{-1}, with water used as the negative control, and the plates were further incubated at 37 °C and 5% CO_2 for 24 h. After treatment, the media were removed, and the cells were washed with phosphate buffer saline (PBS) to eliminate residual compounds. Subsequently, DCFH-DA at a final concentration of 20 μM was added to each well and incubated for 30 min. The cells were then detached using 0.25% (w/v) trypsin, washed with PBS, and transferred to a black plate. Fluorescence intensity was measured at an emission wavelength of 538 nm (excitation at 485 nm) using a fluorescence microplate reader. Cellular antioxidant activity was calculated using the following formula:

$$Cellular\ antioxidant\ capacity\ (\%) = 100 - 100 \times A_s / A_c \quad (2)$$

where: A_s – fluorescence intensity of the sample; A_c – fluorescence intensity of the negative control.

Determination of tyrosinase inhibitory activity. B16F10 melanoma cells were cultured in DMEM with 10% FBS at 37 °C and cell viability was evaluated using the same MTT procedure as for fibroblasts. Tyrosinase activity was assessed based on L-DOPA oxidation. Cells were treated with samples (12.5 mg·mL^{-1}) or water (negative control) for 48 h. After treatment, the medium was discarded, and the cells were lysed with RIPA buffer using a tissue lyser, followed by centrifugation at 12 000 rpm for 20 min. The resulting supernatant was then incubated with freshly prepared 10 mM L-DOPA substrate for 2 h. Subsequently, the absorbance of each well was measured at 475 nm using a microplate reader. Anti-tyrosinase capacity was calculated using the following formula:

$$Cellular\ tyrosinase\ inhibitory\ capacity\ (\%) = 100 - 100 \times A_s / A_c \quad (3)$$

where: A_s – absorbance of the sample; A_c – absorbance of the negative control.

Determination of probiotic growth-promoting activity. *L. acidophilus* ATCC 4356 was cultured anaerobically in 2/3 MRS-1/3 Tryptic Soy (TS) broth at 37 °C. The culture was adjusted to an optical density at 600 nm (OD_{600}) of 0.2 and inoculated with samples (50 mg·mL^{-1}) or water (negative control) in 2/3 MRS-1/3 TS broth. Bacterial growth was monitored by OD_{600} every 2 h for 30 h, and viable counts were determined by plating serial dilutions and reporting colony-forming units (CFU·mL^{-1}).

Determination of total phenolic content. Samples were mixed with 10% Folin–Ciocalteu reagent. Subsequently, 10% sodium carbonate (Na_2CO_3) was added, and the mixture was incubated at 40 °C for 30 min. The absorbance was then measured at 765 nm using a UV spectrophotometer. Gallic acid was used as the standard to construct a calibration curve. Results were expressed as μg gallic acid equivalents per mL (μg GAE·mL^{-1}).

Protein electrophoresis. The samples were combined with 5X protein loading buffer and boiled for 30 min to denature the proteins. Subsequently, the heat-denatured samples (20 μL) were loaded onto a 15% sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel, and the protein bands were detected through Coomassie blue staining.

Statistical analysis. Experiments were conducted with three different kefir samples. One-way analysis of variance (ANOVA), followed by Tukey's post-test, was used to assess statistical differences among the samples in antioxidant and anti-tyrosinase activities and TPC (GraphPad Prism software, version 9.5, Insightful Science LLC, USA). A P-value of < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

pH values, total soluble solid contents, and microbial growth in coconut milk kefir during fermentation. Because pH, total soluble solids, and microbial growth reflect kefir microbial metabolism, their changes were first monitored during coconut milk kefir fermentation. The initial pH (~6.1) and total soluble solids (~11 °Brix) decreased rapidly within 24 h to 4.5

