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The Effect of Aqueous Extract of Coconut Milk on the Growth of Lactobacillus *fermentum* under Oxidative Stress

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Abstract

Oxidative stress negatively affects the growth and survival of lactic acid bacteria (LAB) under aerobic conditions. This effect may limit the technological and probiotic applications of LAB. Plant phenolic compounds possess antioxidant activity and may enhance bacterial tolerance to oxidative stress. The present study investigated the protective antioxidant effects of the aqueous extract of coconut milk (AECM) on *Lactobacillus fermentum* under aerobic conditions. AECM was chemically analyzed to determine its total phenolic content, ortho-diphenol content, and protein content. Antioxidant protection was assessed using two experimental approaches: aerobic growth in the presence of AECM and aerobic growth after overnight pre-treatment with AECM. AECM contained 97.11 µg mL^{-1} total phenolics, of which approximately 16 % were ortho-diphenols. The protein content was 1.11 mg mL^{-1} in the AECM. During aerobic growth on solid medium, the presence of AECM resulted in a significant 16.5 % increase ($p < 0.05$) in colony-forming units (CFU) compared to the controls. Pre-treatment with AECM resulted in a stronger protective effect, with an approximately 49 % increase in CFU during subsequent aerobic growth ($p < 0.05$). No significant differences were observed under anaerobic conditions, indicating that, at the concentrations used, AECM mitigates oxidative stress rather than exerting nutritional support. These findings demonstrated that AECM confers antioxidant protection to *L. fermentum* through both direct and inducible antioxidant mechanisms, and reinforced previous observations on the inducible antioxidant protection of coconut milk phenolics conferred in several LAB species, including *L. fermentum*.

Keywords: Antioxidant protection, Aqueous extract of coconut milk, *Lactobacillus fermentum*, Ortho-diphenols, Oxidative stress

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Introduction

Lactic acid bacteria (LAB) are widely used in food fermentations and as probiotic organisms due to their technological importance, primarily attributed to the production of lactic acid, and the health-promoting properties of intestinal LAB. However, many lactic acid bacteria are sensitive to oxygen exposure. Because of the lack of efficient antioxidant mechanisms, oxygen can negatively affect the growth, survival, and functional performance of LAB through the generation of oxidative stress (Condon, 1987).

Oxidative stress arises from the accumulation of reactive oxygen species (ROS), including superoxide radicals and hydrogen peroxide, which can damage cellular lipids, proteins, and nucleic acids (Imlay, 2013). LAB possess only limited endogenous antioxidant defense systems, and which makes them vulnerable to oxidative damage during aerobic processing, storage, and gastrointestinal transit (Papadimitriou et al., 2016; Pessione, 2012).

Phenolic compounds in food are recognized for their antioxidant properties and their ability to modulate the physiology of gut microbiota (Crozier et al., 2009). Phenolics can reduce oxidative damage to microorganisms by scavenging ROS and chelating oxidative metal ions. They may also induce cellular responses that enhance microbial tolerance to oxidative stress (Cueva et al., 2017; Durazzo et al., 2019).

Coconut milk, derived from the endosperm of *Cocos nucifera* L., is a widely consumed plant-based product, often used as a food additive or thickening agent. It contains a mixture of bioactive compounds, including phenolic compounds, proteins, lipids, and minerals (DebMandal & Mandal, 2011). Several studies, including Alyaqoubi et al. (2015) and Karunasiri et al. (2020), reported measurable antioxidant activity in coconut milk and its aqueous extracts, indicating the presence of phenolic compounds with antioxidant properties.

Ortho-diphenols are phenolic compounds characterized by two adjacent hydroxyl groups on the aromatic ring. These compounds are known to exhibit considerable antioxidant activity due to their enhanced electron-donating ability and metal-chelating properties (Rice-Evans et al., 1996). The presence of ortho-diphenolic structures in phenolic extracts derived from foods is therefore considered an indicator of antioxidant properties (Seneviratne & Dissanayake, 2008).

