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ORIGINAL ARTICLE

Phenolic Compounds from Coconut Milk Reduce Protein Carbonylation in *Escherichia coli* and Lactobacilli

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Abstract

Reactive Oxygen Species (ROS), including superoxide and hydroxyl radicals, can oxidize amino acid residues in proteins, leading to the formation of carbonyl groups and potential loss of protein function. ROS are generated intracellularly as a result of exposure to molecular oxygen, and aerobic organisms possess defence mechanisms, including antioxidant enzymes, to neutralize them. In addition, exogenous antioxidant compounds can contribute to cellular defence by scavenging ROS. Previous research has demonstrated that phenolic compounds display antioxidant properties. In this study, *Escherichia coli* and five *Lactobacillus* spp. were grown overnight in the presence of phenolic compounds extracted from coconut milk, and subsequently subjected to brief sublethal oxidative stress, after which protein carbonylation was measured. A statistically significant reduction in protein carbonylation was observed in bacteria pre-treated with coconut milk phenolics ($p < 0.05$). These results indicate that bacterial cells acquired enhanced antioxidant protection against oxidative stress following growth in the presence of coconut milk phenolics.

***Keywords**: Coconut milk, Escherichia coli, Lactobacilli, Phenolic antioxidants, Protein carbonylation, Oxidative stress,*

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1. Introduction

Proteins, a polymeric form of amino acids, are one of the major groups of biomolecules present in organisms. The unique three-dimensional structure of each protein, which is a factor that determines its function, is formed according to the sequence of amino acid monomers in the polymeric chain and the chemical interactions, such as hydrogen bonds and disulfide bridges between them, which fold it in a pattern unique to each protein. If amino acid monomers undergo chemical changes, the interactions between them are changed accordingly, causing changes in the three-dimensional structure of the protein. The effect of changing a protein's structure on its function depends entirely on the nature and location of the change in the structure, which may be no change at all or partial or complete loss of function (Alberts et al., 2022). One such chemical change possible to occur in amino acid monomers is carbonylation, which would generate carbonyl groups, such as aldehyde, ketone, or lactams, in the structure of the amino acid (Suzuki et al., 2010). Formation of carbonyl groups in amino acids can occur through reactions with Reactive Oxygen Species (ROS), which are toxic byproducts generated in cells under aerobic conditions. (Li et al., 2022). Protein carbonylation can also result from reactions between amino acids and lipid peroxidation products generated by ROS (Halliwell, B. and Gutteridge, J. 2015). Akagawa (2021) has described protein carbonylation as a type of post-translational modification in proteins caused by the action of ROS, where carbonyl groups are irreversibly formed in amino acids of already synthesized proteins.

Organisms, including bacteria, possess antioxidant defence mechanisms that neutralize ROS and prevent oxidative damage to biomolecules, a condition known as oxidative stress. As an aerobic microorganism, *Escherichia coli* contains an antioxidant mechanism, which mainly includes superoxide dismutase and catalase enzymes that neutralise two ROS, namely superoxide anion and hydroxyl free radical, respectively (Imlay, 2013). Lactobacilli are microaerophilic organisms and generally lack key antioxidant enzymes such as superoxide dismutase and catalase, limiting their tolerance to atmospheric oxygen. However, they are able to survive under low oxygen concentrations due to the presence of NADH oxidase and NADH peroxidase (Kang et al., 2013). In addition to the presence of inherent antioxidant mechanisms, certain antioxidant compounds absorbed into cells from outside are also known to augment the antioxidant action in cells. Di Meo et al. (2013) pointed out that polyphenolic compounds can neutralize free radicals, including ROS, by transferring hydrogen atoms or electrons to them, an activity described as scavenging of free radicals.

