Anti-collagenase and Gut Microbiota: Potential Modulatory Properties of Enzymatically Processed By-products from *Cacalia firma* Leaves

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Cacalia firma is known for its unique fragrance, and its young shoots are traditionally used as culinary herbs and for their active constituents. The leaves of *C. firma* and the residual material remaining after enzymatic treatment exhibit both nutritional and therapeutic potentials. Despite this, the application of enzyme-treated *C. firma* leaf residues in functional food development has received limited attention. According to existing research, these residues are abundant in health-promoting compounds, such as antioxidants and polyphenols. Moreover, preliminary *in vitro* studies have suggested their potential to alleviate gut microbiota-modulatory problems. Expanding studies in this field could support the future use of enzyme-treated *C. firma* residues as valuable components in functional food formulations. This study aims to detail the bioactive profiles and nutraceutical potential of various enzyme-treated *C. firma* leaf residues and assess their applicability in health-oriented food products.

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Keywords: Cacalia firma leaf; Gut microbiota-modulatory potential; Enzyme; By-product; Recycling

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INTRODUCTION

Cacalia firma (*C. firma*), which is taxonomically classified as *C. firma* Komar. or *Parasenecio firmus* (Kom.) Y. L. Chen, is a perennial herbaceous species belonging to the Asteraceae family, predominantly distributed in high-altitude forested regions of northeastern Korea and parts of China. The plant typically grows to a height of 1 to 2 m (Yoon *et al*. 2014). Its young shoots, known for their distinctive aromatic profile, are traditionally consumed as herbal food ingredients. Their unique scent is largely attributed to its content of caffeoylquinic acid derivatives (Choi *et al*. 2011). Notably, extracts from *C. firma* have exhibited strong peroxynitrite scavenging activity. Peroxynitrite, a reactive nitrogen species formed from the interaction of nitric oxide and superoxide, is known to induce lipid and protein peroxidation, cytotoxicity, and acute neurotoxic effects (Lee *et al*. 2011).

The critical role of antioxidants in mitigating early-stage oxidative damage has sparked growing interest in identifying potent antioxidant compounds from botanical sources (Kontoghiorghes and Kontoghiorghe 2019). Numerous studies have investigated antioxidant-rich constituents in edible plants, spices, and traditional herbs (Yanishlieva *et al*. 2006), and recent efforts have expanded to include previously underutilized species (Gulcin 2020; Gahtori *et al*. 2024).

Enzymatic processing has been widely employed in the biomaterials and food industries to enhance functional properties such as digestibility, mineral bioavailability, and the release of bioactive peptides (Arte *et al*. 2015). Additionally, fermentation processes—particularly those utilizing lactic acid bacteria—have shown promise in

enhancing the antimicrobial and antioxidative properties of plant-derived substrates (Sabio *et al*. 2021). These microbial enzyme systems, often originating from spontaneous fermentations, are well-adapted to metabolize diverse plant matrices (Radu *et al*. 2016; Hatti-Kaul *et al*. 2018). However, their efficiency can be significantly influenced by intrinsic plant compounds, especially phenolics, which may act as enzyme inhibitors or require specialized microbial tolerance for effective biotransformation (Casa *et al*. 2003; Domingues *et al*. 2022). Notably, enzymatic hydrolysis can activate microbial enzymes such as *β*-glucosidase, cellulase, and tannase, which facilitate the breakdown of complex phytochemicals. This metabolic adaptation supports the detoxification and functional conversion of polyphenols, contributing to the stress resilience and metabolic flexibility of microbial communities (Kumar *et al*. 2023).

Although polysaccharides derived from natural sources have been extensively studied for their bioactive properties (Zong *et al*. 2012; Karaki *et al*. 2016), relatively little attention has been paid to the utilization and characterization of residues generated from enzymatic treatment of plants. In particular, research on the biofunctional potential of such residues from *C. firma* remains limited.

In this study, residues obtained from enzymatic treatment of *C. firma* leaves were isolated and evaluated for their biological activity. This study investigated their antioxidant capacity and their modulatory effects on probiotic growth under in vitro conditions, with a focus on their potential for gut microbiota-modulatory potential functionality and the development of novel health-promoting ingredients.

