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Amino Acid Composition and Bioactive Peptides of Cricket Protein Hydrolysate and Its Effect on the Ileal Microbiome in Quail

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ABSTRACT

This study aimed to investigate whether cricket protein hydrolysate (CPH) supplementation modulates the ileal bacterial community in quails, using 16S rRNA gene amplicon sequencing to characterize changes in bacterial composition and diversity. The research method is divided into two stages. The crickets that had been turned into flour were extracted using 96% ethanol at a ratio of 1:10 (w/v) for 5 days. The cricket flour was hydrolyzed using papain enzyme at a concentration of 1% (w/w under controlled conditions (pH 7, 50 °C) for 6 h. The variables measured were the degree of hydrolysis, amino acids, and peptide analysis. Stage 2 involved application to 200 female quails, five weeks old. The quails were randomly assigned to two dietary treatments with 5 replicates per treatment and 20 quails per replicate, with each replicate considered an experimental unit. The experimental diets were provided for 6 weeks. The variables measured were the abundance of ileal microbiota, determined by 16S rRNA amplicon sequencing. The results demonstrated that CPH had higher levels of ash, carbohydrates, protein, and amino acids, but lower fatty acid content than untreated cricket protein. The degree of hydrolysis of CPH was 26.5%, indicating the extent of protein hydrolysis achieved under the applied hydrolysis conditions. The peptides from cricket protein hydrolysate were dominated by dipeptides, particularly Valyl-lysine, Arginyl-phenylalanine, and L-alanyl-L-tryptophan. Microbiome analysis shows that bacterial diversity in the control feed treatment is higher than in the CPH-treated group. The CPH treatment exhibited a more homogeneous bacterial community and was predominantly enriched with beneficial bacteria, including *Lactobacillus aviarus*, *Bacillus thermolactis*, and *Clostridium* sp. The abundance of basal feed families is dominated by famili *Enterococcaceae* (58%), followed by *Lactobacillaceae* (25%), while CPH is dominated by famili *Lactobacillaceae* (44%) and *Enterococcaceae* (8%). It can be concluded that cricket flour resulting from hydrolyzed protein has the potential to be used as a feed additive that can act as an antimicrobial and improve the microbiota in the digestive tract of quails.

Keywords: antimicrobial; crickets, protein hydrolysis, lactic acid bacteria, gastrointestinal tract

INTRODUCTION

Antibiotics have been extensively used in the livestock industry for therapeutic purposes and as growth promoters. The overuse of antibiotics has a significant impact on livestock product residues and is a major contributor to antimicrobial resistance (AMR), which poses a major threat to global health. Overuse of antibiotics also leads to increased microbial resistance to antibiotics, resulting in the emergence of difficult-to-treat pathogens (Oliveira *et al*., 2024). AMR is responsible for increased morbidity, mortality, and healthcare costs, resulting in nearly 700,000 deaths worldwide each year, and is expected to rise to 10 million deaths per year by 2050. In 2014, the World Health Organization (WHO) listed AMR among the top ten global health threats, a position it maintains nearly ten years later (Baba *et al*., 2022).

The use of safe and environmentally friendly antimicrobial alternatives in livestock production has become increasingly important due to the rising prevalence of antimicrobial resistance (AMR) and restrictions on the use of conventional antibiotics. This has encouraged research into other antibiotic alternatives such as probiotics, prebiotics, phytogenics, and antimicrobial peptides, which are considered

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capable of improving digestive tract health and immunity without leaving residues (Azizi *et al*., 2024). Among these alternatives, antimicrobial peptides (AMPs) have gained considerable attention as a promising solution due to their broad-spectrum activity against pathogenic bacteria, fungi, viruses, and parasites (Liu *et al*., 2024). AMPs improve disease resistance by targeting enteric pathogens such as *Salmonella* and *Clostridium sp*., which are common in poultry systems (Castellanos-Huerta *et al*., 2024). Suppression of pathogenic bacterial growth correlates with improved growth, including average daily weight gain (ADG) and feed conversion ratio (FCR) in poultry production systems (Gao *et al*., 2023).

Bioactive proteins and peptides are potential sources of antimicrobials. Several sources contribute to the pool of potential bioactive peptides, including plants, animals, marine organisms, and, in particular, insects and milk proteins. Insects, which represent the most biodiverse animal taxon on the planet (Patyra & Kwiatek, 2023), produce structurally unique antimicrobial peptides (AMPs) as key components of their innate immune defense against infections (Dinata *et al*., 2024). Due to their broad-spectrum antimicrobial activity and evolutionary adaptation, these molecules have attracted considerable attention as potential next-generation feed additives for sustainable poultry production. Among various insect species, crickets have emerged as promising sources of bioactive peptides because of their high protein content and functional properties. Crickets, particularly *Gryllus assimilis* and *Acheta domesticus*, are widely recognized as protein-rich insects with significant nutritional and biotechnological potential (Magara *et al*., 2021). Crickets are found worldwide, and the highest number of cricket species is usually found in tropical regions with warm temperatures that encourage their development compared to other environments (Magara *et al*., 2021). The advantages of crickets include their potential as a source of protein and bioactive peptides with high protein quality (45% dry matter) (Montowska *et al*., 2019), a balanced profile of essential amino acids, carbohydrates, vitamins, minerals, and rich micronutrient content (Udomsil *et al*., 2025). This protein consistently achieves high digestible indispensable amino acid score (DIAAS) and protein digestibility corrected amino acid score (PDCAAS), reflecting efficient digestibility, and in some cases surpasses the protein quality of yellow mealworms (*Tenebrio molitor*) and is equivalent to or exceeds the protein quality of migratory locusts (*Locusta migratoria*) (Hammer *et al*., 2023; Nachtigall *et al*., 2025). Feeding 45% cricket flour increased body weight in male quail (204.32 ± 5.69 g), 30% cricket flour (226.72 ± 29.45) compared to the control treatment, namely 184.17 ± 3.11 g and 214.55 ± 32.77 g (Kouatcho *et al*., 2022), while feeding broilers with a concentration of 15% showed a lower average final body weight and FCR than the control treatment (Fisher *et al*., 2024).

Although crickets have potential as an alternative protein source, their antioxidant and antibacterial activities have not been optimally expressed, so their use as a feed additive is still limited. One approach that can overcome this problem is the development of proteins through enzymatic hydrolysis to produce bioactive peptides. According to Mudd *et al*. (2022), *in vitro* analysis shows that the application of cricket peptides has greater antioxidant activity than unhydrolyzed proteins and plays a role in anti-inflammatory, antimicrobial, antihypertensive, and anticancer properties (Udomsil *et al*., 2025). Antibacterial peptides have beneficial effects on nutrient digestion, intestinal morphology, and intestinal microflora, thereby improving growth performance in animals and influencing the activity of neutrophils, T lymphocytes, and dendritic cells (Patyra & Kwiatek, 2023). This study aims to explore the hydrolysis of cricket peptides as an alternative feed additive through a comprehensive 16S rRNA gene amplicon sequencing of the quail ileal microbiome, thereby characterizing the bacterial populations and diversity that correlate with quail performance and health.

MATERIALS AND METHODS

Ethical Clearance

This experiment was conducted according to the Animal Care and Research Ethics Committee guidelines at the Biosains Institute, Brawijaya University, Indonesia, under clearance (EC) number (004-KEP-UB-2024).

Cricket Sample

Twenty-eight-day-old brown adult crickets (*Gryllus sp*.) were obtained from a grasshopper farmer in East Java, Indonesia. The crickets were killed using CO_2, dried in an oven at 50 °C for 2 days, and ground into flour.