Coconut milk, the aqueous extract of the endosperm of the nut of the coconut palm (*Cocos nucifera*), is widely used as a food additive to enhance the taste and texture in many regions of the world, including Asia and the Caribbean. Coconut milk is prepared by homogenizing scraped coconut kernel (endosperm) with water and filtering the mixture through cheesecloth to obtain the liquid extract, which was named in this research, and many other research, including Karunasiri et al. (2020), as

domestically prepared coconut milk (DCM). The physical and chemical characteristics of DCM were described by many researchers, including Nadeeshani et al. (2015), and Karunasiri et al. (2020). Those characteristics include the presence of carbohydrates, proteins, and medium-chain saturated fatty acids. HPLC analysis performed by Karunasiri et al. (2020) showed that seven phenolic compounds, namely gallic acid, chlorogenic acid, para-hydroxybenzoic acid, caffeic acid, vanillic acid, syringic acid, and ferulic acid, were present in DCM as minor components. The mixture of those phenolic compounds extracted from DCM showed antioxidant properties *in vitro* in ferric reducing power (FRAP) assay and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay conducted by Karunasiri et al. (2020). Once grown overnight in the presence of a mixture of those compounds, the same authors demonstrated that the yeast, *Saccharomyces cerevisiae* showed enhanced antioxidant capabilities, in the event of induced sub-lethal oxidative stress, in terms of a reduction in the degrees of lipid peroxidation, protein carbonylation, and mitochondrial DNA damage, and an overall increase in the survival rate of the organism, all when compared with the organism that was not previously grown in the presence of the phenolics.

The objective of this research was to determine quantitatively any reduction in the degree of protein carbonylation separately in *Escherichia coli* and selected Lactobacilli by growing the bacteria overnight in the presence of a mixture of phenolic compounds extracted from DCM, and subsequently subjecting them to a brief sub-lethal oxidative stress. Considering that both *E. coli* and Lactobacilli are members of the human intestinal microbiome, the former a commensal and the latter a commensal with probiotic properties, the outcome of an investigation on the potential antioxidant activity of components of coconut milk in foods on those organisms would have implications for the knowledge on the human diet and intestinal microorganisms.

Although the antioxidant properties of phenolic compounds are well documented, limited studies have examined their direct protective effects against protein carbonylation in bacterial systems, particularly in gut-associated microorganisms. Furthermore, the potential role of coconut milk phenolics in enhancing oxidative stress resistance in bacteria remains underexplored. This study addresses this gap by quantitatively evaluating the effect of coconut milk-derived phenolics on protein carbonylation in Escherichia coli and *Lactobacillus* spp.

2. Materials and Methods

2.1. Preparation of DCM, and extraction and quantification of phenolics

Mature coconuts (12-14 months) collected from *Cocos nucifera* L. typica (tall type) coconut trees were used to prepare DCM following the method employed by Karunasiri et al. (2020). 100 g of scraped coconut kernel was mixed with 100 mL of distilled water, homogenized for 3 min using a kitchen blender. The homogenized mixture was squeezed by hand through a cheesecloth to separate the

liquid portion, which was labelled as DCM as previously reported by Karunasiri et al. (2020). To extract phenolic compounds from DCM into an aqueous phase, the lipids were removed by centrifugation at 1080 x g for 10 min. 1 mL of the resultant aqueous supernatant was mixed with 1 mL of methanol and 1 mL of chloroform, vortexed at 30 Hz for 1 min, centrifuged at 380 x g for 10 min, and the top methanol/water layer was separated as the methanolic extract of DCM. For quantification, it was evaporated, and the residue was dissolved in distilled water to reach a concentration of 20.0 mg mL^{-1}. The resultant aqueous extract of DCM phenolics was sterilized for microbiological work by filter-sterilization, using a 0.45 μm bacteriological filter.

2.2. Bacterial strains used to test the antioxidant activity of phenolics of DCM

The same bacterial strains used by Karunasiri et al. (2020) were used in this study. Five lyophilized cultures of Lactobacilli (*Lactobacillus acidophilus* ATCC 4356, *L. plantarum* DMBUK 113080, *L. lactis* ATCC 12315, *L. casei* ATCC 393, and *L. fermentum* ATCC 14931), and one *Escherichia coli* (ATCC 25922), were obtained from the culture collection, DMBUK (Department of Microbiology, University of Kelaniya Culture Collection). The dry culture of *E. coli* was reconstituted in Luria-Bertani (LB) broth, grown overnight at 37 ˚C at 250 rpm, and the cultures of Lactobacilli were grown overnight in deMan, Rogosa, and Sharpe (MRS) broth at 37 °C under anaerobic conditions. All bacterial cultures were then transferred to 20 % glycerol and maintained at -80 °C for further experiments.

