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Development of Fermented Coconut Water and Red Ginger using Next Generation Probiotics as a Potential Biological Activity Agent

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ABSTRACT

Degenerative diseases have increased in prevalence worldwide, predominantly in Indonesia. Some studies found that probiotics have an impact on organic substances that address inflammation and gut microbiota imbalance. This study aims to develop a functional beverage based on coconut water and red ginger fermentation using Next Generation Probiotics (NGP) and characterize the product using metagenomic and metabolomic technologies. This experimental study involved the fermentation of coconut water and red ginger using a probiotic starter identified by 16S rRNA sequencing for 120 hours at room temperature. The analysis included microbial characterization using List the probiotics metagenomic sequencing, metabolite profiling by LC-HRMS, particle size by PSA, and *in vitro* antibacterial and anti-inflammatory activity testing. Metagenomic analysis identified the dominance of the family Acetobacteraceae (45%) and genus *Acetobacter* 942%. Fermentation reduced the tannin content by 28.5% and increased the number of metabolite compounds from 37 to 54. The fermentation results also showed antibacterial activity against *E. coli* CNN 0091 (inhibition zone 7.835 mm), and anti-inflammatory activity reached 1,000%. The combination of coconut water, red ginger, and NGP produced a complex metabolite profile with high therapeutic potential. The findings show significant potential for the prevention of degenerative diseases.

1. Introduction

The prevalence of degenerative diseases such as diabetes, cardiovascular disease, and neurodegeneration continues to increase globally (Kuneš *et al.* 2023; Pandit and Frishman 2024). Cardiovascular disease risk factors such as diabetes contribute to dementia (Hendrickx *et al.* 2021). Some common mechanisms underlying degenerative diseases include inflammation and oxidative stress. Mitochondrial dysfunction and insulin resistance (Mak *et al.* 2020). Fermented functional beverages may help address gut microbiota imbalance, oxidative stress, and inflammation contributing to degenerative diseases (Lee *et al.* 2020; Stiemsma *et al.* 2020).

Coconut water (*Cocos nucifera* L.) contains various nutrients such as amino acids, vitamins, minerals, and carbohydrates that make it an effective fermentation medium (Kumar *et al.* 2021; Xu *et al.* 2022). The content of carbohydrates in coconut water supports the growth of microorganisms and the production of bioactive components during fermentation (Lin *et al.* 2022). Several studies have shown that coconut water can support the development of various probiotic bacteria such as *Lactobacillus casei, Lactobacillus plantarum, Lactobacillus helveticus, Bacillus coagulans,* and *Bifidobacterium longum* (Kantachote *et al.* 2017; Giri *et al.* 2018). Fermentation of coconut water by probiotic bacteria produces various bioactive metabolites, such as vitamin B12, γ-aminobutyric acid (GABA), exopolysaccharides (EPS), and lactic acid, that enhance antioxidant and antibacterial activities (Goveas *et al.* 2021; Wispen *et al.* 2022).

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Red ginger (*Zingiber officinale* var. rubrum) contains bioactive compounds such as gingerol, shogaol, and zingerone that have antioxidant, anti-inflammatory, and antimicrobial activities (Ballester *et al.* 2022; Yücel *et al.* 2022). Adding herbal extracts to fermented beverages can increase their phytochemical content and biological potential, as shown in adding chamomile extract to orange fermented drinks, which increases phenolic content, flavonoids, and antioxidant potential (da Silva *et al.* 2020; Mascarin *et al.* 2023). Fermentation can also reduce the toxicity of herbs and enhance novel effects, as shown in the fermentation of *Polygonatum cyrtonema rhizomes* using *Bacillus subtilis* and *Saccharomyces cerevisiae* that produced functional polysaccharides with significant antioxidant and immunomodulating activities (Lu *et al.* 2023).

Next-generation probiotics (NGPs) are microorganisms that have the potential to provide more specific and practical health benefits than conventional probiotics (Chang *et al.* 2019; Sionek *et al.* 2023). NGPs such as *Faecalibacterium prausnitzii, Akkermansia muciniphila*, and *Bacteroides fragilis* have demonstrated the ability to enhance gastrointestinal immunity, maintain intestinal barrier integrity, and produce beneficial metabolites (Kaźmierczak-Siedlecka *et al.* 2022). The combination of NGPs and prebiotics has the potential to make specific metabolites that play a role in the prevention and management of metabolic diseases (Torres-Sánchez *et al.* 2022), as well as metabolites that have beneficial effects in improving gastrointestinal immunity and increasing the efficacy of immunotherapy (Kalinowska *et al.* 2023).

Fermentation has been shown to increase the content of bioactive compounds in food products, including short-chain fatty acids (SCFAs) that benefit human health (Annunziata *et al.* 2020). Microbial Fermentation can produce bioactive compounds such as pure lactic acid, protein extracts with high biological value, and various functional carbohydrates (Sabater *et al.* 2020). The fermentation process can alter the phenolic profile in plant foods to produce more bioactive compounds (Leonard *et al.* 2021). Fermented soy products, such as tempeh, have bioactive compounds that reduce the risk of chronic diseases and have anticancer, anti-inflammatory, and neuroprotective effects (Mascarin *et al.* 2023).

Metagenomics and metabolomics are technologies that can characterize microbial communities and identify metabolites produced during fermentation. Metagenomic enables the study of microbial communities through amplicon and shotgun sequencing approaches, which can identify the various microorganisms involved and the metabolites produced during fermentation and understand the dynamics of microbial communities and their interaction (Srinivas *et al.* 2022; Sarkar *et al.* 2023). In contrast, metabolomics is the quantitative study of metabolites produced by microbes during fermentation, which can be used to monitor metabolites in real time and provide high-resolution data essential for fermentation process optimization (Li *et al.* 2022a).

Although previous studies have explored the potential of coconut water, red ginger, and NGP separately in developing functional beverages, no study has combined these components in multicomponent fermentation. In addition, the utilization of molecular technologies such as metagenomics and metabolomics in characterizing coconut water and red ginger fermentation products with NGP is still limited. Therefore, this study aims to develop a functional beverage based on coconut water and red ginger fermentation using NGP as a potential biological agent and characterizing fermentation products using metagenomic and metabolomic technologies. This research is expected to contribute to developing innovative functional beverages with superior biological activity for preventing and managing degenerative diseases. The results of this study can also provide a better understanding of the mechanisms of action and bioactive compounds that play a role in the functional effects of coconut water and red ginger fermented beverages with NGP.