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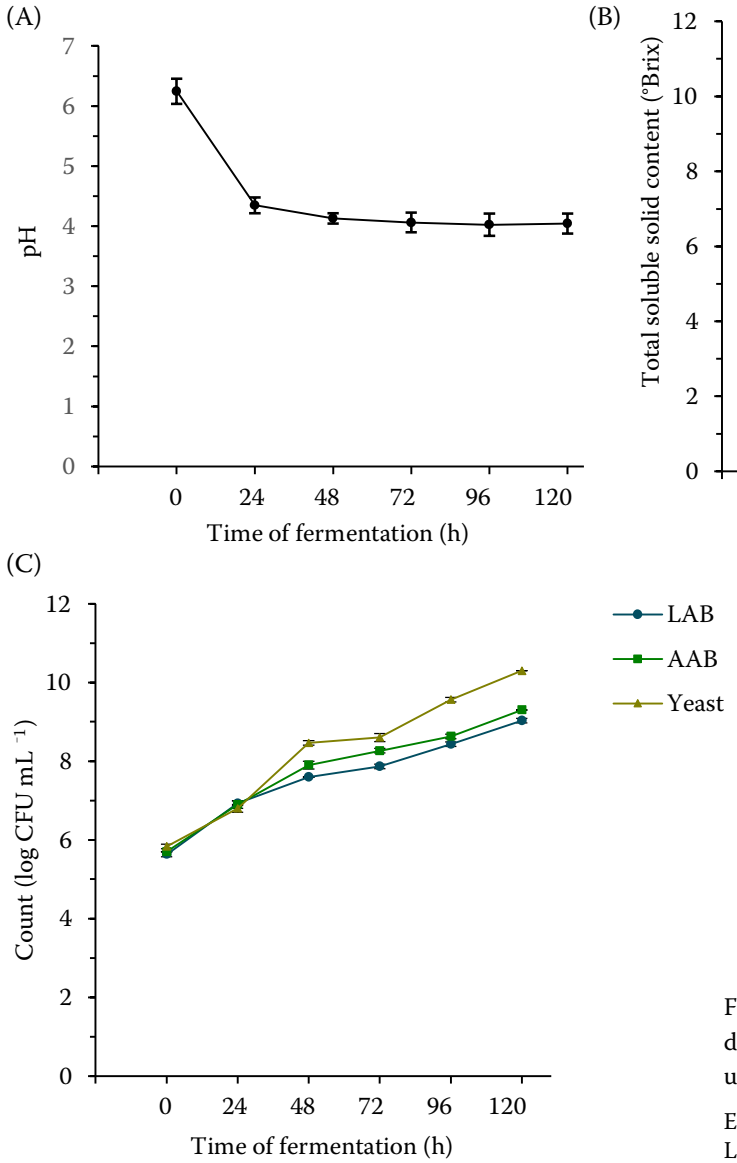

Figure 1. Different variables of coconut milk kefir during fermentation: (A) pH value; (B) total soluble solid content (°Brix); (C) microbial growth

Each data point is presented as mean ± SD (n = 3); LAB – lactic acid bacteria, AAB – acetic acid bacteria

and 8 °Brix, respectively, then remained close to 4.0 and 6.5 °Brix at 120 h of fermentation (Figures 1A and 1B). These decreases result from microbial utilisation of soluble nutrients and their conversion into organic acids, ethanol, and CO_2 (Peng et al. 2025).

Regarding microbial dynamics, yeasts, LAB, and AAB began at similar levels (~ 5–7 × 10^6 CFU·mL^{-1}). Yeasts increased rapidly, reaching ~ 2 × 10^{11} CFU·mL^{-1} at 120 h, while LAB and AAB rose more moderately to 10^{10} and 2 × 10^{10} CFU·mL^{-1}, respectively (Figure 1C). These results indicate that coconut milk supports microbial growth, particularly yeasts. Given that kefir grains harbour diverse probiotic microorganisms, including *Lactobacillus kefiranofaciens*, *L. plantarum*, *L. kefir*, *L. lactis*, *Kluyveromyces marxianus*, and *Saccharomyces cerevisiae*, their robust growth in coconut milk kefir highlights the potential of this fermented product as a rich probiotic source (Prado et al. 2015).