Cricket Flour Extraction

Cricket powder was extracted using a maceration method modified from the research of Ulfah *et al*. (2025) using 96% ethanol with a ratio of 1:10 (w/v) for 5 × 24 hours. The mixture was stirred daily to ensure the powder was completely immersed in the solvent until the solvent appeared almost clear. The extract was filtered using Whatman No. 1 filter paper, and the filtrate was concentrated using a rotary evaporator at a temperature of 50 °C and a rotation speed of 60 rpm.

Preparation of Cricket Protein Hydrolysate

Brown cricket hydrolysis flour was produced by mixing 90 g of dead brown crickets with 180 mL of distilled water (ratio 1:2), then blending. The mashed sample was added with CH_3COOH 1M and 1 M NaOH until the solution pH was 7. PAYA (Enzyme Development Enterprise, Indonesia) brand papain enzyme was added at the recommended dose of 5.65 g (standard dose: 15 g/kg sample) to ensure optimal enzyme activity (Batish *et al*., 2020). The cricket solution

was incubated at 50 °C, pH 7, for 6 hours. The enzyme was then deactivated at 90 °C for 20 minutes. After that, centrifugation was performed at 4000 rpm for 30 minutes to produce a supernatant/cricket protein hydrolyzate. The cricket protein hydrolyzate slurry sample was dried by the freeze-drying method at -73 °C.

Determination of Hydrolysis Degree, Amino Acid, and Antimicrobial Peptide Degree of hydrolysis (DH)

The degree of protein hydrolysis was determined using the method of Islam *et al*. (2022) with several modifications. The degree of protein hydrolysis was determined using formol titration to compare the absorbed nitrogen (N-assimilated) value with the total nitrogen value of the sample. The degree of protein hydrolysis was obtained through the following calculation:
% DH =(N-assimilated)/(N-total material) x 100%

Total nitrogen was calculated using the Kjeldahl method. N-assimilated was obtained using the formol titration method based on the Sörensen method, as described in detail by Taylor. 0.5 mL of hydrolysate was dissolved in distilled water to a volume of 10 mL. The resulting dilution was neutralized with 0.2 N NaOH solution to reach a pH of 8.0. 2 mL of formaldehyde was added to the sample that had been neutralized with 0.2 N NaOH solution to reach a pH of 8.0. The sample was mixed with formaldehyde, and the mixture was titrated with 0.02 N NaOH to reach a pH of 8.0. The amount of product in the form of amino acids was expressed as the absorbed nitrogen value, which was calculated using the following equation
N-assimilated = Vol. NaOH x N NaOH x MW N x DF

where, N NaOH was the normalized NaOH used (0.02 N), MW N was the molecular weight of nitrogen (14), and DF= dilution factor.

Amino Acid Analysis by an HPLC Device

A high-performance liquid chromatography (HPLC) instrument (Shimadzu, Kyoto, Japan) model LC-20AT series equipped with a degasser (DGU-20A), oven (CTO-20A), quaternary pump (LC-20AD), autosampler (SIL-20A) with a 100 μL volume injection loop, and a multi-wavelength detector (SPD-20A) was used. A Shim-pack CLC-C18 column (particle size: 5 μm, L × ID: 150 mm × 4.6 mm) was used for the separation of amino acids. An IBM computer equipped with LC solution software version 1.2 (Shimadzu, Kyoto, Japan) was used for data processing (Eid *et al*., 2022).

Optimal separation conditions were obtained using a Shim-pack CLC-C18 column (particle size: 5 μm, L × ID: 150 mm × 4.6 mm). All AA chromatograms were recorded using mobile phase gradient elution. Elution began with 100% phosphate buffer (pH 7.4, 10 mM) for 10 min and then a linear increase in acetonitrile concentration to 50% over the next 15 min; the mobile phase flow was diverted to waste after 30 min and continued for 2 min in each run before the column was re-equilibrated to its initial conditions for 5 min. The mobile phase solution was filtered, degassed, and pumped at a flow rate adjusted to 1 mL/min, with the column maintained at 25 °C. The injection volume was 10 μL, and the eluent was monitored using UV detection at 225 nm. Needle washing was performed between each injection using a mixture of 80% water–20% acetonitrile, then followed by a mixture of 20% water–80% acetonitrile (Eid *et al*., 2022).

HPLC Method Validation

Amino acid composition of the cricket protein hydrolysate was determined using high-performance liquid chromatography (HPLC) with fluorescence detection. Amino acids were identified by comparing the retention times of sample peaks with those of the corresponding reference standards. Quantification was performed using external calibration curves prepared at concentrations of 0.1, 0.5, 1.0, 2.5, and 5.0 μg/mL. The calibration curves were generated by plotting the peak area against the corresponding amino acid concentration, and the concentration of each amino acid in the sample was calculated using the respective regression equation. The coefficients of determination (R^2) for the calibration curves were evaluated to assess the linearity of the analytical response. The HPLC system was operated using a Shim-pack VP ODS column (5 μm, 150 × 4.6 mm), a column temperature of 40 °C, a flow rate of 1 mL/min, an injection volume of 50 μL, and fluorescence detection at 450 nm (ICH Q2(R2) guidelines).

Antimicrobial Peptide Determination

Peptide sequences with potential AMPs isolated from cricket proteins were identified using Liquid Chromatography–Mass Spectrometry (LC–MS) according to the methods of Singh & Vij (2017) and Kashyap *et al*. (2022). Peptide fractions were redissolved in 0.1% formic acid and analyzed using a nano-liquid chromatography–Quadrupole Time-of-Flight mass spectrometry (nano-LC–QTOF-MS) system (Bruker, Germany). Peptides were separated on a C18 column using a linear gradient of 0.1% formic acid and acetonitrile. Mass spectrometry (MS/MS) data were acquired in positive ion mode and processed using Mascot against the National Center for Biotechnology Information (NCBI) database with a false discovery rate of 1%. The identified peptides were further characterized for biological activity and physicochemical properties using the BIOPEP-UWM database (Bioactive Peptide Database) and the Antimicrobial Peptide Database (APD3) (Kashyap *et al*., 2022).

Feeding Trials of Cricket Protein Hydrolysate on Quails

A total of 200 five-week-old Japanese quail were housed in rectangular wire cages (60 × 37 × 26 cm) at a population density of 20 birds per cage, ensuring uniform space allocation across all treatments. Birds were monitored daily for health and mortality; any

deaths were recorded and excluded from further analysis, with an overall mortality rate of <1% and no differences between treatments. Dietary treatments included: T0: basal diet (control), T1: basal diet supplemented with 10 g/kg of cricket protein hydrolysate, containing 20 amino acids and 41 peptides, administered for 60 days in a completely randomized design. Before the start of the treatments, the quail underwent a 6-day adaptation period to standardize the cage conditions and basal diet intake. The quail were housed in a battery cage system with free access to food and water throughout the experiment. The basal diet contained 20.06% crude protein, 2822.90 kcal/kg metabolizable energy, 0.80% calcium, and 0.45% non-phytate phosphorus, formulated according to nutritional standards for quail production (Table 1). Environmental conditions, including temperature, humidity, and lighting schedule, were maintained according to standard quail husbandry guidelines.

Preparing a Sample of 16S rRNA Gene Amplicon Sequencing

From a total of 200 quails, ileal samples were collected from both basal feed and CPH treatment. For microbiological analysis, healthy quails were randomly selected, and digesta from the ileum were collected per sampling: one tube from basal feed and one tube from CPH treatment. Each tube sample was aseptically collected into sterile 1.5 mL bottles. The samples were transported to the laboratory within 1 day of collection using cooling storage and were immediately subjected to microbiological analysis.

