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Original Article

Evaluating the Potential of a Hydrolyzed Chicken-based Diet as a Supplementary Feed for Enhancing Honey Bee Health, Lifespan, and Gut Microbiome

Bajaree Chuttong [1], Pichet Praphawilai [1,2], Sampat Ghosh [3], Chuleui Jung [4,5], Sukjun Sun [5], Hyeonjeong Jang [5], Ming Cheng Wu [6], Khanchai Danmek [7*]

[1] Meliponini and Apini Research Laboratory, Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University 50200, Thailand
[2] Office of Research and Administration, Chiang Mai University, Chiang Mai University, 239, Suthep, Muang, Chiangmai, 50200, Thailand
[3] Department of Life Science, Sardar Patel University, Balaghat, Madhya Pradesh 481331, India
[4] Agricultural Research Institute, Gyeongkuk National University, Andong GB 36729, South Korea
[5] Department of Plant Medicals, Gyeongkuk National University, Andong GB 36729, South Korea
[6] Department of Entomology, College of Agriculture and Natural Resources, National Chung Hsing University 402202, Taiwan
[7] Biotechnology Program, School of Agriculture and Natural Resources, University of Phayao 56000, Thailand

Corresponding Author: Khanchai Danmek, PhD, Biotechnology Program, School of Agriculture and Natural Resources, University of Phayao 56000, Thailand. Tel: +6654466666 (ext. 3156), E-mail: khanchai.da@up.ac.th

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Abstract

Introduction: This study investigates the potential of animal-derived meat proteins as substitutes for maize pollen in honey bee diets. The research focuses on evaluating their effects on bee health through physiology and microbiome analyses.

Materials and Methods: Meat samples (chicken, pork, and beef) were subjected to enzymatic hydrolysis. The hydrolyzed proteins were incorporated into artificial diet formulations for honey bee feeding trials, with maize pollen serving as the control diet. Honey bees were allocated to each dietary group and monitored to evaluate lifespan and hypopharyngeal gland development. Subsequently, gut microbiome diversity and composition were analyzed to provide a comprehensive assessment.

Results: SEM images showed that enzymatic hydrolysis improved muscle fiber structure, enhancing digestibility, with chicken-based diets showing the best results. Proximate analysis revealed moisture content from 30-48%, carbohydrates from 25-49%, fat from 3-5%, and protein content remained consistent at 13-15%. The hydrolyzed chicken-based diet had the highest amino acid content (26.92 g/100 g). Survival studies showed that bees fed hydrolyzed chicken-based diets had similar survival rates and hypopharyngeal gland acini development to those fed maize pollen diets, outperforming other diets. Microbiome analysis revealed increased microbial diversity, with *Bartonella* and *Lactobacillus* dominating, and *B. apis* most abundant in the hydrolyzed chicken-based diet.

Conclusions: Hydrolyzed chicken-based diets demonstrated equivalent nutritional and microbiome benefits to maize pollen, positioning it as a sustainable and cost-effective alternative for apiculture. Given the occasional scarcity of maize pollen, animal protein emerges as a viable substitute, contributing to greater sustainability within the beekeeping industry.

Keywords: Maize Pollen Alternative, Meat, Artificial Pollen Diet, Honey Bee Health

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Introduction

Honey bees (*Apis mellifera*) rely on pollen as a critical nutritional resource that supports vital biological functions, including growth, physiological development, immunity, and egg production, all of which are essential for maintaining hive population dynamics.[1,2] However, the availability of pollen, a critical nutritional source for honey bees can be disrupted by factors such as seasonal variability and climate change, creating significant challenges for maintaining hive health and productivity. Moreover, contamination of pollen by fungal spores and chemicals poses significant health risks to both individual bees and entire colonies.[3] These challenges underscore the pressing need for alternative feed sources that can effectively replace pollen while ensuring the health and productivity of honey bees. As a result, beekeepers and researchers have been compelled to explore and develop supplemental feeding strategies that meet the nutritional requirements of honey bees.[4,5] Recent studies have proposed various approaches involving plant-based, animal-based, and microbial ingredients, highlighting the potential of these resources to

support hive health and promote sustainability.[5, 6]

In apiculture, hydrolyzed proteins have been explored in the development of artificial diets to replace pollen with promising outcomes. Honey bees fed hydrolyzed protein-based diets exhibited improved growth compared to those consuming traditional pollen-based diets, such as those described by Haydak (1967) (Kumari & Kumar, 2020).[7,8]

Researchers highlighted the positive effects of hydrolyzed protein-based pollen substitutes on feeding behavior, egg production, larval development, and honey storage. These findings underscore the potential of hydrolyzed protein-based diets to enhance various aspects of honey bee health and productivity. Recent innovations have further expanded the scope of artificial pollen diets by incorporating alternative plant-based protein. For example, they developed an artificial pollen diet using maize pollen mixed with leaf protein concentrate (LPC) derived from mulberry leaves (*Morus alba* L.), demonstrating significant contributions to honey bee development and supplemental feeding strategies.[6] These advancements suggest that hydrolyzed proteins, along with novel raw materials like LPC, offer sustainable and effective alternatives to traditional feed production methods.

