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Coconut milk phenolics weakly attenuate oxygen-induced lethality in the anaerobe *Clostridium perfringens*

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Abstract

Clostridium perfringens is an anaerobe that is highly sensitive to molecular oxygen due to the absence of efficient enzymatic systems required to detoxify reactive oxygen species (ROS). However, anaerobes in natural and food-associated environments may encounter transient exposure to oxygen in the presence of extracellular antioxidant compounds, raising the question of whether such environmental antioxidants can influence their short-term survival during oxidative stress. This study investigated whether phenolic components present in the aqueous extract of coconut milk (AECM) could influence the survival of *C. perfringens* during aerobic exposure. Chemical analyses showed that AECM contained measurable phenolic compounds and exhibited free-radical scavenging activity as determined by the Folin–Ciocalteu and DPPH assays. When *C. perfringens* was plated under aerobic conditions, supplementation of the medium with AECM resulted in a modest but statistically significant ($p = 0.012$) increase (~10%) in recoverable colony-forming units compared with control plates lacking AECM. These findings suggest that extracellular antioxidant compounds present in natural substrates may transiently modulate oxidative stress experienced by anaerobes during oxygen exposure.

Keywords: Anaerobes, Aqueous extract of coconut milk, Clostridium perfringens; Oxidative stress, Phenolic antioxidants, Reactive oxygen species

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Introduction

The interaction between living organisms and molecular oxygen is determined by their capacity to neutralize reactive oxygen species (ROS). ROS are generated when oxygen undergoes univalent reduction, in contrast to the controlled tetravalent reduction that occurs during aerobic respiration at the terminal end of the electron transport chain, where oxygen is safely converted to water. When oxygen accepts single electrons due to its high reduction potential, partially reduced oxygen species such as superoxide anion and hydroxyl radicals are generated. These ROS show even stronger oxidizing ability and thus damage cellular macromolecules, including nucleic acids, proteins, membrane lipids, and iron–sulfur enzymes (Imlay, 2013). Because such reactions occur to some extent in all oxygen-exposed cells, every organism living in an aerobic environment, that is, aerobes and facultative anaerobes, must have inherent antioxidant mechanisms to destroy ROS. In addition, exogenous antioxidant compounds, including phenolics, are known to augment antioxidant action in many such organisms. Some bacteria, including lactic acid bacteria, have weak antioxidant mechanisms and therefore can survive only in low-oxygen environments. Clostridia are anaerobes in the sense that they do not possess any inherent antioxidant mechanism, and, as a result, they are rapidly killed by ROS generated upon exposure to oxygen.

Clostridium perfringens is a Gram-positive, spore-forming anaerobe that illustrates this physiology. The organism lacks superoxide dismutase, catalase, and peroxidases, and no exogenously inducible antioxidant response has been described (Smith, 1975; McDonnell & Russell, 2009). The bacterium is widely studied not only because of its role as a human and animal pathogen but also as a model organism for investigating anaerobic metabolism, oxygen toxicity, and oxidative stress responses in anaerobic bacteria (Imlay, 2013; McClane et al., 2016). Given that ROS-mediated oxidative injury is the primary cause of oxygen toxicity in anaerobes, investigating the possibility that exogenous antioxidants might attenuate this effect is scientifically compelling. Coconut milk, the watery slurry of the endosperm of *Cocos nucifera*, which is often used as a dietary additive and food thickening agent in many tropical regions, is known to contain a diverse array of phenolic compounds, including gallic acid, quinic acid, catechins, and ferulic acid. These compounds are recognized as having antioxidant properties, such as free-radical scavenging, hydrogen-atom donation, metal-ion chelation, and interruption of radical-propagation reactions (Shahidi & Ambigaipalan, 2015). The antioxidant roles of phenolics have been extensively studied in food chemistry and human physiology. Previous studies have shown that plant polyphenols can reduce intracellular ROS levels in bacteria exposed to oxidative stressors (Kumar et al., 2017; Karunasiri et al., 2020; Gunawardane et al., 2025). Nevertheless, the potential for exogenous antioxidants to influence the survival of anaerobic bacteria during oxygen exposure has been rarely investigated. Even though exogenous antioxidants are most probably unable to substitute for enzymatic antioxidant systems, the possibility of such antioxidants acting against ROS accumulation, at least partially, cannot be ruled out without an experimental investigation.