Ileal Microbiota DNA Extraction and 16S rDNA Sequencing DNA Isolation

DNA from the ileal digesta was isolated and purified using the QIAamp Fast DNA Stool Mini Kit (Qiagen) with a modified method (Knudsen, 2016; Fadrosh, 2014) and further optimized for this study. Briefly, 200 mg of sample was mixed with 390 mg of glass beads (180 mg of 450–600 μm diameter beads and 210 mg of 106 μm diameter beads) and 1 mL of InhibitEx buffer (Qiagen) preheated to 70 °C. The sample mixed with glass beads and buffer was then vortexed and homogenized using a Bullet Blender (Next Advance) for 5 min, incubated on ice for 30 s, and then heated on a 95 °C heat block for 7 min. The samples were then centrifuged for 2 minutes, and the supernatant was further processed with the QIAamp Fast DNA Stool Mini kit, and then the DNA was eluted with 100 μL of ATE buffer (Qiagen) and stored at −20 °C.

Table 1. Gross composition for experimental layer diet (kg/1000kg)

Ingredients	Layer (kg/1000kg)
Corn	540
Soybean	280
Fishmeal	84
Pollard	72
Vegetable oil	12
Limestone	3.48
MCP	1.9
Salt	2.46
Choline chloride	0.1
Premix	4
Lysine	0.1
Methionine	0.1
CP (%)	20.06
ME (Kcal/kg)	2822.90

16S rRNA Gene Amplicon Sequencing

High-throughput 16S rRNA gene amplicon sequencing was employed to comprehensively characterize the ileal bacterial community, a widely used approach for microbiome profiling in animal-derived biological samples (Rifa'i *et al*., 2025). The V3–V4 region of the bacterial 16S rRNA gene was amplified by PCR using the 341F primer (5′-CCTACGGGNGGCWGCAG-3′) and 785R primer (5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth *et al*., 2013). Dual indexing was subsequently performed for amplicon library preparation prior to sequencing. PCR cycling parameters using Q5 DNA Polymerase (New England Biolabs) were 98 °C for 1 min, 35 cycles at 98 °C for 10 s, 49 °C for 30 s, and 72 °C for 30 s, with a final extension stage of 10 min at 72 °C. The amplicons were then sequenced using Illumina MiSeq 2 × 300 bp paired-end technology.

Bioinformatics and Statistical Analysis

Sequencing data were demultiplexed using Cutadapt (Martin, 2011) and analyzed with Quantitative Insights into Microbial Ecology 2 (QIIME2) (Xia & Sun, 2023) using the DADA2 plugin (Callahan *et al*., 2016) for quality filtering, denoising, and chimera removal. Amplicon sequence variants (ASVs) identified by DADA2 were further clustered with vsearch into Operational Taxonomic Units (OTUs) at a 97% similarity level and taxonomically classified using the SILVA v138.1 and NCBI databases (Quast *et al*., 2012). Downstream statistical analysis and visualization at the OTU and higher taxonomic levels were performed using the RStudio program (RStudio requires R 3.6.0+).

RESULTS

Production Performance and Selection of Samples for Microbiome Analysis

The production performance results provided a basis for selecting the basal diet control and CPH treatment for subsequent microbiome analysis. In particular, CPH treatment showed a numerically lower feed conversion ratio (FCR) than (2.97 ± 0.64 vs. 3.30 ± 0.55), indicating a tendency toward improved feed efficiency. These treatment groups were selected to characterize differences in the ileal bacterial community associated with CPH supplementation using 16S rRNA gene sequencing (Lidiyawati *et al*., 2026a).

Chemical Composition of Cricket Protein Hydrolysate

This research began with enzymatic hydrolysis to produce cricket protein hydrolysate. The percentage of peptide bonds cleaved in the protein hydrolysate is referred to as the degree of hydrolysis (DH). The analysis results of the cricket protein flour, extract, and hydrolysate are presented in Table 2. The results showed that the cricket protein hydrolysate had higher ash, carbohydrate, and protein content, while its fatty acid content was lower.

Table 3 presents the amino acid content of cricket protein extracts and hydrolysates. Based on the comparison of cricket extract and hydrolysate, the amino acid composition was found to be higher in the cricket hydrolysate. This is supported by its high protein content. Protein hydrolysis is the process of breaking down complex proteins into simpler ones, which are then detected as amino acids. The amino acid composition of the cricket protein extract and hydrolysate was nearly identical, but the quantity was higher in the CPH (Cricket Protein Hydrolysate).

Previous results showed that there were differences in amino acid content between the extract and hydrolysate, so peptide testing was only performed on cricket protein hydrolysate (CPH). CPH contains 38 peptides consisting of dipeptides with 16 types of peptides detected, tripeptides with 3 types of peptides, tetrapeptides with 6 types, and the least, pentapeptides with only 2 types of peptides. The results of peptide analysis using the LCMS/MS method are presented in Figure 1 and Table 4.

Peptide yields from cricket protein hydrolysates are dominated by peptides, particularly valyl-lysine (Val–Lys), arginyl-phenylalanine (Arg–Phe), and L-alanyl-L-tryptophan (Ala–Trp). These dipeptides have a combination of cationic and hydrophobic/aromatic residues and contribute to antibacterial activity. The

Table 2. Nutrient composition of brown cricket flour, extract cricket (CE), and protein hydrolysate cricket (CPH)

Sample	Nutrient composition					
	Ash (%)	Moisture (%)	Carbo hydrate (%)	Fat (%)	Crude protein (%)	DH (%)
Cricket flour	4.65	3.50	7.56	21.50	60.74	-
CE	4.75	3.60	8.56	20.50	60.84	-
CPH	7.03	4.50	17.19	0.85	86.5	26.5

Note: CE: Cricket extract with ethanol, CPH: Cricket protein hydrolysate, DH: degree of hydrolysis.

Table 3. Amino acid composition of brown cricket protein extract and hydrolysate protein cricket

No	Amino acids	Treatments	
		CE (g/100 g)	CPH (g/100g)
1	Asparagine	0.37	0.61
2	Threonine	1.87	3.03
3	Serine	1.78	2.89
4	glutamic acid	6.01	9.59
5	Proline	1.44	2.34
6	Glycine	1.86	3.01
7	Alanine	2.36	3.79
8	Valine	4.15	6.65
9	Methionine	1.26	2.05
10	Isoleucine	2.21	3.56
11	Leucine	3.17	5.30
12	Tyrosine	2.10	3.54
13	Phenylalanine	1.52	2.57
14	Histidine	3.06	5.13
15	Lysine	4.28	7.14
16	Arginine	2.81	4.72
17	Tryptophan	0.60	1.01
18	aspartic acid	4.89	7.94
19	Glutamine	0.27	0.49
20	Cysteine	0.42	0.73
	Total IEEA	42.65	70.79

Note: CE: Cricket extract with ethanol, CPH: cricket protein hydrolyzate.