A recent study (Gunawardane et al., 2025) demonstrated that prior exposure to aqueous extract of coconut milk enhanced resistance to oxidative stress in several LAB species, suggesting that pre-treatment with coconut milk phenolics may induce an antioxidant protective response in LAB cellular physiology. However, it remained unclear whether coconut milk aqueous extracts can provide real-time antioxidant protection during aerobic growth and whether similar protective effects occur in other LAB species.

Based on these considerations, we hypothesized that the aqueous extract of coconut milk (AECM), containing phenolic and ortho-diphenolic compounds, enhances the oxidative-stress tolerance of *Lactobacillus fermentum* during aerobic growth through both direct antioxidant activity and inducible cellular responses.

The present study aimed to investigate the antioxidant protective effects of the aqueous extract of coconut milk (AECM) on *L. fermentum* under aerobic conditions. Specifically, the study determined the total phenolic, ortho-diphenolic, and protein content of AECM and evaluated its ability to augment bacterial survival during aerobic growth. To assess this, two experimental approaches were used: (i) aerobic growth in the presence of AECM, and (ii) pre-treatment with AECM before aerobic growth. Through these two approaches, the study sought to understand both direct antioxidant activity and pre-treatment–induced cellular responses leading to increased oxidative-stress tolerance in *L. fermentum*.

Methodology

Preparation of the AECM

Coconut milk was prepared following previously reported procedures with minor modifications (Karunasiri et al., 2020; Gunawardane et al., 2025). Scraped coconut kernel (100 g) from mature coconuts (12–14 months) of *Cocos nucifera* L. typica (tall type) was mixed with distilled water (100 mL), and homogenized (3 min) using a kitchen blender. The slurry was then pressed through cheesecloth, and the liquid fraction was collected as coconut milk.

Coconut milk was frozen overnight to destabilize the emulsion. It was then thawed at room temperature and centrifuged (6000 × g, 10 min) to remove the lipid fraction, to obtain a polar extract while minimizing interference from lipids. This defatted coconut milk was subjected to chloroform-water partitioning to remove lipophilic and non-phenolic compounds with the chloroform phase. The aqueous phase, which contains phenolic compounds, was collected, filter-sterilized using a 0.45 μm bacteriological membrane filter, labelled as the aqueous extract of coconut milk (AECM), and used for subsequent chemical analyses and biological assays.

Determination of Total Phenolic Content of the AECM

To characterize the overall phenolic composition of coconut milk, the total phenolic content of the AECM was determined by the Folin-Ciocalteu assay, following previously reported methods with minor modifications (Karunasiri et al., 2020). The AECM was diluted 20-fold with deionized water to ensure absorbance values were within the range of the calibration curve. An aliquot (150 μL) of the diluted extract was mixed with Folin-Ciocalteu's Phenol reagent (6 μL; HiMedia, India). To provide alkaline conditions for color development, sodium carbonate solution (25 % w/v, 15 μL) was added. The reaction mixture was diluted with deionized water to a final volume of 300 μL and incubated at room temperature for 1 h. The absorbance was then measured at 765 nm using a UV-visible spectrophotometer (Multiskan GO, Thermo Scientific, Finland). Gallic acid monohydrate (HiMedia, India) was used as the calibration standard, and total phenolic content was expressed as gallic acid equivalents (GAE) after correcting for the dilution factor.

Determination of Ortho-Diphenol Content of the AECM

Ortho-diphenol content was determined using a microplate-adapted sodium molybdate (Arnow) colorimetric assay, based on the method originally described by (1937), with minor modifications to accommodate reduced reaction volumes. Since the antioxidant effect of AECM on *L. fermentum* was investigated, its phenolic composition was assessed in terms of ortho-diphenol content, a key contributor to antioxidant activity.

The AECM (15 µL) was first diluted with deionized water (50 µL). Sodium molybdate solution (5 % $Na_2MoO_4 \cdot 2H_2O$, 100 µL; HiMedia, India) and phosphate buffer (0.1 mol L^{-1}, pH 6.5, 50 µL) were added, mixed thoroughly, and incubated at room temperature for 15 min to allow color development. The addition of assay reagents resulted in a final dilution of approximately 15-fold, ensuring the absorbance values remained within the linear range of the assay. Absorbance was measured at 350 nm using a UV-visible spectrophotometer (Multiskan GO, Thermo Scientific, Finland).