2.3. Determining sub-lethal concentration of phenolics for microbiological work

High concentrations of phenolic compounds are bactericidal. Accordingly, for microbiological testing, the concentration of phenolic compounds that is below the bactericidal level was determined following the method used by Karunasiri et al. (2020). The LD50 concentrations of phenolic substances of DCM were determined by exposing bacterial cultures to a concentration series (0.0 – 0.9 mg mL^{-1}) of phenolics, and subsequently counting the number of colonies produced on the relevant medium of solid state. The concentrations of phenolic compounds that were used to grow bacteria overnight in the presence of them were kept below LD50 to prevent possible antibacterial action of phenolics.

2.4. Growth of bacteria with phenolics to introduce antioxidant properties to them

1 mL of sterile LB broth and 1 mL of sterile MRS broth were inoculated separately with loopfuls from glycerol stocks of *E. coli* and *Lactobacillus* spp. respectively. *E. coli* culture was incubated overnight at 37 ˚C on a shaker at 250 rpm, while that of *Lactobacillus* spp. was incubated overnight at the same temperature in an anaerobic jar under oxygen-free conditions. Samples taken from each culture were transferred separately into sterile 1 mL of the relevant broth medium until the optical density reached 0.5. An aliquot of 10 μL of the sterile phenolic solution was added separately into each broth

and incubated overnight at 37 ˚C under the relevant growth conditions of each bacterial strain. Instead of phenolics, 10 μL of distilled water was used in the controls of the experiment.

2.5. Induction of sub-lethal oxidative stress to bacterial cultures grown overnight with phenolics

According to the method described in Karunasiri et al. (2020), the broth cultures maintained overnight with phenolics were subjected to brief oxidative stress. In the process, the cultures were centrifuged at 100 x g for 3 min to harvest the cell pellets, washed with 1 mL of 1 x PBS to wash any residual antioxidants, centrifuged again at 100 x g for 3 min, and resuspended in 1 mL of 1 x PBS. 15 mM ferrous chloride was added, kept for 30 min at room temperature, and then hydrogen peroxide was added to each mixture to reach a final concentration of 2 mM. Expecting the generation of hydroxyl free radicals from hydrogen peroxide by the Fenton reaction and those hydroxyl free radicals causing oxidative stress on bacteria, the samples were kept for 1 hour at room temperature, *E. coli* at 250 rpm, while lactobacilli under anaerobic conditions. In place of hydrogen peroxide, sterile distilled water was used in the control experiments.

2.6. Determination of the degree of protein carbonylation under oxidative stress

The preventive effect of coconut milk phenolics against the carbonylation of proteins caused by hydroxyl free radicals in *E. coli* and Lactobacilli separately was quantitatively analysed by the method described by Reznick and Packer (1994). A colorimetric procedure of measurement of carbonyls, where the total carbonyl groups formed in amino acids of proteins by reacting with ROS were detected and quantified using the reaction between 2,4-dinitrophenylhydrazine (DNPH) and carbonyls, a reaction that produces the colour compounds, hydrazones. 1 mL of each bacterial cell suspension subjected to oxidative stress was centrifuged at 380 x g for 10 min, the cell pellet was resuspended in 1 mL of PBS buffer, centrifuged at 380 x g for 10 min, and the supernatant was removed thereby removing any residue of the oxidant. The cell pellet was subjected to cell lysis by the addition of 100 µL of lysis buffer (1% Tween 20, 0.1% SDS, and 50 mM pH 7 Tris-HCl) and resuspending the cell pellet in it by mixing at 40 Hz for 10 seconds on a vortex mixer. To harvest the proteins in the cell lysate, 250 µL of 30% w/v TCA was added and mixed at 40 Hz for 10 seconds using a vortex mixer, and the mixture was centrifuged at 300x g for 3 min to obtain the protein pellet. In order to cause carbonylation of the proteins, 250 µL of 10 mM DNPH in 2M HCl was added to the protein pellets, mixed, and incubated at room temperature for 60 min in the dark. Excess DNPH from the mixtures was removed by adding 250 µL of 20% TCA, centrifuging at 11000 x g for3 min, and the protein hydrazone pellets were separated, washed three times with a 1: 1 v/v ethanol: ethyl acetate mixture, and kept for 10 min. The mixtures were centrifuged at 11000 x g for 3 min to separate the protein pellets, and the pellets were reconstituted separately in 300 µL of 6 M guanidine hydrochloride solution, a chaotropic agent, to solubilize protein hydrazones. The mixture was then