Cellular antioxidant activity of coconut milk kefir during fermentation. Coconut milk kefir has been recognised for its ability to scavenge free radicals at 24 h of fermentation (Abadl et al. 2023). We further examined this property of coconut milk at extended fermentation durations in normal human fibroblasts. Initially, we evaluated whether coconut milk kefir affects fibroblast viability through an MTT assay. Our findings indicated that coconut milk kefir is non-toxic to fibroblasts. Notably, cell proliferation was slightly enhanced, particularly at the concentration of 50 mg·mL^{-1}, suggesting that coconut milk kefir promoted fibroblast growth (Table 1). Subsequently, we assessed the cellular antioxidant activity of coconut milk kefir by measuring its

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Table 1. Viability of human dermal fibroblasts treated with various concentrations of coconut milk kefir, presented as mean ± SD (n = 3)

CMF concentration ($mg \cdot mL^{-1}$)	CMF0	CMF24	CMF48	CMF72	CMF96	CMF120
	(%)					
3.125	103.13 ± 0.66^{a}	105.76 ± 1.14^{a}	104.79 ± 0.31^{a}	108.62 ± 2.52^{a}	108.73 ± 2.37^{a}	108.19 ± 2.65^{a}
6.25	102.20 ± 4.39^{ab}	105.87 ± 1.27^{a}	104.68 ± 2.56^{a}	109.24 ± 0.18^{a}	109.08 ± 1.53^{a}	114.07 ± 0.42^{b}
12.5	106.41 ± 1.52^{ab}	107.96 ± 0.57^{a}	108.54 ± 1.57^{a}	112.87 ± 1.04^{ab}	115.11 ± 0.84^{b}	117.12 ± 0.48^{bc}
25	107.188 ± 0.77^{ab}	111.90 ± 1.71^{a}	111.59 ± 4.59^{ab}	116.92 ± 1.77^{bc}	117.00 ± 1.58^{bc}	119.90 ± 0.24^{c}
50	109.12 ± 1.10^{b}	121.83 ± 5.25^{b}	117.47 ± 0.52^{b}	118.55 ± 1.14^{c}	121.56 ± 1.57^{c}	125.73 ± 1.86^{d}

$^{a–d}$different letters indicate significant differences among samples within a column; CMF0, CMF24, CMF48, CMF96, and CMF120 – coconut milk kefir at 0, 24, 48, 96, and 120 h of fermentation, respectively

ability to reduce ROS levels in cells treated with H_2O_2. As anticipated, coconut milk kefir decreased ROS levels induced by H_2O_2, demonstrating its antioxidant properties at the cellular level. The most significant reduction was observed in the CMF120 sample, which decreased ROS content by approximately 65% (Figure 2A).

Coconut milk kefir exhibited notable antioxidant activity in fibroblasts exposed to H_2O_2. This effect may be attributed to the presence of bioactive compounds such as phenolics, polysaccharides, and peptides, which can arise from the liberation of bound phenolics into free forms, hydrolysis of glycosides to produce aglycones, or the synthesis of new antioxidant metabolites during fermentation (Zhao et al. 2021). Zhang et al. (2022) reported that *Lactobacillus kefiri* fermentation enhanced the total antioxidant capacity of fibroblasts by upregulating the expression and activity of key antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) (Zhang et al. 2022). It would therefore be valuable for future studies to investigate whether coconut milk kefir exerts its antioxidant effects through similar cellular defence mechanisms.

Interestingly, extending the fermentation time further increases the cellular antioxidant activity, with the most significant effect observed at 120 h of fermentation (Figure 2A). This suggests that extended fermentation promotes the accumulation of antioxidant compounds. During the initial stage, microbial adaptation and growth are relatively slow; however, as fermentation progresses, microbial metabolic activities intensify. This enhanced activity leads to increased production of enzymes such as glucosidases, esterases,