Negative control samples were prepared using distilled water instead of the phenolic-enriched fraction, with all other reagents and volumes unchanged. Caffeic acid (0.1 mg mL^{-1}; HiMedia, India) was used as the calibration standard, and ortho-diphenol content was expressed as caffeic acid equivalents, after correcting for the dilution factor.

Determination of Protein Content of the AECM

Protein content of the AECM was needed to determine the potential contribution of non-phenolic reducing components and to evaluate their possible interference in phenolic quantification and antioxidant activity assays. Bradford colorimetric assay (Bradford, 1976) was used to determine the protein content. The Bradford reagent was prepared by dissolving Coomassie Brilliant Blue G-250 (10 mg; HiMedia, India) in 95 % ethanol (5 mL), followed by the addition of 85 % (w/v) phosphoric acid (10 mL). The solution was diluted with distilled water to a final volume of 100 mL. , Final concentrations of the Bradford reagent components were; 0.01 % (w/v) Coomassie Brilliant Blue G-250, 4.7 % (w/v) ethanol, and 8.5 % (w/v) phosphoric acid.

An aliquot of AECM (30 µL) was mixed with Bradford reagent (1.4 mL) and incubated at room temperature for 5 min. The absorbance was then measured at 595 nm using a UV-visible spectrophotometer (Multiskan GO, Thermo Scientific, Finland). A reagent blank containing all assay components except AECM was used for baseline correction. A standard calibration curve was prepared using bovine serum albumin (BSA) (2 mg mL^{-1}; HiMedia, India). The protein content of the AECM was expressed as BSA equivalents.

Assessment of Antioxidant Protection of AECM during Aerobic Growth of *L. fermentum*

Two experimental approaches were used to evaluate the antioxidant protection conferred by the aqueous extract of coconut milk (AECM) during aerobic growth of *L. fermentum*. The strain used in this study, *L. fermentum* (ATCC 14931), was obtained from the culture collection, DMBUK (Department of Microbiology, University of Kelaniya Culture Collection).

(i) Aerobic Growth After Pre-treatment with AECM

This approach was used to examine whether prior growth in the presence of AECM offers any inducible protection against oxidative stress during subsequent aerobic exposure. To assess this, an overnight pre-treatment experiment was conducted. An inoculum (100 µL) from an overnight culture of *L. fermentum* (OD_{600} = 1.3) was transferred into de Man, Rogosa, and Sharpe (MRS) broth (20 mL) supplemented with AECM (50 %, v/v). In parallel, an MRS broth culture without supplementation was used as a control. Both cultures were incubated (37 °C, 24 h) anaerobically (Anaerobic jar with AnaeroGen™, Oxoid, UK).

Following incubation, cultures were serially diluted up to 10^{-3} to obtain countable colonies. Aliquots (1 mL) from the AECM-supplemented culture were transferred into 20 sterile Petri plates, molten MRS agar was poured, and the contents were mixed gently. Ten plates were incubated aerobically (37 °C, 48 h), while the remaining ten plates were incubated anaerobically under identical conditions. Aliquots from the AECM-free control culture were plated and incubated in parallel following the same procedure.

Comparison of aerobic and anaerobic incubation conditions allowed the differentiation between any antioxidant effect against oxidative stress and baseline growth differences. CFU were enumerated to determine the effect of pre-treatment with AECM on the aerobic growth of *L. fermentum*.

(ii) Aerobic Growth in the Presence of AECM (Solid Medium Assay)

To assess the direct protective effect of AECM during aerobic growth, *L. fermentum* was grown on solid growth medium supplemented with AECM, without prior exposure to AECM. MRS broth medium inoculated with *L. fermentum* and incubated anaerobically (37 °C, 24 h) was serially diluted in sterile diluent up to 10^{-4} in order to obtain countable colonies. Aliquots (1 mL) were transferred into 40 sterile Petri plates. Molten MRS agar supplemented with AECM (50 % v/v) was poured into 20 plates, while the same medium without AECM supplementation was poured into the remaining 20 plates to be used as AECM-free controls.