2. Materials and Methods

2.1. Research Tools and Materials

The tools used in this research are a fermentation jar, glassware, petri dish, inoculating loop, Laminar Air Flow (LAF), UV-Vis spectrophotometer, Bunsen burner, Eppendorf, LC-HRMS, and particle size analysis Nano ZS90.

The materials used in this study include coconut water and red ginger obtained from Pasuruan, East Java. The samples have been identified and authenticated by plant taxonomists from the Microbiology Laboratory, Department of Biology, Brawijaya University. Chemicals included distilled water, tannic acid, casein, Folin-Denis reagent, Na_2CO_3. Commercial starter culture probiotics with a concentration of 10^7 cfu/ml were used to inoculate fermentation media and *E. coli* FNCC 0091 bacteria for the test bacteria.

2.2. Research Methods

2.2.1. Preparation of Probiotic Starter Cultures

The preparation of probiotic starter culture begins with the sterilization process of 100 ml of coconut water at 70°C for 10 minutes. After the temperature decreased to 40°C, 10% (v/v) commercial probiotic starter culture was added to the medium. The incubation process was carried out for 48 hours at room temperature to optimize the growth of probiotic bacteria. This method is a modified standard protocol for adapting probiotic bacteria in a coconut water medium (Nabila *et al.* 2023).

2.2.2. DNA Extraction, Amplification, and 16S rRNA Sequencing

Bacterial genomic DNA from starter cultures was extracted using Quick-DNA Magbead Plus Kit (Zymo Research, D4082). DNA quantification and purity were analyzed using Nanodrop 2000 (Thermo Scientific). Genomic DNA chains were amplified using specific primers, namely 16S rDNA primers V3-V4 parts. PCR amplification used PhusionTM Plus PCR Master Mix (F631S) and KOD Multi & Epi (KME-101). A total of 2 µL amplicons were visualized using Gel Electrophoresis and 1% agarose gel. A 100 bp size (2.5 µL) was used as a reference ladder. The PCR products were subsequently utilized for library preparation and sequencing using the Illumina platform to obtain raw paired-end reads. Adapter and primer PCR sequences were removed using Cutadapt. Repair of erroneous sequences, removal of low-quality sequences, and chimeras using DADA2 (Callahan *et al.* 2016). Taxonomic classification using SILVA database. Data analysis and visualization were performed using R-Studio version 4.2.3, Korona Tools, and PICRUSt2 (Douglas *et al.* 2020). The 16S rRNA gene sequences of probiotic bacterial cultures were analyzed for phylogenetic tree reconstruction using the Mega 11 application with the maximum-likelihood method (Syah 2022).

2.2.3. Fermentation of Coconut Water and Red Ginger using Probiotic Starter Culture

The fermentation process of coconut water and red ginger began by adding 20 g of ginger to fresh coconut water, followed by pasteurization at 70°C for 10 minutes. After the temperature was reduced to 40°C, probiotic starter culture was added as much as 10% (v/v) and put into a closed container. The study was conducted with six variations of fermentation time: 0 h (T1), 24 h (T2), 48 h (T3), 72 h (T4), 96 h (T5), and 120 h (T6) (Giri *et al.* 2018).

2.2.4. Determination of Optimum Condition based on Tannin Content Analysis

The determination of tannin content was carried out using the Folin-Denis method in several steps. The fermentation filtrate was centrifuged for 10 minutes at 3,000 rpm, then 400 µL of sample was mixed with 0.5 mL of Folin-Denis reagent and 1 ml of Na_2CO_3 solution. The mixture was added with distilled water up to 10 ml and incubated for 30 minutes before absorbance measurement at 760 nm. Tannin content was calculated using the tannic acid standard curve and expressed in mg per 100 g weight sample (Zhou *et al.* 2023).

2.2.5. Extraction of Secondary Metabolite Compounds

Extraction of secondary metabolite compounds begins with centrifugation of the fermented filtrate for 10 minutes at 4°C at 10,000 rpm. The supernatant obtained was then filtered using a Waterman GF/D (1.2 µm). This extraction method was chosen based on its effectiveness in separating secondary metabolite compounds from the sample matrix (Wang *et al.* 2024).

2.2.6. Identification of Metabolite Compound Profile of Coconut Water and Ginger Herbal Fermentation

LC-HRMS further processed the optimum condition fermentation extract to identify bioactive compounds (LTQ XL, Thermo Electron Corporation, USA). Detection was performed via direct injection mode with the Electron Spray Ionization (ESI) probe in positive mode. The sample flow rate was kept at 8 µL/min, while the capillary temperature was set at 280°C. The mass was in the range of 50 to 1,000 m/z. As a mobile phase, the ratio of methanol and acetonitrile was 80:20 (v/v) (Shao *et al.* 2022).

2.2.7. Antibacterial Activity

Antibacterial activity testing was performed using a pure strain of Escherichia coli (ATCC 8739). The test consisted of artificial contamination of the preparation using an inoculum of microorganisms. Bacterial suspensions were grown in nutrient broth media at 35°C.

2.2.7.1. Disc Diffusion Method

This method is used to test the susceptibility of bacteria to antibiotics. Testing with discs containing fermentation filtrate was used in this study. A total of 3.8 g of NB powder was dissolved in 100 ml of water and then sterilized in an autoclave at 121°C for

15 minutes. After cooling, about 20 ml of medium was added aseptically into the sterilized Petri dish. Then, inoculate the *Escherichia coli* bacterial suspension of 10^7 cfu/ml for 3 turns at an angle of 60° to ensure homogeneous growth. Filter discs with a diameter of 6 mm were impregnated with the fermentation solution and then placed on nutrient agar plates. The dishes were incubated at 35°C for 24 h. Disk diffusion is based on determining the zone of inhibition proportional to the sensitivity of bacteria and bactericide in the disk after 24 h of incubation (Akbar *et al.* 2023).

2.2.8. Nanoparticle Characterization

Nanoparticle characterization of the filtrate from the optimum fermentation conditions was conducted utilizing a Nano ZS90 particle size analyzer to characterize the filtrate from the ideal fermentation condition (Shamsoddini *et al.* 2025).