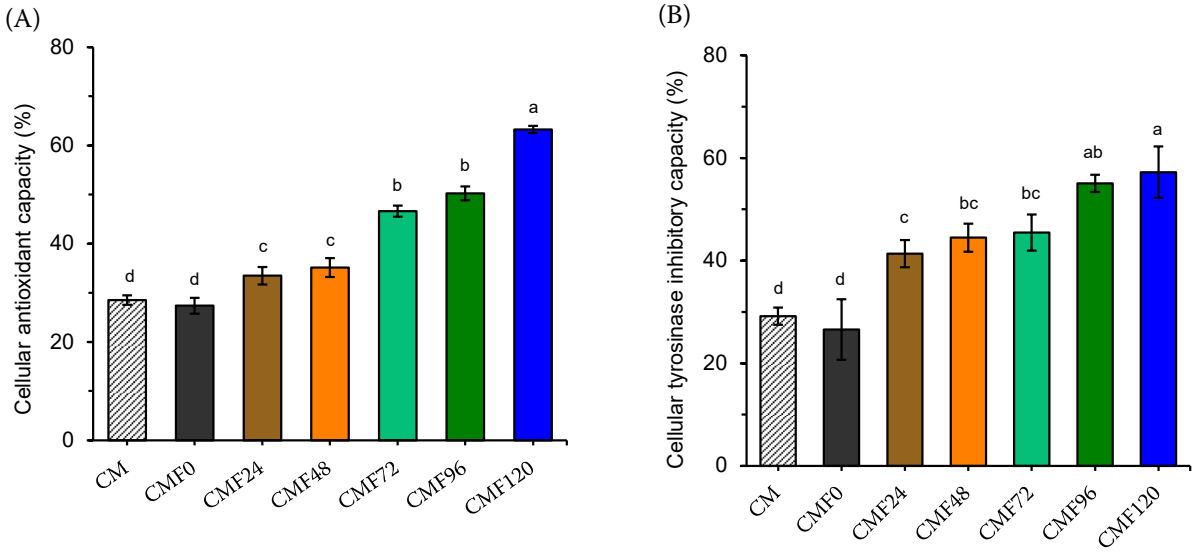

Figure 2. Biological activities of coconut milk kefir during fermentation: (A) cellular antioxidant capacity; (B) cellular tyrosinase inhibitory capacity

$^{a–d}$different letters indicate significant difference among samples ($P < 0.05$); each data point is presented as mean ± SD (n = 3); CMF0, CMF24, CMF48, CMF96, and CMF120 – coconut milk kefir at 0, 24, 48, 96, and 120 h of fermentation, respectively

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and proteases, which break down complex food matrices and facilitate the release or synthesis of antioxidant components, including phenolics and bioactive peptides (Sarıtaş et al. 2024). In the study by Xia et al. (2019), the total phenolic content of soy milk fermented with kombucha increases proportionally with fermentation duration (Xia et al. 2019). Similarly, fermentation of whey with *Lacticaseibacillus rhamnosus* GG and *Streptococcus thermophilus* resulted in higher peptide levels as fermentation time increased (Olvera-Rosales et al. 2023).

Cellular anti-tyrosinase activity of coconut milk kefir during fermentation. To date, there have been no reports of tyrosinase activity inhibition in coconut milk kefir. Considering that kefir-fermented products can exhibit anti-tyrosinase properties, our study aimed to investigate this activity in coconut milk kefir throughout fermentation process by cellular tyrosinase inhibition assay using B16F10 melanoma cells.

As seen in Figure 2B, the unfermented control (CMF0) reduced cellular tyrosinase activity by approximately 30%, which may be attributed to the presence of phenolic compounds and vitamins naturally occurring in coconut milk (Gengan et al. 2025). As fermentation proceeded, tyrosinase inhibition increased markedly, where inhibition rose from 40% in CMF24 to 47% in CMF48 and CMF72, 55% in CMF96, and 60% in CMF120. Moreover, all coconut milk kefir samples showed no cytotoxicity toward B16F10 cells (Table 2). These findings demonstrate that prolonged fermentation significantly enhances the cellular anti-tyrosinase activity of coconut milk kefir.

Tyrosinase inhibitors present in fermented products, including phenolics, aglycones, bioactive peptides, and polysaccharides, exert their effects through multiple modes of enzyme interaction. These compounds can inhibit tyrosinase by binding to the free enzyme and preventing substrate access to the active site (competitive inhibition), interacting exclusively with the enzyme-substrate complex (uncompetitive inhibition), or associating with both the free enzyme and the enzyme-substrate complex (mixed inhibition). In some cases, they bind with equal affinity to both forms of the enzyme, resulting in noncompetitive inhibition (Panzella and Napolitano 2019). As tyrosinase is a key rate-limiting factor in melanin synthesis, regulating tyrosinase activity through inhibitors is vital for addressing skin conditions such as hyperpigmentation, age spots, freckles, melasma, and even malignant melanoma, as well as preventing enzymatic browning in fruits and fungi (Hassan et al. 2023). Our research indicates that coconut milk kefir, particularly those fermented for extended periods, shows promise as a tyrosinase inhibitor with potential applications in pharmaceutical, cosmeceutical, and agricultural fields.