EXPERIMENTAL

Materials

Fresh leaves of *C. firma* were obtained from the Academic Forest managed by Gyeongsang National University (501 Jinjudearo, Jinju-si, Republic of Korea). Immediately after collection, the samples were cryogenically dried using a freeze dryer (Model FD5508, ILSHIN, Korea) operating at –80 °C to preserve their biochemical integrity. The dried leaves were then pulverized using a stainless-steel high-speed grinder (3000 W, 60 Hz, 36,000 rpm), and the resulting powder was passed through a 40-mesh sieve to ensure uniform particle size.

For enzymatic treatment, five commercially available cell wall–degrading enzymes were selected: Cellic® CTec3 HS, Celluclast® 1.5 L, Viscozyme® L, Pectinex® Ultra SP-L, and Amylase AG, all procured from Novozymes A/S (Bagsværd, Denmark).

Enzyme Treatment of *C. firma* under Various Conditions

C. firma was purchased from a farm in Cheongju-si, Chungcheongbuk-do, South Korea, and freeze-dried immediately after purchase using a -80 ℃ freeze dryer (FD5508, ILSHIN, Korea). *C. firma* was separated into leaves, branches, and roots, and the leaves were used in this study. The freeze-dried *C. firma* leaves were powdered using a stainless steel grinder with performances of 3000 W, 60 Hz, and 36,000 r/min. The powdered leaves were passed through a 40 mesh sieve to obtain a uniform powder size. Enzyme treatment of the leaf powder was performed using Cellic CTec3 HS, Celluclast 1.5 L, Viscozyme L, Pectinex ultraSP-L, and Amylase AG. The enzyme treatment conditions were as follows: pH range: pH 3.0 to 6.0, temperature range: 40 to 60 ℃, treatment time: 24 to 72 h, and enzyme treatment concentration: 40 unit/mL. After enzyme treatment, the solution was centrifuged at 4,500 rpm for 10 minutes

(Union 32R Plus, Hanil, Korea) and separated into an enzyme-treated solution and an enzyme-treated residue. While the hydrolyzed solution is typically used after enzyme treatment, this study confirmed the utility of the discarded residue.

Color characteristics of the powdered enzymatic by-products were evaluated using a chroma meter (model CR-400, Konica Minolta, Japan). The analysis was based on the CIE *Lab** color space, where *L** indicates brightness, *a** denotes the red–green spectrum, and *b** represents the yellow–blue axis. Measurements were carried out in three replicates for each sample to ensure consistency.

By-products Extraction after Enzymatic Process

To prepare the ethanolic extract, 1.0 g of dried powder obtained from the enzymatic by-products was mixed with 10 mL of ethanol and shaken in the dark for approximately 2 h using a rotary shaker. Following incubation, the mixture was centrifuged at 1,500 × g for 10 min, and the resulting supernatant was collected for subsequent analytical procedures.

Content of Total Polyphenols

The concentration of total phenolic compounds in the plant extracts was determined using a spectrophotometric assay based on the Folin–Ciocalteu colorimetric method, with minor modifications to the procedure described by Astill *et al.* (2001). Briefly, 0.5 mL of each extract was mixed with an equal volume of Folin–Ciocalteu reagent and subsequently diluted with 7 mL of distilled water. The mixture was thoroughly vortexed and allowed to stand at room temperature for 3 minutes. Then, 2 mL of 20% sodium carbonate solution was added. The reaction was carried out in the dark for 60 minutes, after which the absorbance was measured at 732 nm using a blank control containing no extract. A standard calibration curve prepared with gallic acid was used to quantify the phenolic content, and the results were expressed as milligrams of gallic acid equivalents per gram of sample (mg GAE/g).

Flavonoid Content Analysis

The total flavonoid content was determined by employing the aluminum chloride colorimetric technique. A volume of 500 µL of each sample extract (1,000 µg/mL) was combined with 500 µL of a 2% (w/v) $AlCl_3$ solution. The resulting solution was left to incubate at ambient temperature for 10 minutes with occasional agitation. Absorbance was subsequently recorded at 415 nm using a UV–Vis spectrophotometer (U-3000, Hitachi, Japan) against a reference solution prepared identically but omitting aluminum chloride. Quantification was performed using a standard calibration curve generated with quercetin, and the flavonoid content was expressed as milligrams of quercetin equivalents per gram of dry sample (mg QE/g).