The inoculum and medium were mixed gently to ensure even cell distribution and allowed to solidify. Ten AECM-incorporated plates and ten control plates were incubated (37 °C, 48 h) aerobically, while the remaining ten AECM-incorporated plates and ten control plates were incubated anaerobically under identical conditions.

After incubation, bacterial growth in terms of CFU on plates was quantified. The number of CFU on AECM-incorporated plates was compared with that on AECM-free control plates under both aerobic and anaerobic conditions to assess the protective effect of AECM during aerobic growth on solid medium.

Statistical analysis

All chemical assays (total phenolic content, ortho-diphenol content, and protein content) were performed in triplicate, and results were presented as mean ± standard deviation (SD). For microbiological assays, each experimental condition consisted of replicate plates as described ($n = 10$ per condition). All experiments were conducted once with multiple technical replicates. Statistical comparisons between two groups were performed using an unpaired two-tailed Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

Results

Phenolic, Ortho-Diphenol, and Protein Content of AECM

The aqueous extract of coconut milk (AECM) contained measurable levels of phenolic compounds. The total phenolic content, determined using the Folin–Ciocalteu assay and expressed as gallic acid equivalents (GAE), was 97.11 μg mL^{-1}. Ortho-diphenol content, determined as caffeic acid equivalents, was 15.9 μg mL^{-1}, representing approximately 16 % of the total phenolic content. This indicates that a fraction of the phenolic compounds in AECM bear the ortho-dihydroxyl structural component, which is known to have a stronger antioxidant activity than many other phenolics (Rice-Evans et al., 1996; Karunasiri et al., 2020).

The protein content of AECM, determined by the Bradford assay and expressed as bovine serum albumin equivalents, was 1.11 mg mL^{-1}. This protein concentration was substantially higher than the phenolic concentration, which was 97.11 μg mL^{-1} in gallic acid equivalents. The relatively higher concentration of protein in AECM is consistent with that expected for an aqueous extract of coconut milk, which is not a purified phenolic preparation. Nevertheless, the concurrent presence of substantial phenolic and ortho-diphenolic content indicates that phenolic compounds represent an important antioxidant component of the coconut milk extract, an assumption vindicated in the subsequent experiments of the study.

Effect of AECM on Aerobic Growth of *L. fermentum*

The real-time protective effect of AECM during aerobic growth was evaluated using a solid-medium assay. Figure 1 shows the increase in CFU of *L. fermentum* when the bacterium was incubated with AECM under aerobic conditions.