Growth promoting effect of coconut milk kefir on *L. acidophilus* during fermentation. *L. acidophilus* is a beneficial Gram-positive rod that supports gut health and immunity (Liu et al. 2024). Although coconut milk kefir has been widely studied, its ability to promote the growth of *L. acidophilus* remains unclear (Lakshmi et al. 2017; Abadl et al. 2022, 2023). Therefore, we investigated this potential of coconut milk kefir during fermentation process.

As shown in Figure 3, following 2 h of incubation, the bacterial population entered the exponential growth phase, which continued until approximately 20 h before transitioning into the stationary phase. During the exponential phase, no significant statistical differences were observed among the growth curves across all coconut milk kefir samples. In the stationary phase, noticeable differences in bacterial growth emerged among the samples. The highest proliferation of *L. acidophilus* occurred in the medium supplemented with CMF120, reaching an OD_{600} of 1 (2.36×10^8 CFU·mL^{-1}) after 30 h. In contrast, growth

Table 2. Viability of B16F10 melanoma cells treated with various concentrations of coconut milk kefir, presented as mean ± SD (n = 3)

CMF concentration (mg·mL^{-1})	CMF0	CMF24	CMF48	CMF72	CMF96	CMF120
	(%)					
3.125	98.80 ± 0.86^{a}	96.75 ± 1.10^{a}	104.74 ± 0.65^{a}	102.48 ± 2.45^{a}	108.06 ± 2.89^{a}	107.78 ± 1.66^{a}
6.25	100.42 ± 5.02^{a}	99.79 ± 3.94^{a}	110.82 ± 0.44^{b}	112.02 ± 1.60^{b}	116.26 ± 4.24^{ab}	113.08 ± 0.97^{b}
12.5	104.67 ± 0.32^{a}	109.12 ± 1.59^{b}	113.30 ± 0.37^{bc}	112.45 ± 2.71^{b}	114.85 ± 2.21^{ab}	113.37 ± 1.59^{b}
25	107.64 ± 3.61^{a}	109.90 ± 2.05^{b}	117.19 ± 1.83^{cd}	114.57 ± 3.08^{bc}	118.81 ± 4.78^{b}	119.87 ± 2.12^{c}
50	105.52 ± 3.61^{a}	115.06 ± 3.37^{b}	117.75 ± 2.55^{d}	120.30 ± 2.99^{c}	121.00 ± 3.33^{b}	123.20 ± 2.15^{d}

$^{a–d}$different letters indicate significant differences among samples within a column; CMF0, CMF24, CMF48, CMF96, and CMF120 – coconut milk kefir at 0, 24, 48, 96, and 120 h of fermentation, respectively

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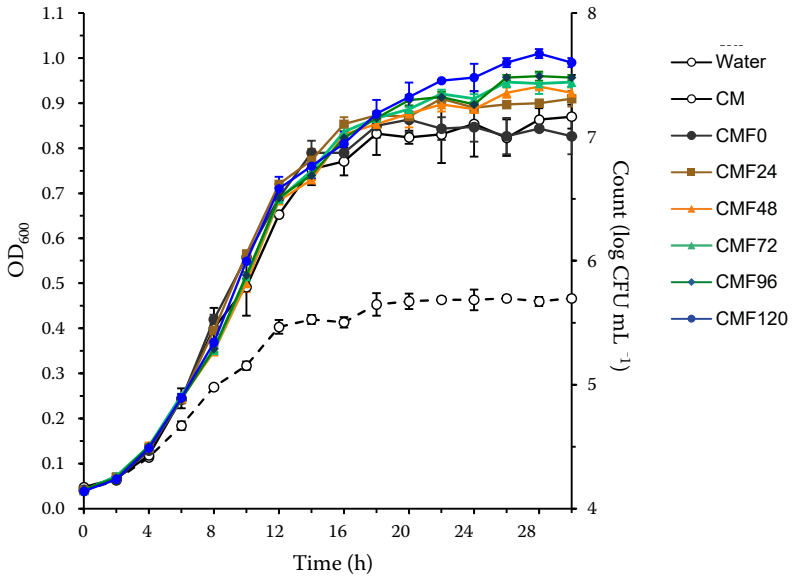