Anaerobes are typically defined by their inability to tolerate molecular oxygen due to the absence or inefficiency of enzymatic antioxidant systems that detoxify reactive oxygen species. However, environments encountered by anaerobic microorganisms in natural and food-associated systems often involve transient exposure to oxygen. Under such conditions, extracellular antioxidants present in the surrounding environment could potentially modulate oxidative stress.

Investigating whether naturally occurring antioxidant compounds can influence the survival of anaerobes during oxygen exposure therefore represents an interesting question in microbial physiology and oxidative stress biology.

This study was designed to determine whether AECM added to the growth medium could provide, at least, partial transient protection against oxidative lethality in *C. perfringens* under aerobic conditions. The findings would provide insight into the role of extracellular antioxidants in microbial oxidative stress physiology.

Methodology

Preparation of AECM

Commercially available liquid coconut milk was obtained from the local market and processed to obtain a phenolic-containing aqueous fraction. Approximately 50 mL of coconut milk was centrifuged (4500 × g, 10 min) to separate the lipid-rich layer, which was removed to yield defatted coconut milk. The remaining aqueous fraction was adjusted to pH 3.5 using citric acid (50% w/v), resulting in the precipitation of some proteinaceous material. The mixture was then centrifuged (4500 × g, 10 min), and the resulting supernatant was collected as the AECM. This procedure enriches the aqueous fraction in low-molecular-weight phenolic compounds, while allowing some residual soluble proteins or peptides also to remain in solution. Accordingly, AECM represents a phenolic-enriched aqueous fraction rather than purified phenolic preparation. It was filter-sterilized using a 0.45 μm bacteriological membrane filter and stored at 4 °C for use in subsequent biological experiments and chemical analysis.

Determination of total phenolic content

Total phenolic content (TPC) was determined using the Folin – Ciocalteu assay assay (Singleton et al., 1999). An aliquot of AECM (0.2 mL) was mixed with aqueous Folin-Ciocalteu reagent (10% v/v, 1.5 mL). After an initial reaction period (5 min), sodium carbonate solution (7.5% w/v, 1.5 mL) was added. The mixture was incubated (room temperature, 30 min) in the dark, after which the absorbance (765 nm) was measured against a reagent blank. A standard calibration curve was prepared using gallic acid (0 -100 mg/L), and TPC was expressed as gallic acid equivalent (GAE) per unit volume of extract.

Chemical determination of antioxidant activity of AECM

The free radical scavenging activity of aqueous extract of coconut milk (AECM) was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. DPPH is a stable free radical exhibiting a deep violet color, which becomes pale yellow upon reduction. The assay is based on the ability of antioxidant compounds to donate a hydrogen atom or an electron to DPPH radicals, resulting in the formation of the reduced hydrazine form (DPPH-H). AECM was tested at concentrations ranging from 62.5 to 1000 μg/mL. AECM samples were mixed with 0.1 mM DPPH solution prepared in methanol and incubated in the dark (room temperature, 30 min). Absorbance was measured at 517 nm (Multiskan GO, Thermo Scientific). Radical scavenging activity was calculated as:

Scavenging activity (%) = $[(A_0 - A_s) / A_0] \times 100$
Where A_0 is the absorbance of the control and A_s is the absorbance of the sample.

Bacterial strain and growth conditions

Clostridium perfringens (American Type Culture Collection, ATCC 13124; Department of Microbiology, University of Kelaniya, DMBUK 10901) was grown and maintained in Reinforced Clostridial Agar (RCA; HiMedia, India) at 37 °C under anaerobic conditions.

A non-inhibitory concentration of AECM (40% v/v), which was determined by anaerobic growth on AECM-supplemented RCA, was used in subsequent biological assays. RCA medium (51 g; HiMedia, India) was dissolved in distilled water (600 mL) and sterilized by autoclaving. After cooling to approximately 70 °C, the filter-sterilized preparation of AECM (400 mL) was aseptically added with continuous mixing to maintain the agar in a molten state during incorporation of the extract. The supplemented medium was then allowed to cool to 45-50 °C before being poured into Petri plates with bacterial inocula.