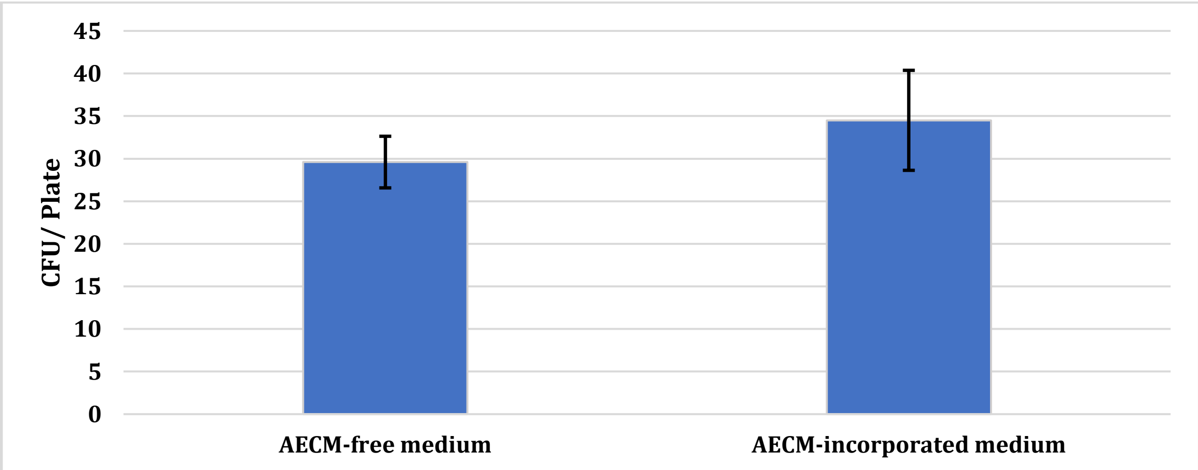

Figure 1. Effect of AECM on aerobic growth of *Lactobacillus fermentum*.
Bars represent mean CFU values, and error bars indicate standard deviation (n = 10). Aerobic growth on AECM-incorporated medium was significantly higher than on AECM-free medium ($p < 0.05$)

Under aerobic conditions, plates supplemented with AECM showed a significantly higher CFU count (34.5±5.88) compared with AECM-free control plates (29.6±3.03). This corresponded to a

16.5 % increase in CFU in the presence of AECM. Statistical analysis using an unpaired two-tailed *t*-test confirmed that this difference was significant ($p < 0.05$).

In contrast, under anaerobic incubation conditions, CFU counts on AECM-incorporated plates were not statistically different from those on control plates. This indicated that AECM does not function as a nutrient or some other growth stimulant under anaerobic, non-stressed conditions, and only confers an antioxidant advantage during aerobic growth, where oxidative stress is expected to limit bacterial survival.

Effect of AECM Pre-treatment on Subsequent Aerobic Growth

Pre-treatment of *L. fermentum* with AECM before aerobic exposure caused a markedly increased protective effect against oxidative stress. Figure 2 shows the effect of pretreatment on the bacterium.

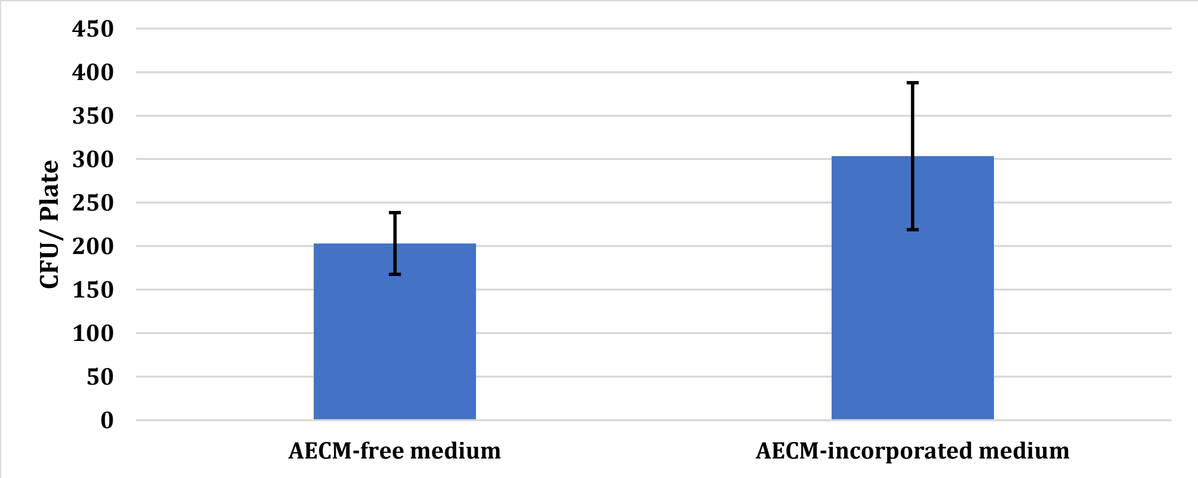

Figure 2. Effect of pre-treatment with AECM on aerobic growth of *L. fermentum*.
Bars represent mean CFU values, and error bars indicate standard deviation (n = 10). Pretreatment with AECM produced significantly higher CFU than no pretreatment ($p < 0.05$)

Cultures derived from overnight growth in AECM-supplemented broth yielded significantly higher CFU counts during subsequent aerobic incubation (303.4 ± 84.41 CFU) compared with cultures derived from AECM-free overnight growth (203.1 ± 34.47 CFU). This represented an approximate 49 % increase in CFU following overnight pre-treatment with AECM. The difference was found to be highly significant ($p < 0.05$).