2.2.9. Anti-inflammatory Activity *In Vitro* by Inhibition of Protein Denaturation

2.2.9.1. Preparation of 1% Casein Solution

Casein as much as 1 g was put in 100 ml. Distilled water was added until the limit mark (Novika *et al.* 2021).

2.2.9.2. Preparation of Positive Control

A total of 50 mg of sodium dichlorophenac was placed into 50 ml of distilled water. Distilled water was added to the final volume. The resulting solution had a concentration of 1,000 µg/ml. The stock solution was then diluted to obtain concentrations of 20, 40, 60, 80 µg/ml.

2.2.9.3. Anti-inflammatory Activity Testing

For each negative control solution, positive control solution, and filtrate from optimum fermentation conditions, as much as 1 ml was put in a test tube and then added with phosphate saline as much as 3.8 ml, then 1% casein solution was added and incubated. The solution was incubated at 37°C for 15 min utes. After that, the solution in the test tube was heated in a water bath at ±70°C for 5 min utes. The sample was cooled at room temperature for 25 minutes, and then the absorbance was measured using a UV-Vis spectrophotometer at 660 nm. The inhibition of protein denaturation using casein was calculated using the equation.

$$\% \text{ Inhibition} = (A_{control} - A_{sample}) \times 100$$

The IC_{50} value is calculated from the linear regression equation between concentration (X) and % inhibition (Y).

2.3. Statistical Analysis

Statistical analysis was performed using SPSS version 26 with a 95% confidence level ($p \leq 0.05$). Data were presented as mean ± standard error, and differences between groups were analyzed using one-way ANOVA. LSD further test was performed to evaluate significant differences between treatment groups. Statistical analysis results were used to confirm the significance of differences between treatments and the validity of the study results.

3. Results

3.1. Metagenomic Analysis

The 16S rRNA gene is a gene for analyzing microbiomes in bacterial identification with a total size of about 1,500 bp. Metagenomic analysis of probiotic bacterial cultures using the Next Generation Sequencing (NGS) 16S rRNA method is shown in Figure 1. The results of the metagenomic analysis revealed a complex microbial community in the coconut-ginger water fermentation product. In addition, some complexities, such as phylogenetic analysis using 16S rRNA gene molecular markers, were also carried out in Figure 2.

3.2. Analysis of Tannin Content

Tannins are phenolic compounds that can coagulate proteins or other organic compounds. Acidic compounds or enzymes can hydrolyze tannins to produce gallic and ellagic acid. The following determines tannin content with the Folin Denis reagent based on the oxidation-reduction reaction of polyphenols reduced to a blue complex compound. Determination of tannin content of coconut-ginger water fermentation is based on variants of fermentation duration to determine the optimum conditions in Figure 3.

The activity of microorganisms during fermentation can change the pH of the medium , indirectly affecting the stability of tannin compounds. The reduction of tannin levels through fermentation is essential in food processing. Reduced tannin levels can increase the nutritional value of ingredients because tannins are known as anti-nutritional compounds that can

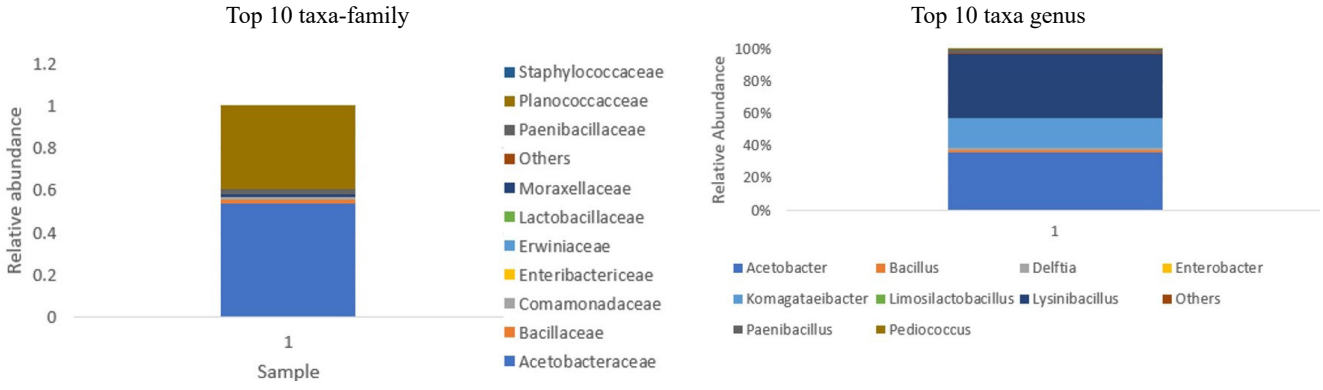

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Figure 1. Relative abundance is at (A) the family level and (B) the genus level