Table 4. Types of peptides contained in cricket protein hydrolysate (CPH)

No	Types of peptides	Peptide name
1.	Dipeptide	L-seryl-L-alanine
2.	Dipeptide	L-alanyl-L-threonine
3.	Dipeptide	Glutamyl glycine
4.	Dipeptide	γ-glutamyl-glycine
5.	Dipeptide	Glutamyl-alanine
6.	Dipeptide	γ-glutamyl-alanine
7.	Dipeptide	Glutamyl-valine
8.	Dipeptide	γ-glutamyl-valine
9.	Dipeptide	Glutamyl-threonine
10.	Dipeptide	γ-glutamyl-threonine
11.	Dipeptide	Alanyl-phenylalanine
12.	Dipeptide	Valyl-lysine
13.	Dipeptide	Arginyl-phenylalanine
14.	Dipeptide	L-alanyl-L-tryptophan
15.	γ-glutamyl dipeptide	γ-glutamyl-leucine
16.	γ-glutamyl dipeptide	γ-glutamyl-isoleucine
17.	γ-glutamyl dipeptide	α-glutamyl-aspartic acid
18.	γ-glutamyl dipeptide	γ-glutamyl-aspartic acid
19.	γ-glutamyl dipeptide	α-glutamyl-lysine
20.	γ-glutamyl dipeptide	ε-(γ-glutamyl)-lysine
21.	γ-glutamyl dipeptide	γ-glutamyl-glutamine
22.	γ-glutamyl dipeptide	γ-glutamyl-glutamate
23.	γ-glutamyl dipeptide	γ-glutamyl-methionine
24.	γ-glutamyl dipeptide	γ-glutamyl-histidine
25.	γ-glutamyl dipeptide	γ-glutamyl-phenylalanine
26.	γ-glutamyl dipeptide	γ-glutamyl-tyrosine
27.	γ-glutamyl tripeptide	γ-glutamyl-valyl-glycine
28.	Tripeptide	SH-tripeptide-4
29.	Tripeptide	Tripeptide-10 (citrulline)
30.	Tripeptide	Glutamyl derivative complex (peak 57)
31.	Tetrapeptide	Tetrapeptide-1
32.	Tetrapeptide	Tetrapeptide-11
33.	Tetrapeptide	Acetyl tetrapeptide-3
34.	Tetrapeptide	Acetyl tetrapeptide-11
35.	Tetrapeptide	NAM-tetrapeptide
36.	Tetrapeptide	Disaccharide tetrapeptide
37.	Pentapeptide	Pentapeptide-4
38.	Pentapeptide	Acetyl pentapeptide-1

antibacterial activity of protein hydrolysates is often multifactorial and synergistic, rather than derived from a single peptide.

Effect of Cricket Protein Hydrolysate on Quails Microbiome Profile

Taxonomic Classification of Illeal Microbiome

The diversity index is an approach to measuring bacterial diversity, dominance, and evenness in samples. The following results of the diversity index analysis are presented in Table 5. The results of the diversity analysis indicate that both treatments showed differences in the gut microbiota of quail. The Shannon index shows that the number of species and the balance of abundance between species in the CPH treatment indicate a more even distribution of microbes than in the basal feed. The Simpson index also shows a very strong level of dominance in the basal feed sample, namely 49%, while the CPH treatment tends to be higher at 51%. This indicates that the CPH treatment shows no species that are too dominant, while the Chao1 index shows that the basal feed sample produces a microbiome with many types compared to the CPH treatment, namely 58% and 42%. This is influenced by the CPH treatment, which eliminates pathogenic bacteria and enriches non-pathogenic bacteria such as *Lactobacillus*.

Phylogenetic Diversity of Ileal Bacteria

Phylogenetic analysis aims to map bacterial clusters according to their abundance. This analytical approach will help understand patterns of descent, diversification, and kinship. The following phylogenetic analysis is presented in Table 6, and Figures 2 and 3.

The results of the phylogenetic analysis, in the form of a circular cladogram, show variation in bacterial diversity between groups A and B, as indicated by differences in the density and color of the rings formed.

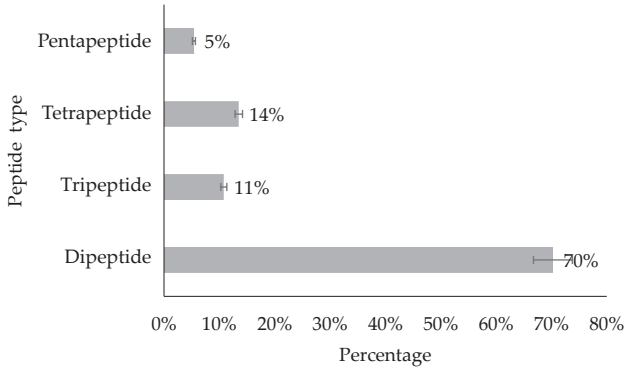


Figure 1. Percentage of peptide types from cricket protein hydrolysate

Treatment B is associated with a higher abundance of Bacillota bacteria, implying its influence promotes fermentation and fiber degradation. This shift indicates an increase in intestinal function and digestive tract homeostasis because it is influenced by the intervention of feed additives that can affect the ileal microbiome. The most dominant group is the *Bacillota phylum* with many branches formed and the resulting clades, while the Pseudomonadota, Actinomycetota, and Bacteroidota phyla show less branching compared to Bacillota. The branching formed by the Bacillota phylum shows variations in the fermentative group genera such as *Lactobacillus*, *Ligilactobacillus*, *Levilactobacillus*, *Enterococcus*, *Streptococcus*, *Ruminococcus*, *Clostridium*, *and Faecalibacterium.*

Based on the results of histogram and Venn diagram analysis (Figure 4) regarding the abundance of bacteria in the quail ileum, contestation was found in the basal feed treatment and the cricket protein hydrolysate treatment. A total of 14 types of bacteria were found in the basal feed treatment, including *Clostridium perfringens*, *Ligilactobacillus aviarius*, *Enterococcus cecorum*, *Escherichia-Shigella*, and *Clostridium botulinum*, while in the cricket protein hydrolysate treatment, 13 types of bacteria were found, including *Ligilactobacillus aviarius*, *Bifidobacterium pullorum*, *Veillonella magna*, *Lactobacillus intestinalis*, and *Escherichia-Shigella*.

The overlap in the Venn diagram indicates similarities among the bacteria found in the basal and adult feed treatments of cricket protein hydrolysate. The amount obtained was approximately 40% of the total population. Although containing the same bacteria, each treatment showed a different total population, such as *Ligilactobacillus aviarius* in the basal feed treatment, which obtained $10^{5.5}$ CFU/mL, while the cricket protein hydrolysate treatment was higher at $10^{5.7}$ CFU/mL. *Clostridium botulinum* and *Streptococcus iniae* bacteria decreased after intervention with cricket protein hydrolysate feed (Figure 4).

PCA Analysis of Quail Ileum Bacteria

Principal component analysis (PCA) was used to visualize data, reduce large and complex datasets, and group species based on similarity. The results of

Table 5. Diversity index of the quail gut microbiota in basal feed and cricket protein hydrolysate (CPH)

Diversity index	Basal feed	CPH
Shannon	3.51	3.6
Simpson	0.76	0.79
Chao1	335	240

Table 6. Ileal bacterial phyla in quail basal diet (A group) and cricket protein hydrolysate (CPH) supplementation (B group)

Color code	Phylum	General Character
P1 (green)	Pseudomonadota (Proteobacteria)	Gram-negative, flexible metabolism, environmentally responsive
P2 (red)	Actinomycetota	Gram-Positive, degradation of complex compounds
P3 (blue)	Bacteroidota	Gram-Negative, polysaccharide degradation, fiber digestion
P4 (orange)	Bacillota (Firmicutes)	Gram-Positive, fermentation, SCFA production