Aerobic survival assay in the presence of AECM

The effect of aqueous extract of coconut milk (AECM) on the aerobic survival of *Clostridium perfringens* was assessed using a colony counting assay. Cultures grown overnight under anaerobic conditions in Reinforced Clostridial Broth (RCB; HiMedia, India) were serially diluted (10^{-5}) in sterile distilled water to obtain a cell density suitable for colony enumeration. Aliquots (1 mL) of the diluted bacterial suspension were transferred to sterile Petri plates, after which molten Reinforced Clostridial Agar (RCA) supplemented with AECM was poured, gently mixed, and incubated aerobically at 37 °C for 24 h. Colony-forming units (CFU) were then enumerated. Identical plates prepared with RCA without AECM-supplementation were used as controls.

Statistical analysis

Aerobic survival of *Clostridium perfringens* grown in the presence and absence of AECM was compared using colony counts obtained from ten paired plates for each condition. Statistical significance was evaluated using a paired, two-tailed Student's t-test. All other assays were performed in triplicate, and results are presented as mean ± standard deviation. A p-value of < 0.05 was considered statistically significant. Statistical differences among concentrations on the DPPH radical scavenging activity were analyzed using one-way ANOVA followed by Tukey's post hoc test. Differences were considered significant at $p < 0.05$.

Results

Total phenolic content (TPC) of AECM

The Folin-Ciocalteu assay showed that the TPC of AECM expressed as gallic acid equivalents was 70.77 mg L^{-1}. It indicates the presence of measurable levels of aqueously extractable phenolic compounds in the AECM, and the value is consistent with previously reported phenolic levels in aqueous coconut milk fractions (Karunasiri et al., 2020; Gunawardane et al., 2025).

Antioxidant activity of AECM evaluated by the DPPH assay

The DPPH assay demonstrated a concentration-dependent increase in radical scavenging activity of AECM (**Table 1**). At 62.5 µg/mL, AECM exhibited 2.32 ± 0.89% inhibition, which increased progressively to 10.67 ± 1.39% at 1000 µg/mL. One-way ANOVA revealed a statistically significant effect of concentration on scavenging activity ($p < 0.05$). Post hoc analysis (Tukey's test) indicated that the highest concentration (1000 µg/mL) differed significantly from the lowest concentrations tested.

Table 1: DPPH radical scavenging activity of AECM

AECM concentration (µg/mL)	% Scavenging (mean ± SD, n = 3)	Significance
62.5	2.32 ± 0.89	c
125	3.48 ± 1.63	c
250	6.03 ± 1.43	bc
500	8.58 ± 0.70	ab
1000	10.67 ± 1.39	a

Values with different letters differ significantly ($p < 0.05$; one-way ANOVA followed by Tukey's test).

Although the antioxidant activity of AECM was markedly lower than that of ascorbic acid used as a positive control, the observed concentration-dependent increase confirms that AECM possesses measurable free radical scavenging capacity.

Protective effect of AECM on the aerobic survival of *Clostridium perfringens*

The effect of AECM on the survival of *Clostridium perfringens* under aerobic conditions was assessed using the plating-based assay. As expected, the exposure to atmospheric oxygen in the absence of AECM resulted in largely reduced survival rates measured in terms of CFU produced on control plates. Across ten replicates, the mean CFU count was recorded as 29.8 CFU per plate (Figure 1).