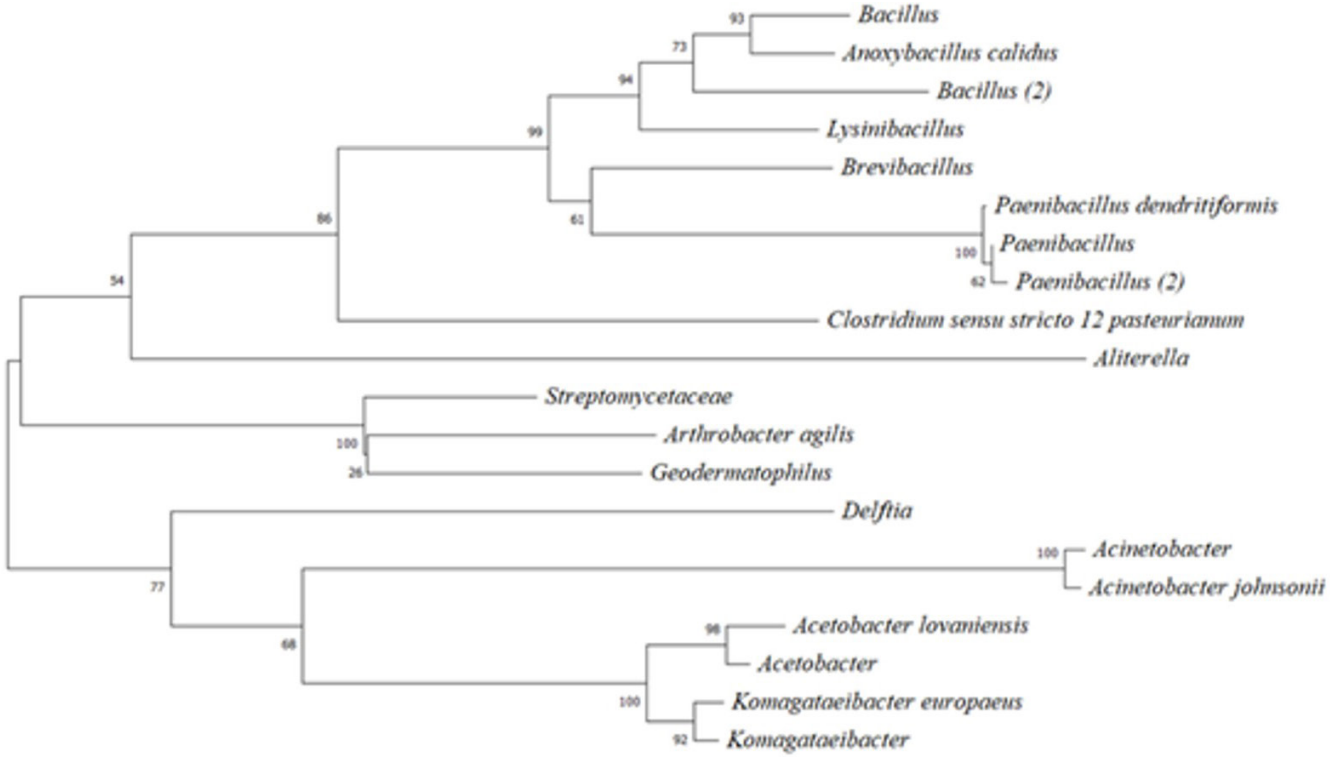

Figure 2. The phylogenetic tree of the top 10 genus was reconstructed using mega 6

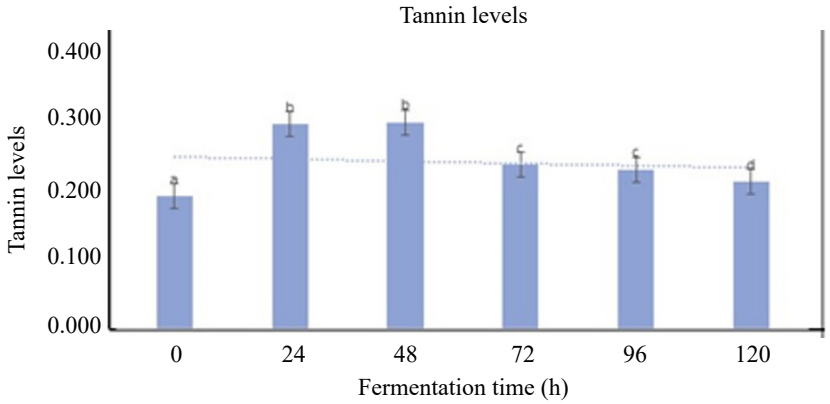

Figure 3. Graph of the decrease in tannin levels from coconut-red ginger water fermentation based on variations in fermentation duration. Values marked with different lowercase letters indicate significant differences ($p<0.05$)

inhibit the absorption of proteins and minerals. The fermentation process can also produce beneficial bioactive compounds from the degradation of tannins. Understanding the kinetics of tannin degradation during fermentation can help in optimizing the food processing process, which is applied in Table 1 below.

3.3. Identification of Metabolite Compound Profile

Metabolomic analysis using LC-HRMS identified the number of metabolite compounds from 37 compounds in non-fermented samples to 54 after fermentation. A total of 37 metabolites were identified in the non-fermented sample, dominated by natural phenolic compounds from ginger, such as citral, ferulic acid, and pipecolinic acid. After fermentation, the number of metabolite compounds increased to 54. Table 2 shows the main compounds with high biological potential identified in the optimum condition fermentation samples.

Based on Figure 4, it can be seen that the LC-HRMS analysis indicates the presence of several essential metabolite compounds from the fermentation of coconut-ginger water. One of the main compounds identified was 6-Gingerol, nicotinic acid, and α-Pinene-2-oxide.

3.4. Antibacterial Activity

Antibacterial activity was determined against *Escherichia coli* bacteria using the disc diffusion method. The positive control used was chloramphenicol. The favorable control treatment showed a more significant inhibition zone diameter than the test samples in Table 3. The diameter of the chloramphenicol inhibition zone was 20.13 mm against *Escherichia coli* bacteria. This occurs because chloramphenicol has a broad spectrum against gram-positive and negative bacteria.

Table 1. The tannin content of ginger, coconut water, and fermented coconut water and ginger, and without fermentation

Sample	Tannin levels (%) ± SD
Ginger Extract	0.413±0.030
Coconut Water	0.108±0.007
Coconut water + Ginger (Without fermentation)	0.298±0.012
Coconut water + Ginger (Optimum Condition)	0.213±0.029

Table 2. Component compounds from coconut water-ginger fermentation using probiotic starter culture at optimum conditions

Calc. MW	Area (max)	m/z cloud best match	Compound	Formula
294.18169	6.98E+07	61.5	6-Gingerol	C17H26O4
123.03159	6.39E+07	95.9	Nicotinic acid	C6H5NO2
152.11945	8.92E+07	76	α-Pinene-2-oxide	C10H16O

3.5. Nanoparticle Characterization

Nanoparticle characterization produced innovative findings with uniform particle size distribution in the 2,000-3,000 nm range in fermentation samples using a particle size analyzer (PSA) test. This measurement aims to determine the particle size in the fermented coconut-ginger water solution using the Dynamic light scattering test method shown in Figure 5.

3.6. Anti-inflammatory Activity

Protein denaturation inhibition activity of coconut-ginger fermented water was carried out to determine anti-inflammatory activity. The results of the anti-inflammatory activity test of fermented coconut-ginger water can be seen in Figure 6.