DPPH Radical Scavenging Assay

To evaluate radical scavenging activity, 2.0 mL of 0.2 mM DPPH solution prepared in methanol was mixed with 400 µL of the sample supernatant and 1.6 mL of deionized water. The mixture was briefly vortexed and incubated at room temperature for 30 minutes in the dark. As a control, a solution containing 2.0 mL of methanol and 2.0 mL of DPPH reagent was prepared under identical conditions. The absorbance was subsequently measured at 517 nm using a UV–Vis spectrophotometer (U-3000, Hitachi, Japan).

ABTS Free Radical Scavenging Assay

ABTS radicals were generated by mixing equal volumes of 7 mM ABTS stock solution and 2.4 mM potassium persulfate solution, followed by incubation in the dark at room temperature for 12 hours to allow radical formation. The resulting $ABTS^{+}$ solution was then diluted with ethanol until an absorbance of 0.70 ± 0.02 at 734 nm was obtained. For the assay, 6.0 mL of the diluted radical solution was mixed with 40 µL of the sample supernatant, and the absorbance was immediately measured at 734 nm using a UV–Vis spectrophotometer (U-3000, Hitachi, Japan), with ethanol used as the blank.

Evaluation of the Activity of Enzymatic Process By-products against Gut Lactobacilli

To evaluate the gut microbiota-modulatory potential activity of the enzymatically processed *C. firma* leaf residues, eight different strains of intestinal lactic acid bacteria were selected for the assay. The specific bacterial strains utilized in this investigation are listed in Table 1.

Table 1. Lactobacillus Used to Test the Efficacy of Gut Microbiota-Modulatory Potential

No.	Lactic Acid Bacteria	KCTC	Resource Type	Temperature (ºC)
1	*Lactococcus gasseri*	3181	Bacteria	37 ºC
2	*Lactobacillus casei*	13086	Bacteria	37 ºC
3	*Lactobacillus acidophilus*	3140	Bacteria	37 ºC
4	*Lactobacillus delbrueckii subsp. bulgaricus*	3635	Bacteria	37 ºC
5	*Enterococcus faecium*	13225	Bacteria	37 ºC
6	*Enterococcus faecalis*	2011	Bacteria	37 ºC
7	*Bacillus subtilis*	1023	Bacteria	30 ºC
8	*Bacillus coagulans*	3625	Bacteria	37 ºC

The lactic acid bacterial strains used in this study were obtained from the microbial seed bank of the National Institute of Agricultural Sciences, under the Rural Development Administration of Korea (https://genebank.rda.go.kr/microbeMain.do). Each of the eight strains was initially suspended in MRS broth (Difco 288130, BD, USA), streaked onto MRS agar plates (Difco 288210, BD, USA), and incubated at 37 °C under controlled conditions. The detailed compositions of the media used for bacterial cultivation are provided in Table 2.

Table 2. Components and Properties of MRS Broth and MRS Agar used in Lactic Acid Bacteria Cultures

Media	MRS broth	MRS agar
Composition		
Proteose peptone	10.00	10.00
Beef extract	10.00	10.00
Yeast extract	5.00	5.00
Dextrose	20.00	20.00
Polysorbate 80	1.00	1.00
Ammonium citrate	2.00	2.00

Sodium acetate	5.00	5.00
Magnesium sulfate	0.10	0.10
Manganese sulfate	0.05	0.05
Dipotassium phosphate	2.00	2.00
Agar		15.00
Properties		
pH	pH 6.5 ± 0.2	pH 6.5 ± 0.2
How to use	55 g/L	70 g/L

To evaluate the growth-promoting effect of *C. firma* enzymatic residues on lactic acid bacteria, 200 mg of enzyme-treated by-product was incorporated into 10 mL of MRS agar and poured into sterile Petri dishes. A single colony of each lactic acid bacterial strain, pre-suspended in 1.0 mL of MRS broth, was used as the inoculum. Subsequently, 100 µL of the bacterial suspension was evenly spread over the surface of each prepared plate. The cultures were incubated at 37 °C for 72 h, and bacterial growth was assessed by counting the visible colonies formed on the medium.