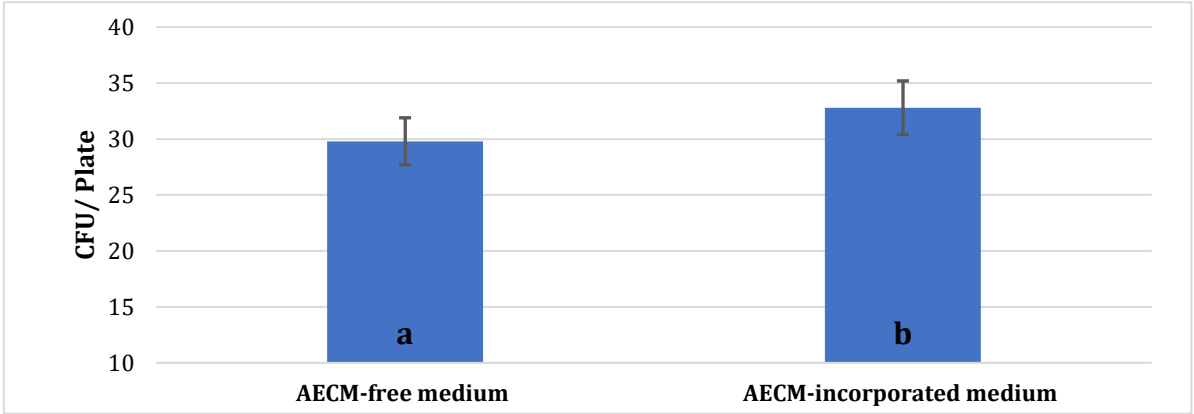

Figure 1. Effect of aqueous extract of coconut milk (AECM) on the aerobic survival of *Clostridium perfringens*.

Colony-forming units (CFU) recovered from aerobic pour plates prepared with Reinforced Clostridial Agar either without AECM (control) or supplemented with AECM (40% v/v). Bars represent mean CFU per plate ± SD ($n = 10$). Bars with different letters indicate statistically significant differences ($p < 0.05$; paired two-tailed Student's *t*-test).

In contrast, ten plates with the medium supplemented with AECM (40% v/v) produced a higher number of colonies with a mean CFU value of 32.8 CFU per plate under aerobic growth conditions (**Figure 1**). This corresponds to an approximate 10% increase in the survival rate relative to the parallel controls. Statistical analysis using a paired two-tailed t-test indicated that this difference was statistically significant ($p = 0.012$; n = 10). The observation, however, probably reflects short-term survival rather than sustained proliferation. In parallel anaerobic control experiments, CFU counts on plates with and without AECM were comparable, confirming that PECM at the concentration tested was neither inhibitory nor growth promotive under anaerobic conditions.

The results demonstrated that, although *C. perfringens* remains highly sensitive to oxygen, the presence of AECM during the aerobic growth moderately attenuates oxygen-induced cell death.

Discussion

The present study investigated whether AECM that contains phenolic compounds can offer any advantage to the survival of the anaerobe *Clostridium perfringens* under aerobic conditions. As expected from its known physiology, *C. perfringens* showed extremely poor survival when exposed to atmospheric oxygen, reflecting the well-known sensitivity of anaerobes to reactive oxygen species generated during oxygen exposure (Imlay, 2013; Smith, 1975). Irrespective of this basis of strong oxygen sensitivity, the observation that supplementation with AECM provided an increase, albeit small, in aerobic survival is biologically meaningful and informative.

Chemical analyses confirmed that AECM contains measurable levels of phenolic compounds and displays free-radical scavenging capacity, consistent with the well-established antioxidant properties of plant-derived phenolics (Shahidi & Ambigaipalan, 2015; Kumar et al., 2017). Nevertheless, as AECM is not a purified phenolic solution, it should be noted that the presence of residual proteins, peptides, or protein-phenolic complexes in it, and their possible contribution to the antioxidant activity, cannot be excluded. The concentration-dependent increase in DPPH radical scavenging activity displayed by AECM indicated that AECM preparation exhibits a modest free-radical scavenging capacity. These initial findings established that the AECM used in the subsequent biological assays had measurable antioxidant capacity capable of interacting with reactive oxygen species, as previously observed for several plant-derived phenolic compounds in microbial systems (Karunasiri et al., 2020; Gunawardane et al., 2025).

In aerobic survival assays, supplementation of the growth medium with AECM resulted in an approximately 10% and statistically significant ($p = 0.012$) increase in the number of CFU relative to aerobic controls lacking AECM. It reflects a genuine biological effect in terms of enhanced short-term survival during exposure to oxygen, rather than random variation.

Although the results are consistent with attenuation of ROS-mediated oxidative stress by antioxidant components present in AECM, the present study did not directly measure intracellular ROS levels or oxidative damage markers in *C. perfringens*. Therefore, the proposed mechanism should be interpreted as a plausible explanation rather than definitive mechanistic proof. Nevertheless, the demonstrated radical scavenging capacity of AECM in the DPPH radical

scavenging assay, together with the well-established role of ROS in mediating oxygen toxicity in anaerobes, supports the interpretation that antioxidant components of AECM may have contributed to the observed increase in survival (Imlay, 2013; Shahidi & Ambigaipalan, 2015).