4. Discussion

4.1. Metagenomic Analysis

Figure 1 shows that the relative abundance is dominated by the Acetobacteraceae family (45%) at the family level and the Acetobacter genus (42%) at the genus level. This finding aligns with Kim *et al.* (2023), who reported similar dominance in traditional fermented beverages, where Acetobacteraceae accounted for 40-50% of the total microbial population. In these samples, at least 10 different bacterial genera were detected, including bacterial genera such as *Bacillus* (15%), *Delftia* (12%), and *Limosilactobacillus* (8%), indicating the significant probiotic potential of these fermented products, exceeding the diversity reported by Seo (2024) who only identified 5 genera in fermented coconut water. This genus diversity indicates the complexity of the microbial community in probiotic cultures. These various bacterial genera may indicate the potential for diverse probiotic functions. The presence of several genera of lactic acid bacteria, such as *Limosilactobacillus* and *Pediococcus,* corroborates these cultures' probiotic properties. The dominance of lactic acid bacteria indicates the ability to produce organic acids that can enhance the therapeutic properties of the product, as reported in their study on fermented beverages (Kang *et al.* 2020). The diversity of microbes indicates the complexity of interactions between species that contribute to the characteristics of

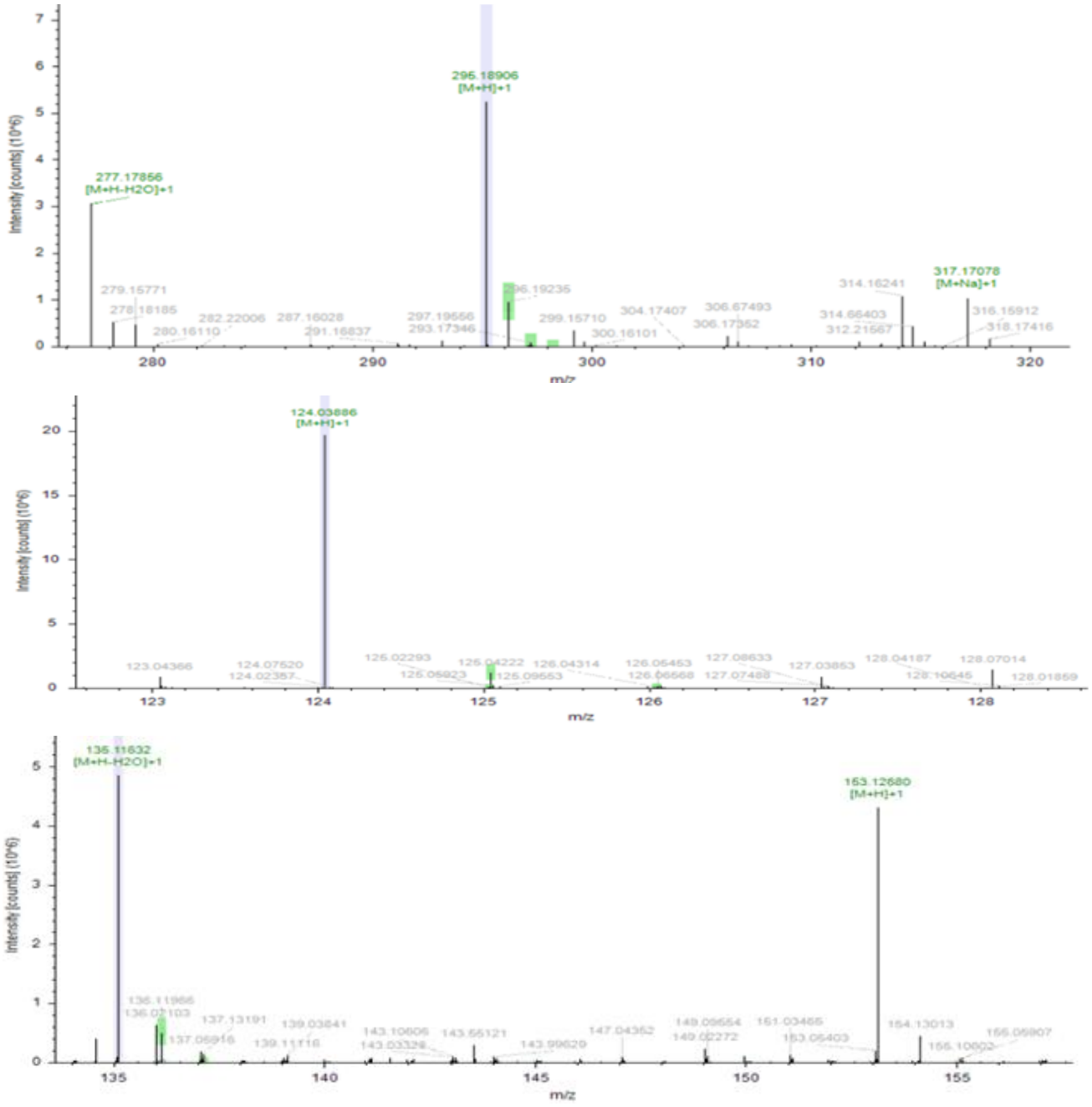

Figure 4. LC-HRMS spectra of (A) 6-Gingerol, (B) nicotinic acid, (C) α-Pinene-2-oxide

Table 3. Antibacterial activity based on inhibition zone diameter (mm)

Sample	Antibacterial activity (mm)
Ginger Extract	4.835
Coconut Water	0
Coconut water + Ginger (Without fermentation)	4
Coconut water + Ginger (Optimum Condition)	7.835

the final product. The family Lactobacillaceae detected in a proportion of 5% adds to the probiotic value of the product, given that this family is known to have beneficial effects on digestive health. The balance of proportion among these bacterial genera is essential to maintain the stability and probiotic function of the culture. The presence of well-known probiotic genera