Collagenase Inhibitory Activity

The first step was to add 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg (0.3 mg/mL) and collagenase (0.2 mg/mL) to a 0.1 M Tris-HCl solution (pH 7.5) containing 4 mM $CaCl_2$ to prepare the substrate solution and enzyme solution. The enzyme solution was diluted 8-fold, and 125 µL of substrate solution, 75 µL of enzyme solution, and 50 µL of sample were mixed and reacted at 37°C for 20 minutes. After the reaction, 250 µL of 6% citric acid was added to stop the reaction, followed by the addition of 750 µL of ethyl acetate. The last step was to transfer 200 µL of the supernatant to a 96-well plate and measure the absorbance at 320 nm to evaluate the collagenase inhibitory activity.

Statistical Analysis

Statistical analysis of the experimental data was performed using one-way ANOVA in SAS software (version 9.2; SAS Institute Inc., Cary, NC, USA). Post hoc comparisons among treatment means were conducted using Duncan's multiple range test. All measurements were performed in triplicate or more, and the results are presented as the mean ± standard deviation (SD). A p-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Evaluation of Chromaticity of Enzymatic Process By-Products of *C. firma* Leaves

Chromatic variation in the enzyme-treated by-products of *C. firma* leaves was evaluated by comparing visual attributes across different enzyme treatments. Figure 1 presents the color characteristics of the residues following enzymatic processing. A general decrease in L^* values was observed in most treatment groups after 72 hours of incubation, with statistically significant reductions ($p < 0.001$) particularly noted in samples treated with Pectinex Ultra SP-L and Viscozyme L. The reduction in L^* values indicates a shift toward a darker appearance, suggesting that enzymatic treatment affects brightness. This darkening effect is likely associated with the presence of phenolic compounds and their oxidative polymerization, which contribute to color transformation, as previously reported by Athanasiou *et al*. (2024).

Consistent with the findings of Lotfi *et al*. (2015), enzymatic treatment was shown to influence color properties. The *a** values remained relatively stable after enzymatic processing, likely due to the absence of red pigments in the substrate matrix. In contrast, *b** values significantly increased ($p < 0.001$), indicating an enhancement in yellow chromaticity.

The observed increase in the *b** value is presumed to result from the enzymatic degradation of *C. firma* leaf components, leading to noticeable changes in color characteristics. Previous studies have reported that enzymatic modification—particularly *via* lactic acid bacterial fermentation followed by acidification—is associated with the formation of cis-isomeric compounds, which are positively correlated with enhanced yellow coloration (Liu *et al*. 2025). In line with these findings, the present study demonstrated a significant increase in the *b** parameter, suggesting that the intensified yellowness in the enzyme-treated by-products may be attributed to structural transformations involving cis-isomer formation.