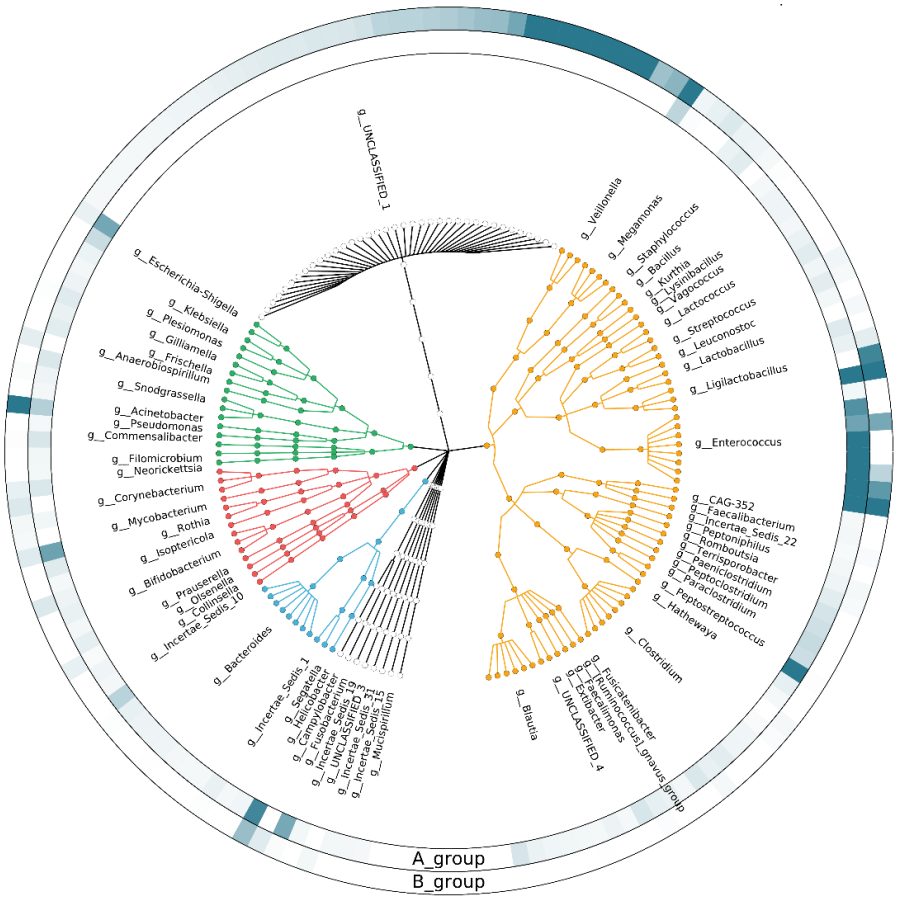


Figure 2. Circular phylogenetic cladogram showing the bacterial community in the ileum of Japanese quails receiving the basal diet (A group) and cricket protein hydrolysate (CPH) supplementation (B group). Note : ● p1: Pseudomonadota, ● p2: Actinomycetato, ● p3: Bacteroidota, ● p4: Bacillota

PCA analysis are presented in Table 7, and Figures 5 and 6. The PCA results (Figure 5) show that most bacterial samples are concentrated on the left side of the Dimension 1 (Dim1) axis, which explains 75% of the variation in the data. This indicates that the main differences between bacterial samples are primarily influenced by the first component, with a tendency for most quail ileal bacterial species to have similar microbiota characteristics in terms of composition or abundance.

The results of the factor loading (Table 7) of the PCA analysis of 19 identified types of quail ileum bacteria showed different results based on the PCA analysis, namely the variation value of DM1 was 75% while DM2 was 25%. This indicates that DM1 and DM2 can explain the distribution of the data because they account for more than 50% of the cumulative variance. *Ligilactobacillus aviarius* bacteria have the highest DM1 value of all ileum bacterial species. Furthermore, *Ente*rococcus *cecorum*, *Clostridium chauvoei*, *Enterococcus*, and *Clostridium perfringens* bacteria showed high DM1 values, which indicate the most striking character differences compared to other species. Meanwhile, the DM2 value is the opposite of the DM1 value, where *Enterococcus cecorum* bacteria are ranked highest, followed by *Lactobacillus_aviarius*. The dots on the left are most likely from one treatment group, indicating that the treatment produced a relatively homogeneous bacterial community and was significantly different from the other treatments. However, species that were far from the overall species distribution had high population sizes, such as *Ligilactobacillus aviarius* and *Enterococcus cecorum*.

DISCUSSION

Characteristics of Brown Cricket Protein Extract and Brown Cricket Protein Hydrolysate

The hydrolysis degree of 26.50% indicates a moderate degree of hydrolysis. The degree of hydrolysis also affects the iron-binding capacity of the protein hydrolysate. A moderate degree of hydrolysis is beneficial for producing peptides with high iron-binding capacity, as seen in soy protein hydrolysate (Zhang *et al*., 2021). In addition to demonstrating a moderate degree of hydrolysis, cricket flour also exhibits high amino acid content after HPLC analysis. This is influenced by the enzymatic hydrolysis process. Controlled enzymatic hydrolysis of crickets produces hydrolyzed protein with improved protein functionality (Hall *et al*., 2017).

Cricket flour (*Gryllus assimilis*) contains several amino acids such as histidine, threonine, sulfur-containing amino acids, and aromatic amino acids (de Oliveira Filho *et al*., 2020), while research by Soares Araújo *et al*.