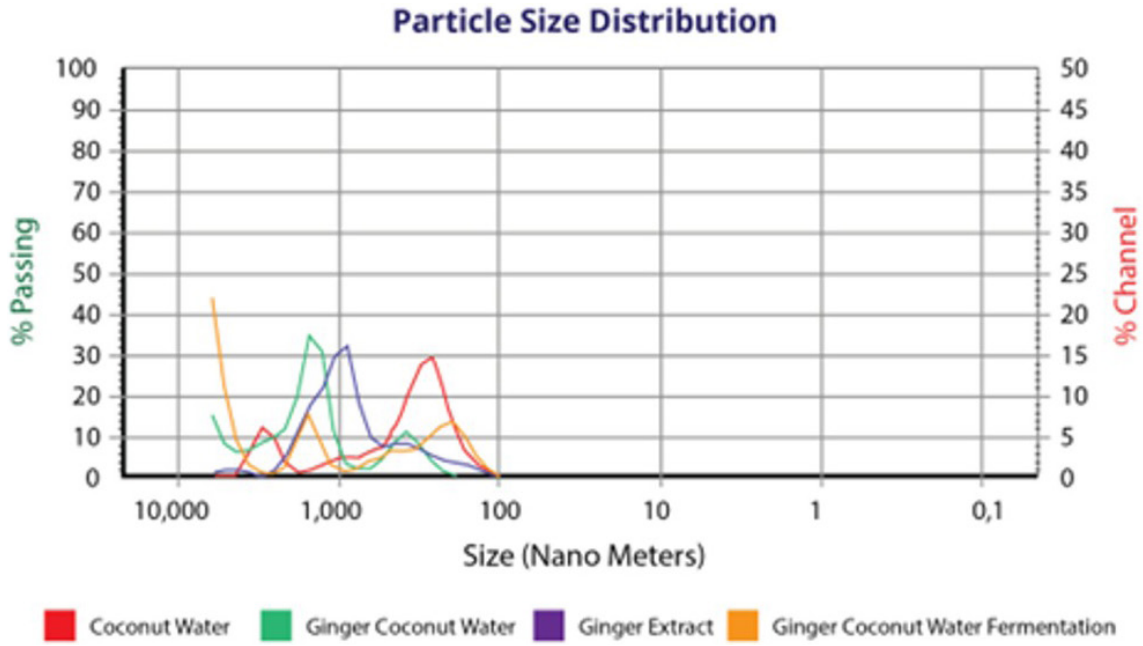

Figure 5. Comparison chart of particle size distribution

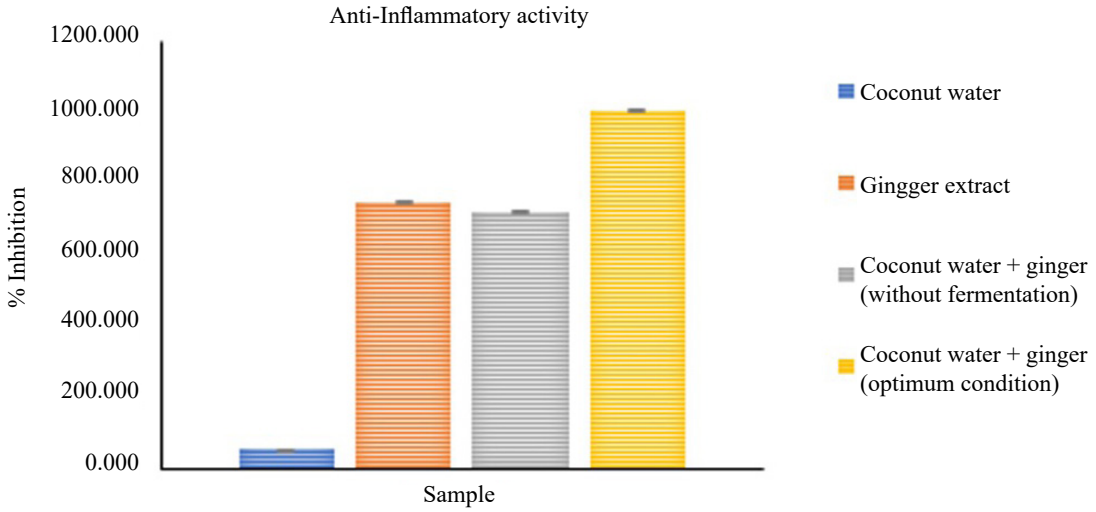

Figure 6. Comparison chart of anti-inflammatory activity based on inhibition of protein denaturation

such as *Bacillus* and *Limosilactobacillus* indicates the potential application of these cultures in probiotic products. Combining these genera can provide diverse health benefits when applied as probiotics.

Based on the results of phylogenetic tree analysis using the neighbor-joining method, there is a close relationship between several groups of probiotic bacteria. The *Bacillus* group and its relatives form a solid cluster with a high bootstrap value (73-100%), indicating a strong evolutionary relationship (Lebaka *et al.* 2018). This grouping aligns with the research of (Ma *et al.* 2021), who also found phylogenetic closeness among *Bacillus, Anoxybacillus*, and *Paenibacillus* in 16S rRNA-based analysis. This evolutionary closeness can explain the similarity between physiological and biochemical characteristics between these bacterial groups. These results strengthen the understanding of the evolution and diversification of probiotic bacteria in microbial ecology (Huang *et al.* 2021).

The degradation ability of rhizome bioactive compounds by the identified bacterial groups shows significant biotechnological potential. Bacteria from the *Bacillus* and *Streptomycetaceae* genus showed extracellular enzyme activities that can break down

complex compounds such as curcumin and gingerol (Cheng *et al.* 2019). The biotransformation ability of rhizome compounds by the identified bacterial groups showed promising potential in producing compounds with enhanced biological activity. The *Bacillus* group can biotransform curcumin into metabolites with more potent antibacterial activity against pathogens such as *Staphylococcus aureus* and *Escherichia coli* (Adamczak *et al.* 2020). Research by Cheng *et al.* (2019) showed that gingerol biotransformation products by Streptomycetaceae produced compounds with higher anti-inflammatory activity through inhibition of the NF-κB pathway. Secondary metabolites produced from the biotransformation process of shogaol by Acinetobacter showed increased immunomodulatory and antioxidant activities compared to the original compound (Yang *et al.* 2020).

The biological activities of the rhizome biotransformation products also show interesting synergistic effects. The study by Kim (2019) revealed that combining biotransformed metabolites from various bacterial groups produced more potent antibacterial and anti-inflammatory effects than single use. Biotransformation products by bacterial consortia exhibited more complex immunomodulatory activities with the ability to modulate multiple proinflammatory cytokines (Fei *et al.* 2023). This discovery leads to the development of more effective combination therapies for various pathological conditions. In addition, biotransformation products also show potential as preventive agents in the body's defense system.