When separate inocula from both pre-treated and untreated cultures were incubated anaerobically, CFU counts were comparable, indicating that AECM pre-treatment did not significantly affect baseline growth under anaerobic, non-oxidative conditions. These findings suggested that prior exposure to AECM induces a sustained physiological state in bacterial cells, that enhances tolerance to oxidative stress during subsequent aerobic growth.

Table 1 summarizes the antioxidant protective action offered by AECM against oxidative stress in *L. fermentum* caused by exposure to an aerobic environment.

Table 1: Effect of AECM on CFU of *L. fermentum* under aerobic conditions

Aerobic conditions	CFU (mean ± SD)	% increase in CFU *vs* control	*p* value
No AECM in the medium (control)	29.6 ± 3.02	16.50 %	0.030
AECM in the medium	34.5 ± 5.87		
No AECM pre-treatment (Control)	203.1 ± 34.47	49.40 %	0.004
AECM pre-treatment	303.4 ± 84.41		

Discussion

Chemical analysis of AECM revealed the presence of moderate levels of total phenolics, out of which approximately 16 % was ortho-diphenolic compounds, which are known to be formidable antioxidants due to the presence of adjacent hydroxyl groups, which facilitate electron donation and metal chelation (Rice-Evans et al., 1996). The presence of this phenolic sub-fraction therefore, provides a credible chemical basis for the antioxidant effects recorded in the bacterial assays. While proteins and peptides present in AECM may also contribute to overall reducing capacity, the significant ortho-diphenol content of the extract signifies a major role for phenolics in antioxidant protection. Nevertheless, the complex composition of AECM suggests that synergistic interactions between phenolics and other components cannot be excluded and merit further investigation.

The present study demonstrated that AECM confers significant antioxidant protection to *L. fermentum* under aerobic conditions. This protection was observed both when AECM was present during aerobic growth and when bacteria were pre-treated with AECM before aerobic exposure, suggesting the possibility of involvement of at least two complementary protective mechanisms. One mechanism apparently requires the presence of AECM during aerobic growth, while the other mechanism seems to induce a protective physiological effect that persists during the subsequent aerobic growth on the following day.

The presence of AECM during aerobic growth resulted in a relatively small but statistically significant increase in CFU counts. This finding suggests the presence of a mechanism involving immediate antioxidant scavenging or reducing activity by phenolic constituents of AECM during growth under aerobic conditions.

Pre-treatment with AECM generated a substantially stronger protective effect, with nearly a 50 % increase in CFU observed during subsequent aerobic growth. This sustained protection indicates that AECM exposure may induce adaptive cellular responses in *L. fermentum*, such as up-regulation of endogenous antioxidant defenses, improved redox homeostasis, or enhanced repair of oxidative damage, mechanisms that have been described in phenolic–microbe interactions (Cueva et al., 2017). Such responses may include up-regulation of inherent antioxidant defenses or enhanced repair of oxidative damage.

The sustained protection observed following AECM pre-treatment is consistent with earlier findings reported by Gunawardane et al. (2025), who demonstrated enhanced oxidative-stress tolerance in five species of LAB, including *L. fermentum*, after prior exposure to aqueous coconut milk extracts. The present study confirmed the observation and also showed that AECM confers direct protection as well when present during aerobic growth. This suggests the possibility of both inducible cellular responses and immediate antioxidant mechanisms offered by AECM.

Conclusion

The aqueous extract of coconut milk (AECM) contains phenolic compounds, including a substantial ortho-diphenolic fraction, that confers significant antioxidant protection to *L. fermentum* during aerobic growth. Protection was observed both when AECM was present during aerobic exposure and following prior pre-treatment, indicating the involvement of both immediate antioxidant effects and sustained, inducible cellular responses. These findings are consistent with earlier observations in LAB and shed further light, extending the evidence for phenolics in coconut milk as promoters of tolerance against oxidative stress among LAB.

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Conflict of interest statement

The authors declare that there was no conflict of interest in conducting this research work.

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