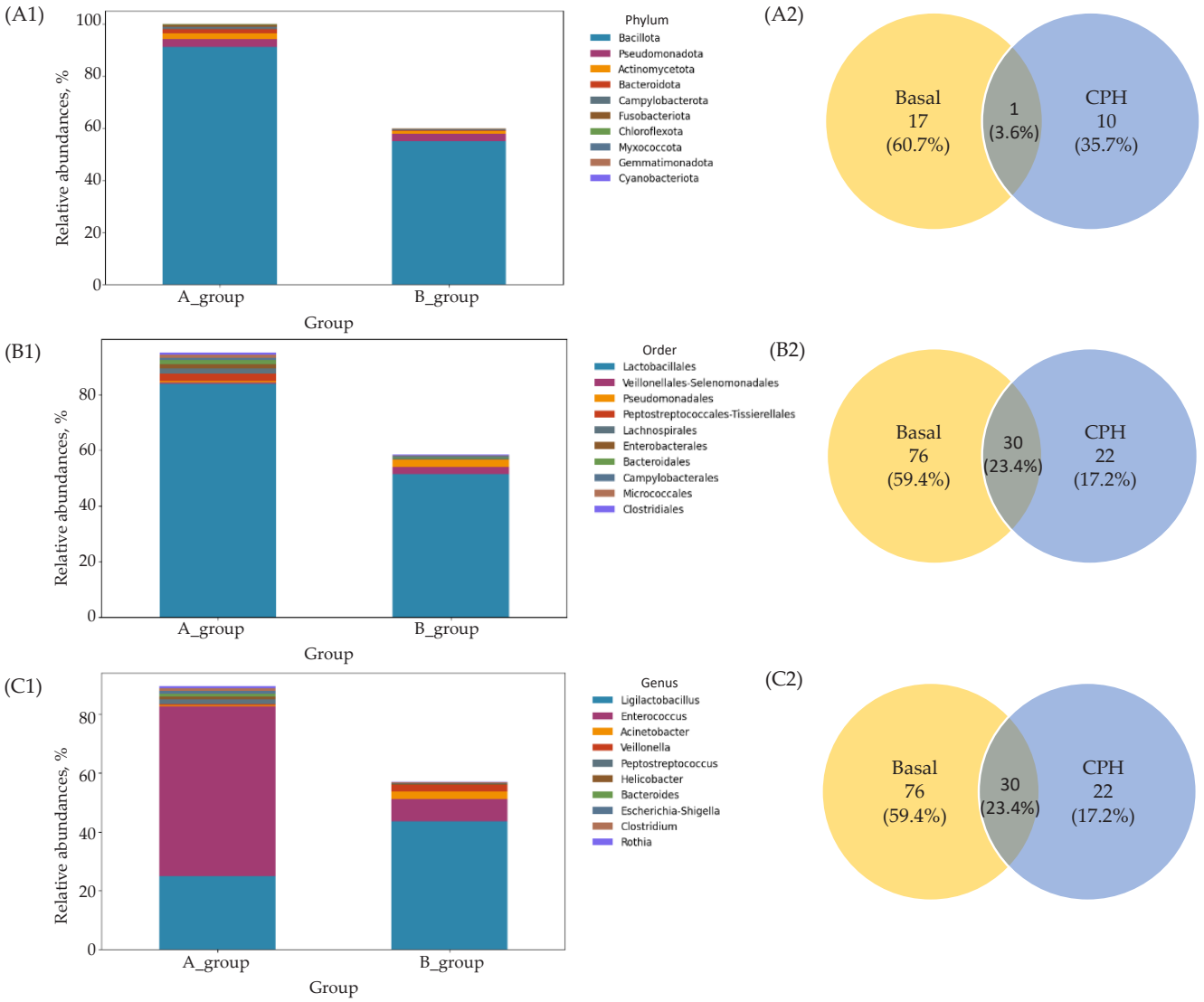


Figure 3. Taxonomic classification of the 16S rRNA gene sequence at the (A) phylum, (B) order, and (C) genus level for the basal diet (Group A) and cricket protein hydrolysate (CPH) supplementation (Group B) samples

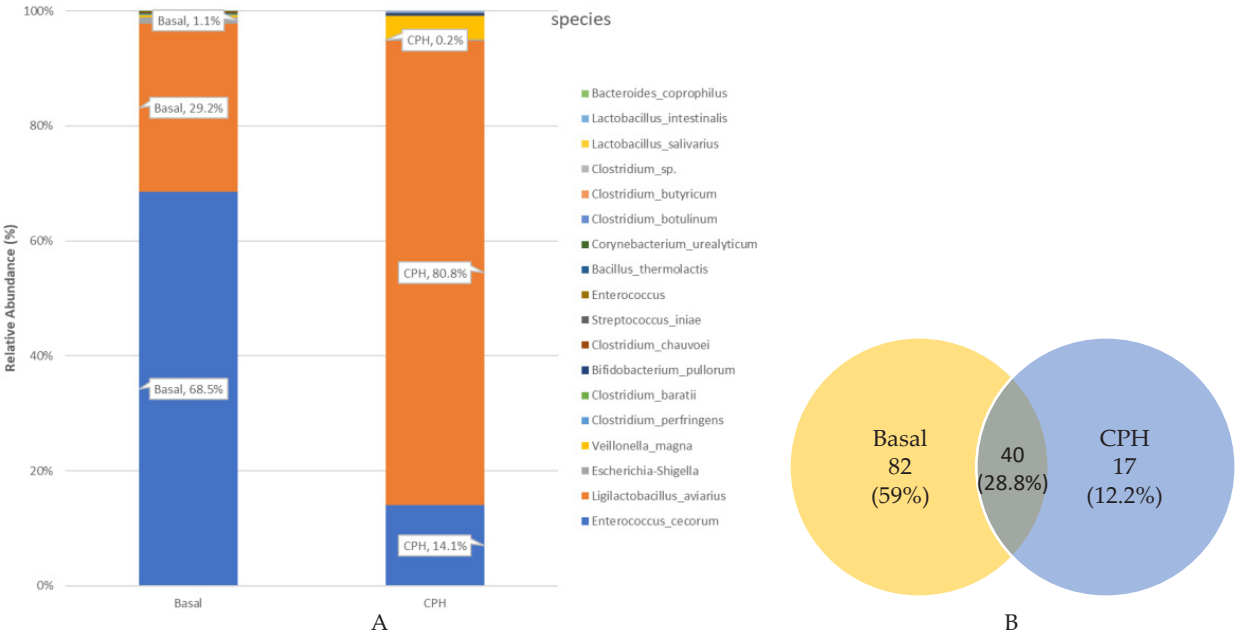


Figure 4. Top 20 abundance of quail ileum bacteria = A (relative abundance of species) and B (Ven diagram)

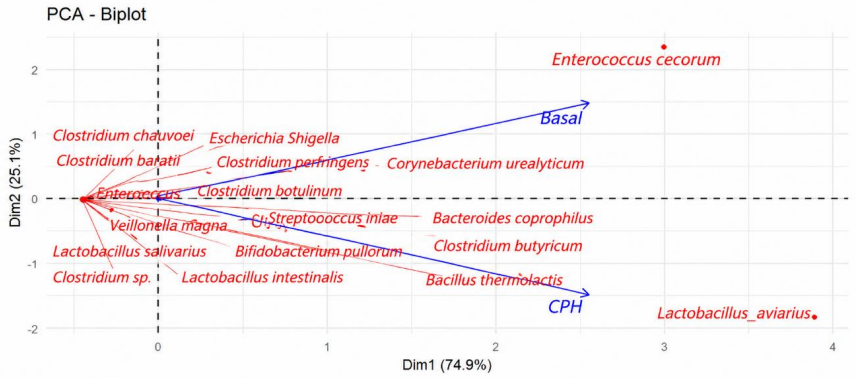


Figure 5. Principal component analysis (PCA) visualization results of bacteria and treatment vector direction

Table 7. Principal component analysis (PCA) coordinates of the predominant bacterial taxa in the quail ileum

Individuals (the first 10)	Dist	Dim.1	Dim.2
Enterococcus cecorum	3.808	2.997	2.348
Lactobacillus_aviarius	4.299	3.874	1.865
Escherichia Shigella	0.395	0.395	0.014
Veillonella magna	0.32	0.274	0.166
Clostridium perfringens	0.439	0.439	0.015
Clostridium baratii	0.442	0.441	0.017
Bifidobacterium pullorum	0.433	0.432	0.032
Clostridium chauvoei	0.445	0.444	0.02
Streptococcus iniae	0.44	0.44	0.025
Enterococcus	0.446	0.445	0.021

Note: Dist = Euclidean distance of each bacterial taxon from the origin in the two-dimensional PCA space; Dim.1 = coordinate of each bacterial taxon on the first principal component (PC1); Dim.2 = coordinate of each bacterial taxon on the second principal component (PC2).

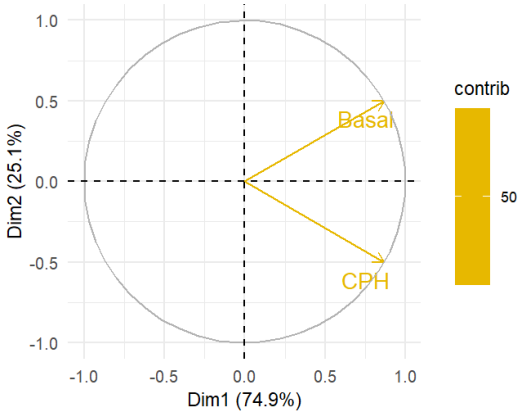


Figure 6. Principal component analysis (PCA) loading plot of the basal and cricket protein hydrolysate (CPH) treatments in quails

(2019) stated that the limiting amino acid for *Gryllus assimilis* is methionine. A similar finding was reported by Khatun *et al.* (2021), who stated that the amounts of methionine and cysteine in *Gryllus assimilis* are limited. Insect extraction with ethanol can improve protein quality compared to using water and produces lower insect fat compared to organic solvents (Laroche *et al*., 2019).