4.2. Analysis of Tannin Content

Figure 3 shows the graph of the results of tannin content analysis carried out during the fermentation process for 120 hours. At the fermentation time of 0 hours (the beginning of fermentation), the measured tannin content was about 0.190%, which is the initial tannin content in the material. The Folin-Denis method is a colorimetric method that measures the total phenol content, which includes tannins. Measurement using this method is based on the oxidation-reduction reaction, in which the Folin-Denis reagent will react with tannin compounds to produce a blue color whose absorbance can be measured. Tannase enzyme activity produced by microorganisms plays an essential role in tannin degradation (de Las Rivas *et al.* 2019). After fermentation lasted for 24 hours, there was a significant increase in tannin levels reaching around 0.300%. This increase can be explained through the enzymatic activity of microorganisms that break down complex bonds in the material, thus liberating previously bound tannin compounds. In the 24-48 hours, tannin levels remained stable at 0.300%, indicating a balance between tannin formation and degradation processes. The stability of the measurement in this period also shows the consistency of the Folin-Denis method in measuring tannin levels.

After fermentation lasted more than 48 hours, there was a consistent decrease in tannin levels until the end of observation at 120 hours. This decrease indicates that 120 hours of fermentation time is the optimal duration to decrease tannin levels significantly. This pattern developed the findings of Hawashi *et al.* (2019), who reported a decrease in tannins in vegetable fermentation but with a shorter optimal time of 96 hours. The total reduction of 28.5% in the fermented sample compared to the non-fermented sample (0.298%) indicates the effectiveness of the process in reducing these anti-nutritional compounds with a 20% reduction (Chaji and Jahanara 2024). The fermentation process causes a decrease in tannin levels through several biochemical mechanisms involving the activity of microorganisms. Microorganisms that grow during fermentation produce tannase enzymes that can hydrolyze tannins into simpler compounds. This tannase enzyme breaks the ester bonds in hydrolyzed tannins into gallic acid and glucose (Ananda *et al.* 2019). This enzymatic activity is an adaptive response of microorganisms to the presence of tannins in the fermentation substrate. This tannin breakdown process also allows microorganisms to utilize the degradation products as a carbon source for growth.

Table 1 shows the results of tannin content analysis from several samples, including red ginger, coconut water, and a combination of both under different conditions. The red ginger sample has the highest tannin content of 0.413%±0.030, which indicates that red ginger naturally contains significant tannin compounds. When red ginger was combined with Coconut Water without fermentation, the tannin content was 0.298%±0.012. This resulted in a natural dilution of the Red Ginger tannin content by the Coconut Water. Meanwhile, the combination of Coconut Water and Ginger under optimum conditions (through the fermentation process) showed a tannin level of 0.213%±0.029, lower than the mixture without fermentation. This difference indicates that the fermentation process reduced the tannin content in the mix ture. These results suggest that the fermentation process effectively reduces tannin

levels by about 28.5% compared to the mixture without fermentation. This decrease is likely due to the activity of microorganisms during the fermentation process that can break down tannin compounds. The fermentation process under optimum conditions can be an effective method to produce products with lower tannin levels. This finding can be essential in developing ginger and coconut water-based beverage products that are more acceptable to consumers.

4.3. Identification of Metabolite Compound Profile

Based on the results shown, it can be seen that the LC-HRMS analysis indicates the presence of several essential metabolite compounds from the fermentation of coconut-ginger water. One of the main compounds identified was 6-Gingerol, nicotinic acid, and α-Pinene-2-oxide. These results enrich the findings of Benmeziane *et al.* (2018), who identified similar bioactive compounds in fermented ginger products but with different concentrations. The increased nicotinic acid concentration suggests increased nutritional value through fermentation, a phenomenon not reported in previous studies. Biotransformation of compounds during fermentation results in a more complex metabolite profile than the initial raw material. The fermentation process altered and enhanced the bioactive potential of the coconut water and ginger combination. LC-HRMS analysis enabled more accurate and comprehensive identification than conventional methods used in previous studies. The presence of 6-Gingerol in significant concentrations indicated that the fermentation process successfully retained the main bioactive compounds of ginger. The identified α-Pinene-2-oxide compound adds a new dimension to understanding coconut-ginger water fermentation products.

The combination of these three compounds in fermented products shows promising synergistic potential. A study by Yang *et al.* (2023) revealed that the interaction between 6-Gingerol and nicotinic acid could increase both bioavailability. Analyses by Marco *et al.* (2017) showed that combining these three compounds provided more potent antioxidant effects than when used individually. Shahbazi *et al.* (2021) reported that the anti-inflammatory effect of the combination was more effective than using each compound. Gupta *et al.* (2023) observed that the stability of these three compounds in fermented products was better than in conventional extracts. Hughes *et al.* (2021) also found that the fermentation process could increase the bioaccessibility of these three compounds simultaneously. The discovery of this unique metabolite profile opens up opportunities for developing functional products with specific health benefits. The presence of these compounds in fermented products suggests metabolite transformation during the fermentation process that may increase the bioactive potential of the final product. The increased concentration of these compounds indicates that the fermentation process successfully optimized the extraction and biotransformation of bioactive compounds from ginger.

4.4. Antibacterial Activity

Based on Table 3, red ginger showed significant antibacterial activity with an inhibition zone of 4.835 mm against *E. coli* bacteria. Meanwhile, coconut water as a single sample showed no antibacterial activity, as indicated by the inhibition zone value of 0 mm. This shows that red ginger has potential as a natural antibacterial agent. In addition, the results also showed a significant difference between the mixture of coconut water and red ginger without fermentation compared to the optimum conditions involving the fermentation process. The mixture without fermentation produced an inhibition zone of 4 mm, while the optimum condition with fermentation produced a much larger inhibition zone of 7.835 mm.

The almost two-fold increase in the inhibition zone (from 4 mm to 7.835 mm) indicates that fermentation significantly increased the antibacterial effectiveness of the mixture. This increase is higher than the results of Li *et al.* (2022b), who reported a 50% increase in antibacterial activity in the fermentation of plant materials. The synergistic effect between coconut water and ginger, enhanced by fermentation, resulted in more potent antibacterial activity than either component alone. The optimum conditions of fermentation allow for favorable biochemical transformations, such as producing bacteriocins or other antimicrobial compounds by fermentative microorganisms. The fermentation process can also enhance the extraction of active compounds from red ginger, which, combined with the nutrients from coconut water, create a more effective antibacterial system. Secondary metabolites produced during fermentation contribute to increased antibacterial activity. The fermentation process also optimizes the extraction and bioconversion of antibacterial compounds from ginger. Compared to the

conventional extraction method reported by Bidwell *et al.* (2023), fermentation showed higher effectiveness in producing antibacterial compounds. The resulting zone of inhibition suggests potential application as a natural preservative in food products.