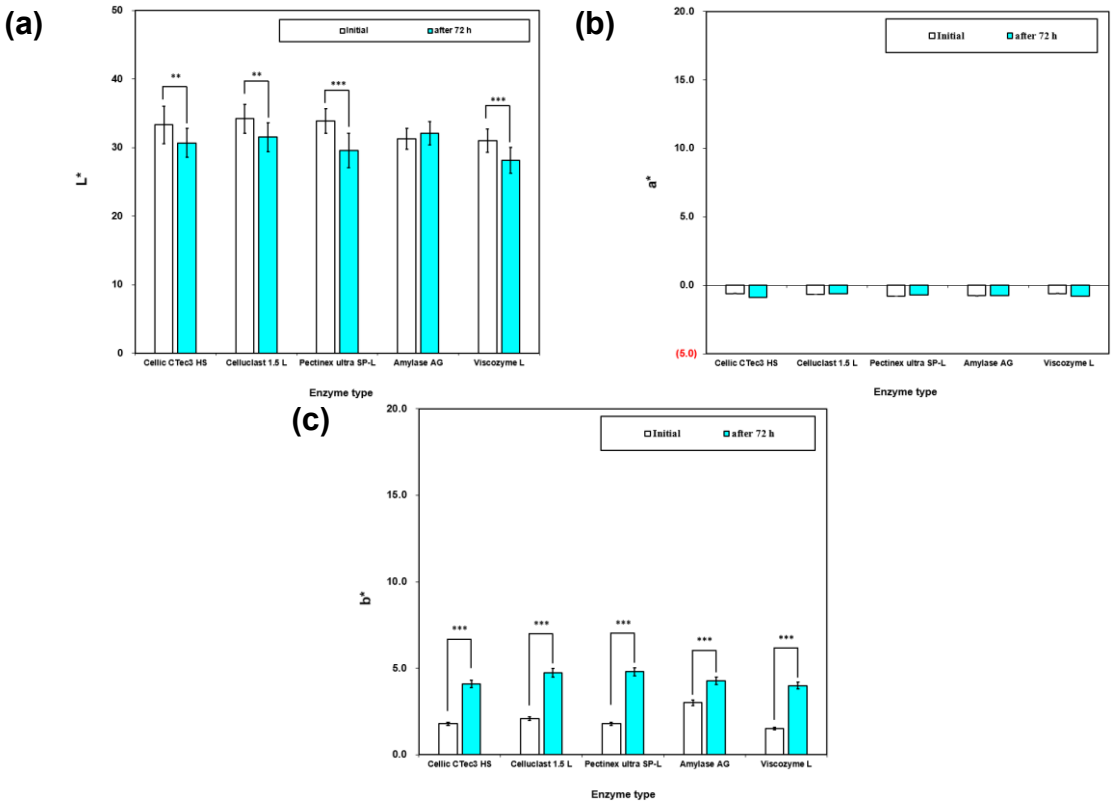

Fig. 1. Color intensity of enzymatic process by-products according to the enzyme type treated ((a): *L** value, (b): *a** value, (c): *b** value; fixed variables: Temperature 50 °C, pH 5, treatment time: 72 h; **p < 0.01, ***p < 0.001)

Bioactivity of Enzymatic Processing By-products

The effects of enzymatic treatment conditions on the polyphenol and flavonoid content, as well as the DPPH and ABTS radical scavenging capacities of *C. firma* leaf residues, are summarized in Table 3. Given the chemical complexity of plant-derived samples, it is widely accepted that multiple analytical assays are required to comprehensively assess antioxidant activity (Tan and Lim 2015). Among the tested enzymatic protocols, the residue treated with Viscozyme L under conditions of pH 5.0, 50 °C, and 72 hours exhibited the highest level of bioactivity. Previous studies have reported that Viscozyme-treated extracts often display stronger antioxidant properties than those treated with Pectinex, which is likely due to an increased abundance of low molecular weight flavonoids in their aglycone form (Hwang *et al*. 2023). Notably, the

solid fraction remaining after enzymatic hydrolysis also retained considerable antioxidant potential, suggesting that by-products from enzymatic processing may serve as valuable functional materials.

The DPPH radical scavenging activity of Piper betel leaf ethanol extract has been reported to range from 7 to 71% (Rathee *et al*. 2006). Kiritsakis *et al*. (2010) reported 7 to 90% DPPH radical scavenging after sequential extraction of olive leaves with petroleum ether, dichloromethane, and methanol. DPPH radical scavenging activity of leaf extracts from *Dorystoechas hastata* was 3 to 70%, and the extracts were obtained using diethyl ether, ethanol, and water (Karagözler *et al*. 2008). This study is interesting because it showed 24% DPPH radical scavenging using residues discarded after enzyme treatment without chemical solvents.

Table 3. Biologically Activity in Enzymatic Process By-products of *C. firma* Leaves under Various Conditions

Treatment Condition		Cellic CTec3 HS	Celluclast 1.5 L	Pectinex ultra SP-L	Amylase AG	Viscozyme L
Temperature (ºC)	pH					
Total Polyphenol Content (mg GAE/g)						
40 ºC	pH 3	2.84	2.91	2.90	2.92	2.91
	pH 4	2.88	2.88	2.93	2.90	2.91
	pH 5	2.89	2.89	2.92	2.89	2.91
	pH 6	2.87	2.86	2.88	2.89	2.90
50 ºC	pH 3	2.89	2.93	2.89	2.93	2.93
	pH 4	2.89	2.91	2.88	2.91	2.92
	pH 5	2.90	2.90	2.89	2.92	2.91
	pH 6	2.92	2.87	2.87	2.90	2.91
60 ºC	pH 3	2.92	2.92	2.93	2.90	2.88
	pH 4	2.89	2.89	2.94	2.90	2.93
	pH 5	2.88	2.89	2.93	2.94	2.89
	pH 6	2.88	2.92	2.92	2.90	2.87
Flavonoid Content (mg QE/g)						
40 ºC	pH 3	0.237	0.243	0.242	0.243	0.243
	pH 4	0.240	0.240	0.244	0.242	0.243
	pH 5	0.241	0.241	0.244	0.241	0.243
	pH 6	0.239	0.239	0.240	0.241	0.242
50 ºC	pH 3	0.241	0.244	0.241	0.244	0.244
	pH 4	0.241	0.242	0.240	0.243	0.243