centrifuged at 11000 x g for 3 min. The absorbance of the supernatant, which carries protein hydrazones, was measured at 370 nm using a UV-visible spectrophotometer (Multiskan Go, Thermo Scientific, Finland). The carbonylated protein contents were calculated using the following formula:

Carbonylated proteins (nmol mL^{-1}) = {(As -Ac)/ X} x 100

As = Absorbance of the sample

Ac = Absorbance of the control (guanidine solution)

X = Extinction Coefficient for DNPH (0.011 μM^{-1})

2.7. Statistical analysis

All experiments were performed in triplicate (n = 3). Data are presented as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by post hoc Tukey's test to compare differences between groups. A p-value ≤ 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Protein carbonyl formation in E. coli grown with phenolic compounds of coconut milk

When *E. coli* was grown in the presence of phenolic compounds extracted from DCM, there was a significant reduction in protein carbonylation under the subsequent sub-lethal oxidative stress induced by hydrogen peroxide with ferrous chloride. While *E. coli* grown without phenolics subsequently showed the highest degree of protein carbonylation, increasing phenolic concentration in the medium showed an increasingly reduced carbonylation (**Fig. 1**). The difference between *E. coli* grown without phenolics and those grown with 0.9 mg mL^{-1} phenolics was statistically significant (Mean = 9.90 and 7.37 nmol mL^{-1}, respectively; $p = 3.3 \times 10^{-4}$). It showed that *E. coli* acquired a significant antioxidant activity against protein carbonylation during its growth with DCM phenolics.

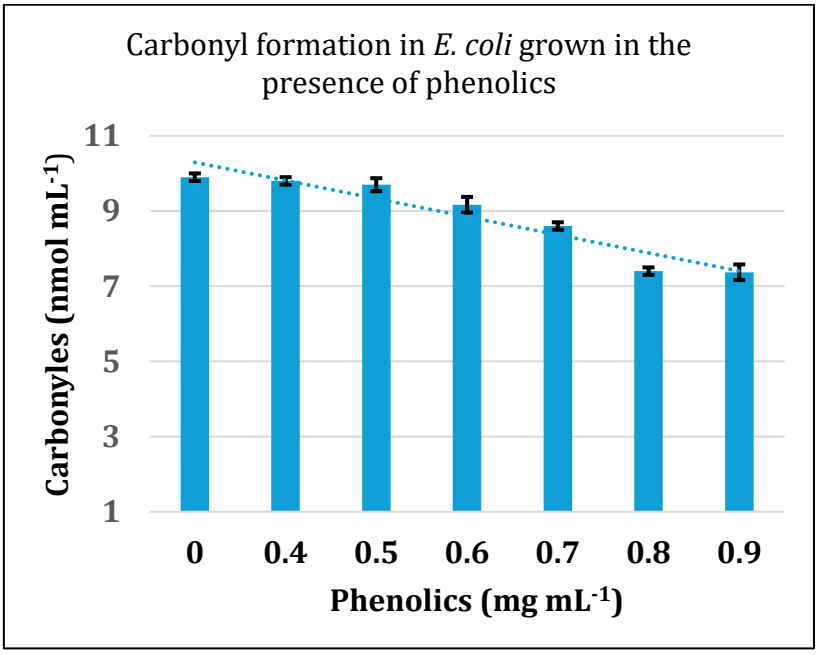

Figure 1. Prevention of protein carbonylation in *E. coli* by phenolic compounds extracted from DCM. Bars with different letters (a, b) indicate statistically significant differences ($p < 0.05$). Values represent mean ± SD (n = 3).
(*Increasing concentration of phenolics increasingly reduced the degree of carbonylation*)