In the pour-plate method, as the inoculum is mixed with molten agar rather than spread on the surface, a fraction of cells is embedded within the agar matrix, where oxygen diffusion is restricted (Madigan et al., 2018). This microenvironment may permit a limited survival of anaerobic bacteria even during aerobic incubation. In the aerobic assay of this study, it was observed that even the CFU produced in the plates were colonies trapped within the medium, where the oxygen penetration is expected to be limited, rather than those on the surface of the medium. Within this low-oxygen niche, the presence of AECM may have been able to reduce the oxidative stress sufficiently, reducing ROS-mediated cell death.

An alternative explanation for the observed effect is that AECM may have modified the extracellular redox environment of the medium rather than directly scavenging intracellular ROS. Phenolic compounds and other reducing agents present in plant-derived extracts may lower the redox potential of the surrounding medium or interact with reactive oxygen species before they reach the bacterial cell. In such a scenario, the protective effect would occur extracellularly, reducing oxidative stress experienced by the cells during the initial exposure to oxygen. The present experimental design does not allow a clear distinction between intracellular ROS scavenging and extracellular redox buffering, and both mechanisms remain plausible explanations for the modest increase in survival observed in this study. The present assay, therefore, evaluates survival during transient oxygen exposure followed by recovery within a microaerobic niche in the agar matrix. The observed increase in colony recovery in AECM-supplemented plates should therefore be interpreted as attenuation of oxygen-induced lethality during this initial stress period rather than the acquisition of oxygen tolerance and true aerobic growth capability.

In organisms such as *C. perfringens*, which lack major enzymatic antioxidant defenses, including superoxide dismutase and catalase, even modest reductions in oxidative burden may permit limited short-term survival (Imlay, 2013; McDonnell & Russell, 2009). This limited magnitude of the effect highlights the inability of exogenous antioxidants to substitute for efficient intracellular enzymatic antioxidant systems required for full tolerance to oxygen exposure, which in bacteria primarily depend on above mentioned enzymes that detoxify reactive oxygen species generated during oxygen metabolism (Imlay, 2013; McDonnell & Russell, 2009).

Beyond its clinical importance as a pathogen, *C. perfringens* has frequently been used as a model organism for studying anaerobic physiology and oxidative stress in bacteria. Understanding how exogenous compounds influence the survival of anaerobes under oxygen exposure may therefore have broader relevance for microbiological research. Natural phenolic compounds have increasingly been investigated for their ability to modulate oxidative stress in biological systems, including microorganisms (Shahidi & Ambigaipalan, 2015; Kumar et al., 2017). The present findings suggest that food-derived phenolics, such as those present in coconut milk, may influence the survival dynamics of anaerobic bacteria in environments where transient oxygen exposure occurs. The present findings, therefore, contribute to a broader question in microbial physiology: whether environmental antioxidants present in natural substrates can transiently modulate oxygen stress in anaerobes.

Conclusion

This study demonstrates that an aqueous extract of coconut milk (AECM) contains measurable levels of phenolic compounds and exhibits measurable chemical antioxidant activity, as shown by Folin–Ciocalteu and DPPH radical scavenging assays. When applied at a non-inhibitory concentration, AECM produced a small but statistically significant enhancement in the aerobic survival of the anaerobe *Clostridium perfringens*. This effect represents attenuation of oxygen-induced lethality.

The improved survival of the bacterium under aerobic conditions is attributed to the ability of AECM to reduce oxidative stress within microenvironments of restricted oxygen diffusion within the agar matrix. However, the contribution of residual proteins, peptides, or protein-phenolic complexes to the observed antioxidant activity cannot be ruled out.

Overall, these findings indicate that exogenous antioxidant components can transiently buffer ROS-mediated damage in anaerobic bacteria, although they cannot replace the absence of endogenous enzymatic antioxidant systems. This work augments the understanding of microbial oxidative stress physiology and highlights the limited yet meaningful role that natural antioxidants may play at the interface between anaerobic microorganisms and the aerobic environment.

Future studies should investigate the specific phenolic components responsible for the observed protective effect and directly measure intracellular ROS levels or markers of oxidative damage to clarify the mechanism involved. Such investigations may be particularly relevant to fields of food microbiology, anaerobic microbial physiology, and the development of natural antioxidant strategies for controlling oxidative stress in microbial systems.

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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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