	pH 5	0.242	0.241	0.241	0.243	0.242
	pH 6	0.243	0.239	0.239	0.242	0.242
60 ºC	pH 3	0.243	0.243	0.244	0.242	0.240
	pH 4	0.241	0.241	0.245	0.242	0.244
	pH 5	0.240	0.241	0.245	0.245	0.241
	pH 6	0.240	0.243	0.244	0.242	0.239
DPPH Free Radical Scavenging (%)						
40 ºC	pH 3	23.67	24.26	24.15	24.32	24.27
	pH 4	23.97	23.97	24.42	24.18	24.27
	pH 5	24.10	24.07	24.36	24.10	24.27
	pH 6	23.95	23.85	23.98	24.11	24.20
50 ºC	pH 3	24.06	24.42	24.12	24.44	24.42
	pH 4	24.10	24.22	24.01	24.27	24.30
	pH 5	24.18	24.13	24.10	24.30	24.24
	pH 6	24.34	23.93	23.89	24.17	24.23
60 ºC	pH 3	24.33	24.34	24.40	24.15	24.01
	pH 4	24.09	24.08	24.52	24.19	24.39
	pH 5	24.01	24.10	24.46	24.53	24.07
	pH 6	23.97	24.33	24.37	24.17	23.88
ABTS Free Radical Scavenging (%)						
40 ºC	pH 3	18.94	19.41	19.32	19.46	19.42
	pH 4	19.17	19.17	19.54	19.35	19.41
	pH 5	19.28	19.26	19.49	19.28	19.42
	pH 6	19.16	19.08	19.19	19.29	19.36
50 ºC	pH 3	19.25	19.53	19.30	19.55	19.54
	pH 4	19.28	19.37	19.21	19.42	19.44
	pH 5	19.35	19.31	19.28	19.44	19.39
	pH 6	19.47	19.15	19.11	19.34	19.38
60 ºC	pH 3	19.47	19.47	19.52	19.32	19.21
	pH 4	19.27	19.26	19.62	19.35	19.51
	pH 5	19.20	19.28	19.56	19.63	19.25
	pH 6	19.18	19.46	19.49	19.34	19.10
SEM (±)		0.000	0.000	0.000	0.000	0.000
CV (%)		0.748	0.724	0.877	0.545	0.644

CV: Coefficient of variation in percentage

Effect of Enzymatic Processing By-Products on Activity of Lactic Acid Bacteria

Table 4 presents the results of the lactic acid bacteria activity assay in response to treatment with enzyme-processed *C. firma* leaf residues. Among the eight bacterial strains evaluated, the sample treated with Viscozyme L showed the most pronounced enhancement in bacterial growth. The high level of bioactivity observed in the Viscozyme L-treated by-product is presumed to be due to the presence of bioavailable polyphenols and flavonoids, which may act as growth-promoting substrates for lactic acid bacteria, as supported by the findings of Zhang *et al*. (2025).

Table 4. Influence of Enzyme Type on Viable Cell Number of Lactic Acid Bacteria in *C. firma* Enzyme-treated Dried By-product