Based on the results of LC-HRMS analysis, a combination of metabolite compounds shows a strong synergistic effect in inhibiting *E. coli* growth. Yu *et al.* (2022) reported that this combination produced a Fractional Inhibitory Concentration Index (FICI) value of 0.35 against *E. coli*, indicating a strong synergistic interaction. Meanwhile, Zhang *et al.* (2024) used confocal microscopy to show that this combination caused more extensive membrane damage to *E. coli*. Razaviamri *et al.* (2021) reported the kill rate of *E. coli* decreased from 24 hours to 6 hours when the three compounds were combined. Hughes *et al.* (2021) confirmed that this combination inhibited *E. coli* biofilm formation by up to 95% at lower concentrations than individual use.

4.5. Nanoparticle Characterization

Figure 5 shows the particle size distribution of the four samples: Coconut Water, Ginger Coconut Water, Ginger Extract, and Fermented Ginger Coconut Water. Measurements were taken using a Particle Size Analyzer (PSA) ranging from 0.1 to 10,000 nanometers. The X-axis shows the particle size in nanometers on a logarithmic scale. In contrast, the left Y-axis shows the percentage of particles passing (% Passing), and the right Y-axis shows the percentage of size distribution (% Channel). Each sample is shown in a different color for easy identification, with Coconut Watermarked in red, Ginger Coconut Water in green, Ginger Extract in purple, and Fermented Ginger Coconut Water in orange. The measurement results show that each sample has a different particle size distribution pattern.

The fermentation process of Ginger Coconut Water produces a typical particle size distribution pattern, where there is a significant change compared to the sample before fermentation. The fermentation process produces various secondary metabolite compounds essential in nanoparticle synthesis through green synthesis. During fermentation, microorganisms produce extracellular enzymes such as nitrate reductase and NADH-dependent reductase that can reduce metal ions into nanoparticles (Yang *et al.* 2022). Biomolecules produced during fermentation, including proteins, polysaccharides, and organic acids, can form a protective layer around the formed nanoparticles, preventing agglomeration and resulting in uniform size distribution (Sivasankaran *et al.* 2024). It was identified that polyphenols and flavonoids produced during fermentation have dual capabilities as reducing agents and stabilizers due to hydroxyl groups that can donate electrons and form bonds with the nanoparticle surface (Afonso *et al.* 2024). Based on the results of Dey *et al.* (2023) found that exopolysaccharides produced by lactic acid bacteria during fermentation can serve as a natural template in the formation of nanoparticles with controlled morphology.

Metabolomics analysis using LC-HRMS in Table 2 shows that variation in fermentation conditions affects the profile of metabolites that play a role in nanoparticle synthesis. The duration of fermentation affects the concentration and type of metabolites produced, with optimal production of reducing biomolecules occurring after 48-72 hours of fermentation (Yang *et al.* 2023). Studies using LC-MS conducted by Agarbati *et al.* (2024) identified a significant increase in the production of phenolic compounds and bioactive peptides during the stationary phase of microbial growth.

The stability of nanoparticles synthesized using fermentation metabolites showed advantages over conventional synthesis methods. Agarbati *et al.* (2024) reported that nanoparticles stabilized by fermentation metabolites have better colloidal stability with a zeta potential of -35 mV to -45 mV. The nanoparticles showed better dispersibility in biological media than those synthesized using synthetic stabilizing agents (Rajoka *et al.* 2020).

4.6. Anti-inflammatory Activity

Based on Figure 6, anti-inflammatory activity was compared using the inhibition percentage in several samples. The samples tested consisted of pure coconut water, ginger extract, coconut water, ginger without fermentation, and a mixture of coconut water and ginger under optimal conditions (with fermentation). Anti-inflammatory activity was measured by observing the ability of the samples to inhibit protein denaturation. Pure coconut water showed the lowest anti-inflammatory activity, with a percentage inhibition of about 50%. Ginger extract showed a significant increase in activity, with the inhibition percentage reaching about 750%. The combination of coconut water and unfermented ginger showed similar results to the pure ginger extract, with an inhibition percentage of about 700%. Most interestingly, the combination of coconut water and ginger under optimal conditions (with fermentation)

showed the highest anti-inflammatory activity, with the percentage inhibition reaching almost 1,000%.

The results showed that the combination of coconut water and fermented ginger had higher anti-inflammatory activity than the other samples. This increase in activity is in line with research conducted by de Carvalho and Conte-Junior (2024), who reported that fermentation can increase the bioactivity of compounds in natural materials. According to Kim *et al.* (2023), complex compounds are broken down into simpler compounds by microorganisms during fermentation. This process can produce new secondary metabolites that have higher biological activity. The high anti-inflammatory activity in fermented samples can also be attributed to the increased content of phenolic compounds and flavonoids during fermentation, as reported in the study by Adebo and Medina-Meza (2020).

The increased anti-inflammatory activity through inhibition of protein denaturation in the fermented sample indicates the potential of developing this product as a natural anti-inflammatory alternative. This result is supported by the research of Shao *et al.* (2022), which demonstrated that fermented products have better bioavailability than non-fermented materials. The inhibition percentage reaching almost 1,000% in the fermented samples indicated promising effectiveness, although further research is needed to isolate and characterize the bioactive compounds responsible for the activity. According to Alam *et al.* (2021), the molecular mechanism behind the enhanced anti-inflammatory activity in fermented products involves modulation of the NFκB pathway and inhibition of the COX-2 enzyme. Some limitations in this study need to be considered for future research. Based on the guidelines proposed by Gao *et al.* (2021), a more in-depth analysis regarding the standardization of the production process is also essential to ensure product quality and activity consistency.

This study highlights significant advancements in developing functional beverages through the fermentation of coconut water and red ginger using Next Generation Probiotics (NGP). Metagenomic analysis revealed the dominance of beneficial microbes, while fermentation enhanced bioactive compounds and reduced undesirable components. The product demonstrated notable antibacterial and anti-inflammatory activities, showcasing its potential for preventing and managing degenerative diseases. The innovative combination of local natural ingredients with modern fermentation and NGP technology resulted in a beverage with biological properties. This research represents a novel approach to creating health-promoting functional drinks.

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Collate acknowledgments in a separate section at the end of the article before the references and do not, therefore, include them on the title page as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance, proofreading the article, etc.).

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