The PAYA enzyme hydrolysis process degrades proteins into easily digestible amino acids, which are detected in the amino acid counts in the protein hydrolysate. The papain enzyme is capable of hydrolyzing cricket proteins, thereby increasing bioavailability. The enzyme used in the hydrolysis process directly affects the profile of the released peptides and their bioactivity (de Matos *et al*., 2021). Alkaline pH induces changes in the unfolded protein structure, with the structure opening up, exposing hydrophobic groups and sulfhydryl (-SH) groups (Moment & Dietz, 2021). Extraction, solvent extraction, ultrasonication, and enzyme hydrolysis methods can be applied to improve the functionality and bioactivity of insect proteins (Hall *et al*., 2017; Lv *et al*., 2025).

The higher concentrations of free amino acids in cricket protein hydrolysate (CPH), particularly glutamic acid, aspartic acid, lysine, leucine, valine, isoleucine, arginine, threonine, and tryptophan, may have contributed to the differences in ileal microbial composition observed in quails. Free amino acids function not only as essential nutrients for host metabolism but also as substrates that support microbial growth and influence the composition of the intestinal microbial community. Consequently, the greater availability of amino acids released during protein hydrolysis may have provided a favorable nutritional environment for beneficial bacteria. This may explain the higher relative abundances of *Lactobacillus aviarius, Lactobacillus salivarius, Enterococcus cecorum, Bacillus thermolactis, and Clostridium butyricum* observed in the CPH group. Functional amino acids such as threonine and glutamate have been reported to promote intestinal health by supporting mucin synthesis and enterocyte metabolism, respectively. In addition, aromatic amino acids, including tryptophan, phenylalanine, and tyrosine, serve as precursors of microbial metabolites that regulate host–microbiota interactions. Collectively, these findings suggest that the enriched amino acid profile of CPH may have created a favorable intestinal environment that supported the proliferation of beneficial bacteria and promoted a healthier ileal microbial community (Taylor-Bowden *et al*., 2024; McCann & Rawls, 2023; Chalvon-Demersay *et al*., 2021).

In addition to its high free amino acid content, the peptide-rich composition of CPH may also have contributed to the observed modulation of the ileal microbiota. Dietary protein hydrolysates contain low-

molecular-weight peptides that can serve as microbial substrates and influence microbial metabolism, thereby promoting the production of metabolites involved in maintaining intestinal homeostasis (Hu *et al*., 2024; Wijesekara *et al*., 2024). In the present study, the greater relative abundances of *Lactobacillus*, *Enterococcus*, *Bacillus, and Clostridium butyricum* in the CPH group may be associated with the availability of these bioactive peptides, which could have supported the growth and metabolic activity of beneficial bacteria. In addition to the enhanced free amino acid profile, the peptide-rich characteristics of CPH may have created a favorable intestinal environment that promoted beneficial microbial populations and contributed to the observed changes in the ileal microbiota.

Diversity of Microbiome

Bacteria in the digestive tract play a crucial role in nutrient utilization in feed. A healthy digestive tract is characterized by a balanced microbiota, with lactic acid bacteria playing the most important role. The role of bacteria identified in the Bacillota phylum has the potential to ferment structural carbohydrates, namely high in fiber such as cellulose, hemicellulose, pectin, and lignin, and can produce short-chain fatty acids (SCFA) such as acetic acid, propionate, and butyrate which can be converted as an energy source, can maintain the integrity of the intestinal mucosa, maintain intestinal adhesion, improve the immune system, support the growth of good bacteria, and increase the efficiency of energy utilization. The Bacteroidota phylum is known as a bacteria that plays a role in degrading complex polysaccharides and dietary fiber, such as those found in the genera *Bacteroides*, *Alistipes*, and *Prevotella*. The Actinomycetota phylum is a group of probiotic bacteria that plays a role in maintaining intestinal health, increasing the population of LAB bacteria, and improving nutrient utilization in the intestine, such as the genera *Bifidobacterium* and *Corynebacterium*. High abundance of Bacillota increases total feed intake, especially through starter feed intake, which results in high final body weight on the 43rd day of feeding (Deryabin *et al*., 2024). Bacillota belong to a group of large-genome bacteria that use more than 9% of their genome to encode new biosynthetic gene clusters (BGCs) (Baltz, 2017) that produce novel bioactive compounds (Baltz, 2021). These bioactive molecules belong to different chemical classes, such as erpenes, polyketides (PKs), non-ribosomal peptides (NRPs), lasso peptides, bacteriocins, thiopeptides, ectoins, and melanins (Viju *et al*., 2021). The findings of Burrows *et al*. (2025) show that the genus *Lactobacillus* is most abundant in the jejunum and ileum, indicating its central role in fiber degradation and anaerobic fermentation.

The identification results also revealed pathogenic bacteria, namely members of the Pseudomonadota phylum, including *Escherichia-Shigella, Klebsiella, Pseudomonas,* and *Acinetobacter*. The numbers formed in the phylogenetic branch appeared lower than those in the Bacillota phylum, indicating that the cricket peptide hydrolysis intervention could reduce the population of pathogenic bacteria and increase the population of LAB bacteria associated with immunity and health. Generally, pathogenic bacteria increase when environmental stress or disruption of the microbiota balance occurs. The complex microbial ecosystem in the chicken digestive tract is often dominated by lactic acid bacteria in the proximal intestine (Dedenum and jejunum) (Yadav & Jha, 2019), including the genera *Lactobacillus, Bifidobacterium, Enterobacteriaceae*, and *Klebsiella* which initiate feed digestion (Fathima *et al*., 2022) while *Lactobacillus, Enterococcus, Clostridia, Streptococcus, Bacteroides, coliforms, Faecalibacterium, Ruminococcus* are often abundant in the distal intestinal microbial community (Ileum, cecum, colon, and cloaca) (Kumar *et al*., 2018) these findings are strengthened by the intervention of giving cricket protein hydrolysate showing lactic acid bacteria concentration.

Correlation between Chemical Composition CPH and Microbiome Profile in Quails

The competition for nutrients in the digestive system leads to increased peptide hydrolysis in lactic acid bacteria. This is influenced by the synergy between peptides and gut microbiota. According to Li *et al*. (2021), antimicrobial peptides (AMPs) are a class of small-molecule peptides consisting of 15–100 amino acids that are an important part of the body's innate immune system and can fight the invasion of pathogenic microorganisms. Peptides from insects can be used as antibacterials, antioxidants, antifungals, and antivirals (Xia *et al*., 2017). The presence of peptides added to feed can attack pathogenic bacteria (Zeiders & Chmielewski, 2021) by destroying cell membranes, lowering pH, cleansing the digestive tract, and increasing the population of lactic acid bacteria (Jangpromma *et al*., 2022).

Patyra and Kwiatek (2023) claim that insects are the organisms producing the most peptides. One insect produces 15–20 peptides, the concentration of which in the hemolymph increases rapidly during infection. The antibacterial activity of peptides binds to the cell membrane and inserts itself into the hydrophobic core of the membrane. This causes pores and leakage of cytoplasmic material and a decrease in membrane potential. Peptides damage the cell membrane, disrupting cell ionic homeostasis and causing dysfunction and bacterial cell death (Patyra & Kwiatek, 2023). The antibacterial activity of peptides can attack both Gram-negative and Gram-positive bacteria, but this study shows that the ability of lactic acid bacteria to survive is due to their thick peptidoglycan cell walls, indicating that lipopolysaccharide (LPS) has hydrophobic (lipid A) and hydrophilic components, while peptidoglycan tends to be hydrophilic (polar).