Lactic Acid Bacteria	Control (only MRS agar)		Cellic CTec3 HS		Celluclast 1.5 L		Pectinex ultra SP-L		Amylase AG		Viscozyme L	
	Ave.	S. D	Ave.	S. D	Ave.	S. D	Ave.	S. D	Ave.	S. D	Ave.	S. D
Lactococcus gasseri	2.7	0.2	4.1	0.1	2.6	0.2	5.3	0.2	3.3	0.1	6.2	0.5
Lactobacillus casei	2.5	0.1	3.5	0.3	3.1	0.5	6.8	0.2	5.2	0.3	7.1	0.2
Lactobacillus acidophilus	1.5	0.2	1.5	0.2	1.2	0.1	2.0	0.2	1.7	0.1	5.8	0.3
Lactobacillus delbrueckii subsp. bulgaricus	2.0	0.3	2.4	0.4	2.0	0.2	3.4	0.1	3.6	0.3	5.8	0.4
Enterococcus faecium	3.3	0.1	3.8	0.1	3.1	0.1	9.8	0.2	3.7	0.2	9.6	0.3
Enterococcus faecalis	4.2	0.2	4.7	0.3	3.8	0.1	8.3	0.2	5.4	0.2	9.1	0.1
Bacillus subtilis	4.5	0.1	7.3	0.2	4.8	0.1	6.0	0.3	4.6	0.2	9.8	0.1
Bacillus coagulans	3.5	0.2	4.5	0.3	3.9	0.1	7.4	0.4	5.6	0.2	7.7	0.3

The effect of varying concentrations of Viscozyme L-treated *C. firma* enzymatic residues on lactic acid bacterial activity was evaluated, and the results are summarized in Table 5. Bacterial growth showed a positive correlation with the concentration of by-products, reaching its peak at 200 mg per 10 mL of MRS agar medium.

Table 5. Effect of Enzyme Treatment Residue Dosage on Viable Cell Number of Lactic Acid Bacteria

Lactic Acid Bacteria	Control (only MRS agar)		mg /10 mL MRS Agar									
			100 mg		200 mg		300 mg		400 mg		500 mg	
	Ave.	S. D	Ave.	S. D	Ave.	S. D	Ave.	S. D	Ave.	S. D	Ave.	S. D
Lactococcus gasseri	2.7	0.2	3.1	0.1	6.2	0.2	6.2	0.2	6.3	0.1	6.5	0.5
Lactobacillus casei	2.5	0.1	4.5	0.3	7.1	0.5	7.0	0.2	7.2	0.3	7.5	0.2
Lactobacillus acidophilus	1.5	0.2	2.8	0.2	5.8	0.1	5.9	0.2	6.0	0.1	6.3	0.3
Lactobacillus delbrueckii subsp.	2.0	0.3	4.1	0.4	5.8	0.2	5.8	0.1	5.9	0.3	6.2	0.4

bulgaricus												
Enterococcus faecium	3.3	0.1	3.6	0.1	9.6	0.1	6.7	0.2	6.8	0.2	7.2	0.3
Enterococcus faecalis	4.2	0.2	6.5	0.3	9.1	0.1	9.2	0.2	9.3	0.2	9.6	0.1
Bacillus subtilis	4.5	0.1	6.2	0.2	9.8	0.1	9.8	0.3	9.8	0.2	10.5	0.1
Bacillus coagulans	3.5	0.2	5.5	0.3	7.7	0.1	7.6	0.4	7.6	0.2	8.0	0.3

At higher concentrations (*e.g.*, 300 mg), no further enhancement in bacterial activity was observed, suggesting that 200 mg/10 mL is the optimal supplementation level for promoting lactic acid bacterial proliferation under the tested conditions. This study evaluated gut microbiota-modulatory potential through in vitro experiments. Further *in vivo* testing is needed to clearly verify the anti-constipation effect.

The observed antioxidant, anti-collagenase, and gut microbiota-modulatory effects of the enzymatically treated *C. firma* leaf residues may be attributed to several interrelated molecular mechanisms. Enzymatic hydrolysis likely enhanced the release of polyphenols and flavonoids in their aglycone forms, which are known to possess increased bioavailability and stronger free radical scavenging capabilities. These compounds can directly neutralize reactive oxygen species (ROS) and inhibit oxidative stress pathways *via* donation of hydrogen atoms or electrons, thereby contributing to the antioxidant potential observed in both DPPH and ABTS assays (Parcheta *et al*. 2021). In terms of gut microbiota-modulatory activity, phenolic compounds are known to influence the composition and growth of beneficial gut bacteria such as *Lactobacillus* and *Bifidobacterium* spp. The breakdown of complex polyphenolic structures during enzymatic treatment may generate low-molecular-weight metabolites that serve as prebiotic substrates, promoting the selective proliferation of probiotic strains (Makarewicz *et al*. 2021). The anti-collagenase activity observed in Viscozyme L-treated samples is also likely due to the presence of specific phenolic compounds, which are known to chelate metal ions at the active sites of matrix metalloproteinases, such as collagenase, thereby inhibiting their enzymatic activity (Borkakoti 2000). Collectively, these mechanistic insights support the hypothesis that enzymatic processing not only improves the extractability of bioactives but also enhances their functional properties through structural modification and biotransformation.