3.2. Protein carbonyl formation in Lactobacillus spp. grown with phenolic compounds of coconut milk

The five species of Lactobacilli tested in this study also showed that the phenolic compounds extracted from DCM could reduce the degree of protein carbonylation in those bacteria. **Table 1** gives the amounts of protein carbonyls formed in each *Lactobacillus* sp. grown separately in the presence of increasing concentrations of CDM phenolics. While all the tested Lactobacilli produced the highest amounts of carbonyls under oxidative stress after growth in the absence of phenolic compounds, those grown with a higher concentration of phenolic compounds showed lower amounts of carbonyls.

Table 1. The effect of phenolic compounds on protein carbonylation

	Protein carbonyl formation in Lactobacilli (nmol mL^{-1}) ± SD				
Phenolics (mg mL^{-1})	***L. acidophilus***	***L. plantarum***	***L. lactis***	***L. casei***	***L. fermentum***
0	5.09 ± 0.23	5.03 ± 0.11	5.03 ± 0.25	5.10 ± 0.13	4.81 ± 0.55
0.4	4.93 ± 0.05	4.82 ± 0.14	4.86 ± 0.04	4.78 ± 0.11	4.86 ± 0.12
0.5	4.53 ± 0.11	4.49 ± 0.08	4.58 ± 0.22	4.28 ± 0.07	4.77 ± 0.10
0.6	3.24 ± 0.10	3.81 ± 0.08	3.12 ± 0.22	3.30 ± 0.17	3.63 ± 0.46
0.7	2.85 ± 0.24	3.23 ± 0.11	2.37 ± 0.14	3.08 ± 0.15	3.19 ± 0.23
0.8	1.80 ± 0.14	1.75 ± 0.23	1.55 ± 0.14	1.44 ± 0.12	1.69 ± 0.05
0.9	0.43 ± 0.10	0.53 ± 0.06	0.45 ± 0.09	0.46 ± 0.10	0.44 ± 0.11

Fig. 2 illustrates the lowering degree of carbonylation in Lactobacilli subjected to oxidative stress after growth with increasing concentration of phenolic compounds in the medium. The mean carbonyl content of *Lactobacillus* spp. grown without phenolics (Mean ≈ 5.01 nmol mL^{-1}) was significantly higher than that of those grown with 0.9 mg mL^{-1} phenolics (Mean ≈ 0.46 nmol mL^{-1}; $p < 0.05$), showing that the growth in the presence of DCM phenolics significantly enhanced the antioxidant activity of the bacteria to face against the oxidative stress to which they were subsequently subjected.