The heatmap in Figure 7 shows a pattern of absolute abundance of pathogenic bacteria indicated by a color change from red to white in the basal feed and cricket peptide hydrolysis treatments. The basal feed treatment showed that pathogenic bacteria dominated and their populations were very high, such as *Clostridium perfringens*, *Clostridium baratii*, *Clostridium chauvoei*, and *Enterococcus cecorum*. While the CPH group

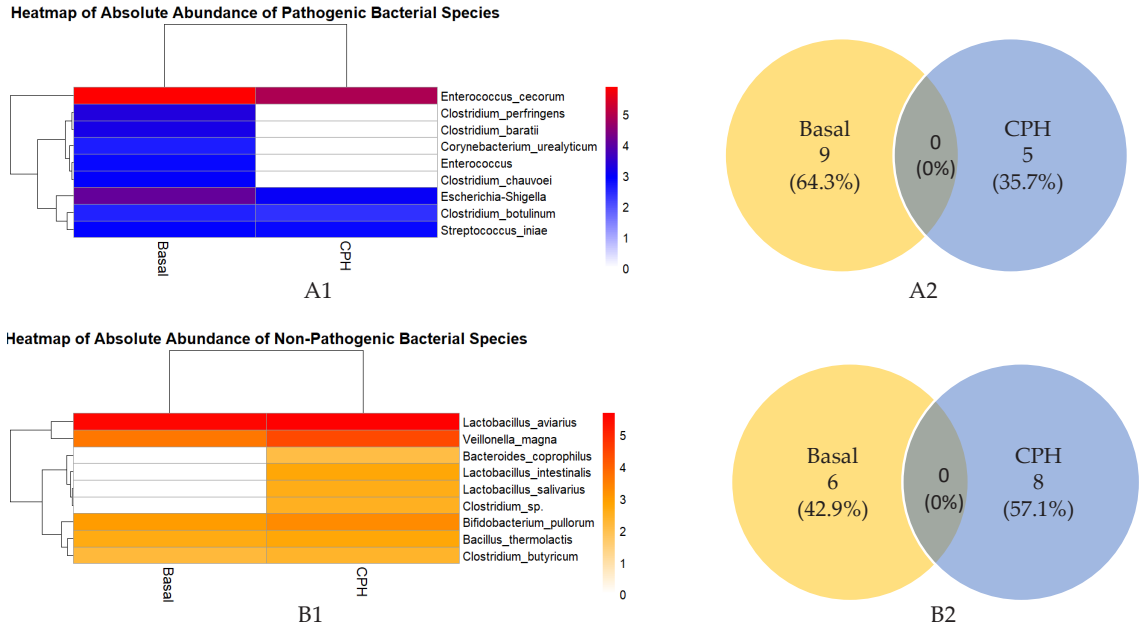


Figure 7. Heatmap of abundance of pathogenic and non-pathogenic bacteria. (A1= Dendrogram analysis of pathogenic bacteria, A2= Venn diagram of pathogenic bacteria, B1= Dendrogram analysis of non-pathogenic bacteria, B2= Venn diagram of non-pathogenic bacteria)

showed a significant decrease in white color dominance in Enterococcus, Corynebacterium urealyticum, Clostridium chauvoei, and Clostridium baratii, it still showed moderate abundance in Escherichia-Shigella, Clostridium botulinum, and Streptococcus iniae. The clustering pattern in the dendrogram shows that the CPH group tended to have more homogeneous and reduced types of bacteria and had suppressive properties.

The findings suggest an enhancement in intestinal health and microbial equilibrium. The heatmap in Figure 5 illustrates an increased abundance of several bacterial taxa following CPH supplementation. Notably, these taxa were primarily beneficial or non-pathogenic, indicating a healthier gut ecosystem. This positive microbial shift likely facilitated improved nutrient utilization and immune homeostasis. These observations align with our previous findings, which demonstrated that CPH supplementation enhanced hematological status by increasing red blood cell counts and reducing white blood cell counts, while also improving internal egg quality. Collectively, these results imply that CPH functions as a natural immunonutrient by modulating gut microbiota, strengthening host immunity, and supporting poultry health and productivity. (Lidiyawati *et al*., 2026b).

The Venn diagram also confirms the heatmap findings that the number of pathogenic bacteria found tended to decrease in the CPH treatment, at 44.4%, compared to 100% in the basal treatment. However, conversely, non-pathogenic bacteria dominated in the CPH treatment, at 100%. This confirms that the CPH treatment can reduce the population and types of pathogenic bacteria and increase the population of non-pathogenic bacteria associated with digestive tract health.

The PCA plot showed that samples clustered on the left side were likely derived from the same treatment group, indicating that the treatment produced a relatively homogeneous bacterial community structure that was clearly differentiated from the other treatment groups. In contrast, bacterial species located farther from the central cluster, such as *Ligilactobacillus aviarius* (*L. aviarius*) and *Enterococcus cecorum*, contributed strongly to the observed variation due to their relatively higher abundance. This finding suggests that the treatment may selectively influence the proliferation of specific bacterial taxa. According to Galicia *et al*. (2023), the genus *Ligilactobacillus* comprises host-adapted, homofermentative bacteria commonly found in the gastrointestinal tracts of vertebrates (Zheng *et al*., 2020). In addition, *L. aviarius* has been reported to encode an alpha-glucanotransferase that converts amylose starch into isomalto-/malto-polysaccharides (IMMP) (Meng *et al*., 2018), suggesting its potential role in carbohydrate metabolism and gut microbial adaptation.

These findings complement our previous study, which demonstrated that dietary supplementation with brown cricket extract and protein hydrolysate improved quail performance (Lidiyawati *et al*., 2026a). The present study extends those observations by demonstrating that these beneficial effects may be associated with higher concentrations of amino acids, the presence of bioactive peptides, and favorable modulation of the ileal microbiota. Supplementation with cricket protein hydrolysate improved hematological parameters associated with physiological condition and immune responses, supporting the present microbiome findings. The increased abundance of beneficial lactic acid bacteria and reduced pathogenic bacterial populations may contribute to enhanced intestinal health and systemic immune function. The synergistic relationship between antimicrobial peptides, gut microbiota modulation, and hematological improvement suggests

that cricket protein hydrolysate has strong potential as a functional feed additive and sustainable alternative to antibiotic growth promoters (AGPs) in poultry production systems.

CONCLUSION

Protein hydrolysis increased the concentrations of several amino acids, particularly glutamic acid, valine, lysine, and aspartic acid, and identified 36 peptides predominantly composed of dipeptides. The resulting cricket protein hydrolysate was subsequently evaluated as a dietary feed additive in quails. Supplementation with cricket protein hydrolysate (CPH) promoted a more homogeneous gut microbiota characterized by increased lactic acid bacteria and reduced pathogenic bacteria compared with the basal diet. These microbiome changes were consistent with improvements in physiological, immunological, and production performance variables, highlighting the potential of CPH as a functional and sustainable alternative to antibiotic growth promoters (AGPs) in poultry production. However, further studies are needed to determine the optimal supplementation dosage and to evaluate its long-term effectiveness and stability under commercial-scale production conditions.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this manuscript, the authors used Paperpal and ChatGPT (OpenAI) to assist with English language editing and to improve the readability and clarity of the text. The authors critically reviewed and revised all AI-assisted suggestions before incorporation into the manuscript and accept full responsibility for the accuracy, originality, and integrity of the work.

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