Collagenase Inhibitory Activity

The collagenase inhibitory activity of *C. firma* leaves treated with Viscozyme L enzyme at pH 5, 50 °C, and 72 h was 10 times higher than that of untreated *C. firma* leaves (Fig. 2). This condition was selected because it had the highest antioxidant activity and was most effective for intestinal health among various enzyme types and treatment conditions. The leaves showed higher collagenase inhibitory activity than previously studied plants such as *Acalypha indica*, *Bacopa monnieri*, *Flueggea leucopyrus*, and *Tephrosia purpurea*, thereby demonstrating their efficacy in wrinkle improvement (Ito *et al*. 2018).

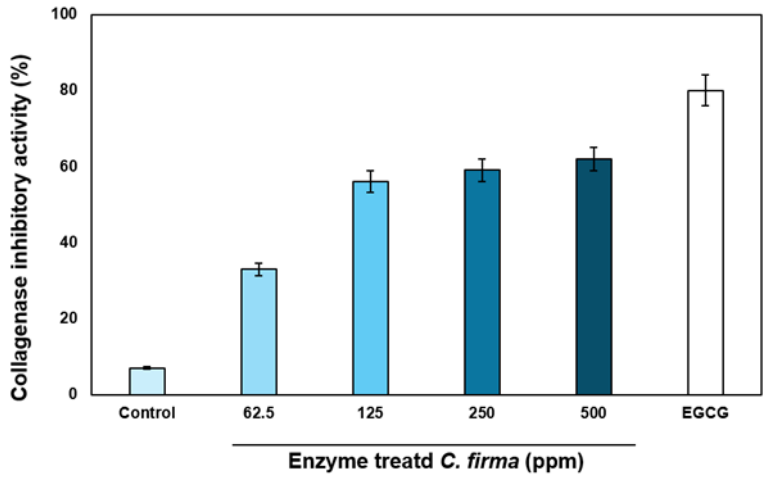

Fig. 2. Collagenase inhibition activity of enzyme treated C. firma. Control: Non-enzymatic treated C. firma

This study has highlighted the potential of enzyme-treated natural resource residues—without the need for chemical processing—as sustainable and cost-effective candidates for industrial applications in functional foods, cosmetics, and gut health supplements, and potentially for clinical use in antioxidative and anti-aging interventions. The findings of this study suggest that the enzymatically processed residues of natural resources—previously considered as waste—can retain or even concentrate bioactive properties, including antioxidant activity, gut microbiota-modulatory potential, and anti-collagenase effects. These results hold substantial promise for the development of high-value functional ingredients across multiple sectors. In the functional food industry, such residues may serve as cost-effective and eco-friendly sources of dietary supplements or nutraceutical additives aimed at mitigating oxidative stress and modulating intestinal health. In the cosmetic field, the anti-collagenase activity of these residues indicates potential applications in anti-aging formulations, particularly in skin care products designed to maintain extracellular matrix integrity. Furthermore, the absence of chemical extraction steps in the processing workflow enhances the biocompatibility and safety profile of the final materials, increasing their feasibility for clinical translation.

CONCLUSIONS

1. The residues obtained from enzymatic processing of *C. firma* leaves were found to exhibit promising biological activities, including beneficial effects on selected probiotic strains.
2. These findings suggest their potential application as natural antioxidants and gut microbiota-modulatory potential agents in the development of functional food products.
3. In addition, this study offers valuable insight into the feasibility of utilizing *C. firma*-derived residues, providing a scientific foundation for the value-added use of plant-based enzymatic by-products.
4. This study not only highlights the functional potential of enzyme-treated *C. firma* leaf residues but also underscores their industrial relevance in food technology and nutraceutical development.

5. The effective reuse of enzymatic by-products aligns with global waste valorization strategies by transforming agricultural residues into high-value materials, thereby promoting a circular bioeconomy, and these results provide a foundation for the scalable utilization of enzyme-processed plant waste in the development of eco-friendly, functional food and cosmetic products.

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Data Availability

All datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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