Figure 3. The growth stimulating effect of coconut milk kefir on *L. acidophilus* during fermentation

Each data point is presented as mean ± SD (n = 3); CMF0, CMF24, CMF48, CMF96, and CMF120 – coconut milk kefir at 0, 24, 48, 96, and 120 h of fermentation, respectively; OD_{600} – optical density at 600 nm

in media supplemented with CMF24, CMF8, CMF72, and CMF96 was moderate, with OD_{600} values between 0.9 and 0.96 (9.66×10^7–1.63×10^8 CFU·mL^{-1}). The lowest bacterial growth was observed in the medium with CMF0, with an OD_{600} below 0.9 (3.79×10^7 CFU·mL^{-1}). Nonetheless, some growth-promoting activity was detected in the CMF0 sample, as OD_{600} readings from 20 h onward were higher than those in the water-supplemented control. Although there is no existing literature on the proliferative effects of coconut milk on *L. acidophilus*, the observed activity in this study may be attributed to the substantial amount of dietary fibre found in coconut milk (Hanafi et al. 2022).

In our study, the addition of coconut milk kefir enhanced the growth of *L. acidophilus*, especially in products fermented for longer periods. This may be due to increased levels of prebiotic substances such as phenolic compounds and peptides produced by microorganisms in kefir grains. These components may stimulate the production of short-chain fatty acids (SCFAs), which offer various health benefits such as anti-inflammatory, immune-modulating, anti-obesity, anti-diabetic, and anticancer (Alves-Santos et al. 2020, Ashaolu and Ashaolu 2021, Xiong et al. 2022). Further investigations are necessary to explore how coconut milk kefir supplementation impacts gut microbiota composition and SCFA production in living gut models.

Total phenolic content of coconut milk kefir during fermentation. Phenolic compounds are vital components that contribute to the antioxidant activity, anti-tyrosinase effect, and prebiotic property of fermented products (Wróblewska et al. 2023). In light of this, our study aimed to assess the levels of these compounds in coconut milk kefir over an extended fermentation period. The TPC of CMF0 was measured at more than 900 µg GAE·mL^{-1}. Surprisingly, it steadily declined throughout fermentation, reaching levels of 500–600 µg GAE·mL^{-1} at 24, 48, 72, 96, and 120 h of fermentation (Figure 4A).

Decreases in phenolic compounds during fermentation may result from their degradation through metabolic pathways, the hydrolysis of glycosidic or ester bonds by microbial glycosidases and esterases, or their interaction with other components within the food matrix (Apostolidis et al. 2011; Adebo and Medina-Meza 2020). In the present study, the TPC declined regardless of fermentation duration; however, this trend contrasts with the observed enhancement in cellular antioxidant activity, anti-tyrosinase effects, and *probiotic* growth-promoting properties of coconut milk kefir over time. This discrepancy suggests that, despite the reduction in overall TPC, certain individual phenolic compounds with higher bioactivity may increase in concentration. A similar phenomenon has been reported in fermented persimmon, where total phenolic levels remained relatively stable while gallocatechin content increased, correlating with enhanced antioxidant activity (Zou et al. 2017). Alternatively, these findings may indicate that phenolic compounds are not the sole contributors to the improved bioactivities, and that other bioactive constituents formed during fermentation play a more significant role.

Protein profile of coconut milk kefir during fermentation. SDS-PAGE showed that coconut milk