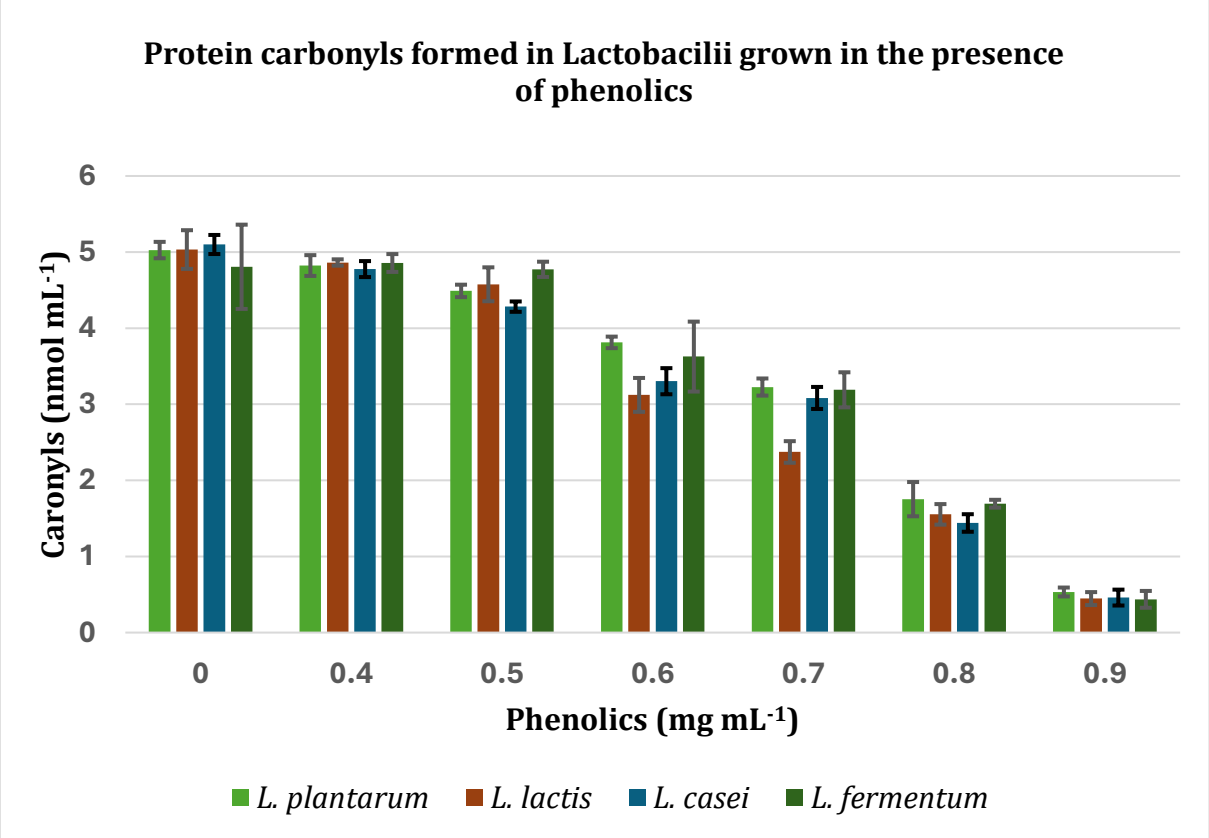

Figure 2. Prevention of protein carbonylation in *Lactobacillus* spp. by phenolic compounds extracted from DCM. Bars represent mean ± SD (n = 3). Values at 0 mg mL^{-1} differ significantly from those at 0.9 mg mL^{-1} ($p < 0.05$).
(*Increasing concentration of phenolics increasingly reduced the degree of carbonylation*)

Both *E. coli* and most Lactobacilli are intestinal organisms, and it should be noted, therefore, that the phenolic compounds present in coconut milk, once consumed, may reach the intestinal environment where those bacteria live. The results showed that those phenolic compounds have the potential to prevent oxidative damage, in terms of protein carbonylation, in those bacteria under oxidative stress conditions. Accordingly, *E. coli* and Lactobacilli, two of the most abundant bacterial members of the intestinal flora, may benefit from the consumption of coconut milk.

4. Conclusions

The mixture of phenolic compounds extracted from DCM, once absorbed into cells by overnight growth in the presence of them, showed separately in *Escherichia coli* and *Lactobacillus* spp. a significant increase ($p < 0.05$) in antioxidant protection, in terms of decreased degree of carbonylation of proteins under sub-lethal oxidative stress briefly induced on those bacteria by hydrogen peroxide together with ferrous chloride.

These findings suggest that coconut milk-derived phenolic compounds can reduce protein carbonylation in bacterial systems under controlled experimental conditions. Further studies are required to determine whether similar effects occur in the human intestinal environment.

Conflicts of interest: The authors have no conflicts of interest regarding this publication.

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