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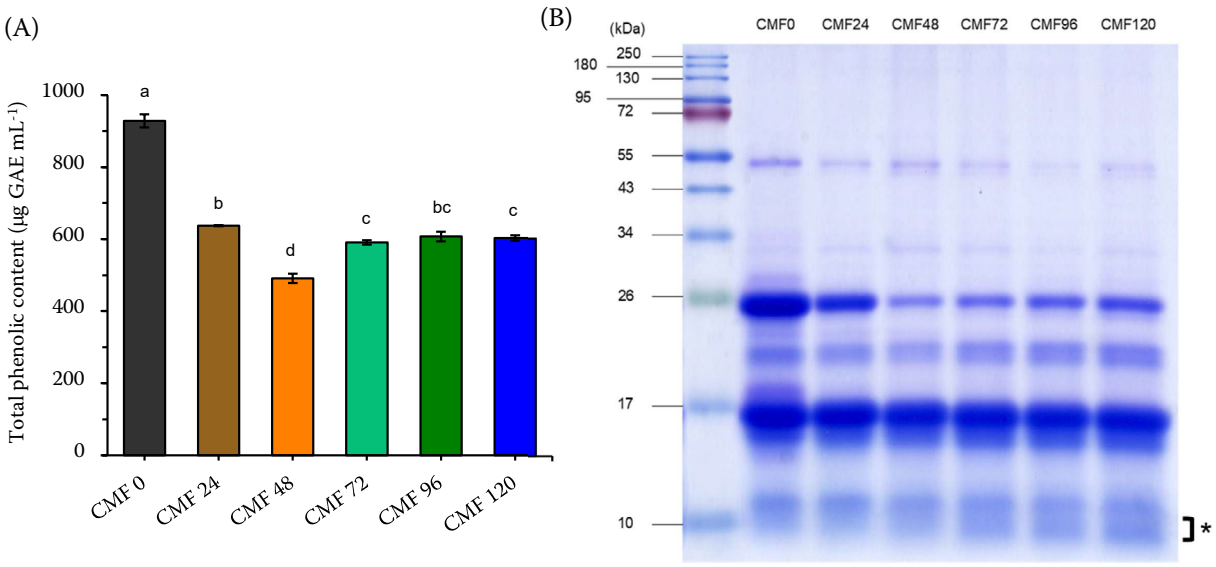

Figure 4. Whole figure caption: (A) the total phenolic content and (B) protein profile of coconut milk kefir during fermentation

[a–d]different letters indicate significant difference among samples ($P < 0.05$); each data point is presented as mean ± SD ($n = 3$); *newly produced small proteins and peptides; CMF0, CMF24, CMF48, CMF96, and CMF120 – coconut milk kefir at 0, 24, 48, 96, and 120 h of fermentation, respectively

proteins mainly ranged from 10 to 55 kDa, with major bands at approximately 55, 26, 22, 17, and 10 kDa. These likely correspond to various globulins: the 11S globulins (cocosin) around 55 kDa, which consist of acidic (~ 34 kDa) and basic (~ 22 kDa) subunits; the 7S globulins also near 55 kDa, composed of subunits approximately 26 kDa, 22 kDa, and 17 kDa; and other peroxisomal enzymes in the 22–34 kDa range (Ma et al. 2022). After kefir fermentation, the 55 kDa and 26 kDa proteins were markedly degraded within 24 h, while lower-molecular-weight proteins were less affected. Additionally, there was a notable increase in proteins and peptides smaller than 10 kDa, especially in the CMF120 sample (Figure 4B). These findings indicate that coconut milk proteins are vulnerable to hydrolysis by kefir microorganisms, resulting in the formation of smaller proteins and potentially peptides through proteolytic activity.

Kefir grains contain diverse lactic acid bacteria species, including *L. plantarum*, *L. helveticus*, *L. kefiranofaciens*, and *L. paracasei*, which can hydrolyse milk proteins to generate bioactive peptides (Prado et al. 2015; Raveschot et al. 2018). These peptides have been shown to possess antioxidant, antibacterial, antihypertensive, and immunomodulatory activities (Raveschot et al. 2018). It is probable that these bacteria contribute to peptide formation in coconut milk kefir, with higher peptide levels during extended fermentation potentially linked to the observed time-dependent increases in cellular antioxidant, anti-tyrosinase, and prebiotic effects in our study. Further research is required to identify the bioactive peptides present in coconut milk kefir and to explore their biological effects in *in vivo* models.

CONCLUSION

Our research indicates that fermenting coconut milk with kefir modifies its biological activities and bioactive compounds. The antioxidant, anti-tyrosinase, and probiotic growth-stimulating activities improved with extended fermentation times, reaching their peak at 120 h of fermentation. Throughout the fermentation process, total phenolic content decreased, while the production of small proteins and potential peptides increased in a manner dependent on fermentation duration. Additional research is needed to optimise a formulation using coconut milk kefir fermented for 120 h to leverage its potent biological properties and peptide content.

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