Another promising protein ingredient to produce an artificial pollen diet is hydrolyzed protein derived from chicken meat. This protein is particularly attractive due to its small molecular size, which enhances digestibility and nutrient absorption in animals. Previous studies have demonstrated the use of hydrolyzed chicken-based diets in various livestock, such as pig, broiler chicken, and fish (*Nile tilapia*),[10,11] as well as insects like house cricket (*Acheta domesticus*) and field cricket (*Gryllus bimaculatus*).[12] Moreover, poultry farming demonstrates rapid production rates and high yields, effectively addressing the increasing consumer demand on an annual basis.[13] This positions chicken meat as a viable alternative animal protein source for beekeepers seeking to replace traditional pollen with an animal-based diet. The incorporation of hydrolyzed chicken protein into honey bee diets offers a sustainable and efficient option, particularly for beekeepers aiming to provide cost-effective solutions that enhance honey bee health and productivity.

Although the use of chicken meat in creating artificial pollen diets for honey bees has not been extensively explored, developing such diets requires a careful blend of ingredients. This formulation must ensure an optimal nutritional profile that promotes honey bee growth, longevity, and colony expansion.[14,15] It may be similar in nutritional value to commercially available artificial pollen substitutes, as indicated by previous studies on their nutritional content.[16,17] The aim of this study is to assess the efficacy of animal-derived proteins as a protein substitute for pollen in honey bee nutrition. The hypothesis is that hydrolyzed chicken meat can effectively replace pollen in artificial diets, supporting honey bee growth, health, and lifespan to a similar or superior extent compared to proteins derived from other sources such as beef or pork. Furthermore, this study will examine the influence of a hydrolyzed chicken-based diet on the gut microbiota of honey bees. By exploring the use of animal-derived proteins as pollen replacements, this research seeks to advance sustainable and efficient beekeeping practices, addressing critical challenges in apiculture during periods of natural pollen scarcity.

Materials and Methods

Preparation of Samples

Chicken breast, along with two economically viable protein sources, including pork hock and beef shank, were procured from a supermarket in Andong City, North Gyeongsang Province, South Korea, in August 2024. This selection aimed to mitigate the risk of contamination from pathogenic microorganisms commonly associated with raw meat. Additionally, maize pollen was obtained from a local beekeeping supply store in Chiang Mai Province, Thailand. All samples were meticulously stored at 4 °C to ensure their integrity until subsequent use in experimental procedures.

Preparation for Animal Meat-based Diets

Three types of meat included chicken breast, pork hock, and beef shank were pretreated with 2.0% (w/w) of a commercially available acid protease enzyme purchased from Thailand under conditions of 60 °C for 3 hours. After pretreatment, the enzymatic activity was subsequently inactivated by boiling the samples in hot water for 15 minutes. The treated samples were then homogenized to achieve a uniform texture, resulting in hydrolyzed meat, which was used for subsequent experimentation. Three different types of animal meat-based diets, each containing approximately 15% protein derived from hydrolyzed meat, were prepared using the following composition: 60.0% (w/w) hydrolyzed meat, 35.0% (w/w) sucrose, 3.0% (w/w) palm oil, 1.0% (w/w) xanthan gum, 0.50% (w/w) vitamin mix, and 0.50% (w/w) mineral mix. The three diets were homogenized using microwave heating for 3 minutes and then transferred into prepared containers to serve as animal meat-based diets. Additionally, a control group consisting of a maize pollen-based diet (patty) was prepared by mixing maize pollen with 50% (w/w) sucrose in a 2:1 ratio.

Characterization of Samples and Diets

The microstructures of untreated and protease-treated meats were analyzed using Scanning Electron Microscopy (SEM). Prior to examination, the samples were gold sputter-coated and observed using SEM (Prisma E, Thermo Fisher Scientific, USA). The proximate analysis of the artificial pollen diets, including crude protein, crude fat, crude fiber, ash, carbohydrate, and energy content, was determined

according to the guidelines of the Association of Official Analytical Chemists (AOAC) procedures.[18] Amino acid content analysis using a Sykam Amino Acid Analyzer S633 (Sykam GmbH, Bayern, Germany) equipped with a Sykam LCA L-07 column, adhering to the standard procedures of the AOAC.[19] For sample preparation, hydrolysis was carried out using 6 N HCl for 24 hours at 110 °C under a nitrogen atmosphere, followed by concentration in a rotary evaporator. The concentrated samples were then reconstituted with a sample dilution buffer (physiological buffer, 0.12 N citrate buffer, pH 2.20) provided by the manufacturer, and the hydrolyzed samples were analyzed for their amino acid composition.

Preparation of Honey Bee Colonies

Honey bee colonies were obtained from the bee lab (36°32'37.3"N, 128°48'02.8"E) at the apiary located in Gyeongkuk National University, Andong City, North Gyeongsang Province, South Korea. The colonies were carefully maintained according to established beekeeping protocols. Sealed brood frames were removed from the beehive and transferred to an insect growth chamber maintained in complete darkness, with a controlled temperature of 33 ± 1 °C and a relative humidity of 60 ± 1%.[6] The chamber conditions were maintained until the pupae metamorphosed into adult honey bees.

Honey Bee Lifespan

The experiment involved three treatments, each using animal meat-based diets containing hydrolyzed meat derived from chicken breast, pork hock, and beef shank, with a maize pollen-based diet serving as the control. Each treatment consisted of 30 newly emerged adult honey bees, housed in 600 ml transparent polypropylene cages with lids containing 40 ventilation holes, each no larger than 5.0 mm in diameter, to ensure adequate airflow. Syringes filled with water and a 50% (w/w) sucrose solution were attached to the cage lids as liquid feeders. Two grams of each type of diet were placed on plastic plates within the cages and wrapped in waxed paper, perforated with small holes to maintain softness, moisture, and suitability for bee consumption. All water, syrup, and diets were monitored and replenished every three days. Daily observations were recorded to assess the longevity and lifespan of adult worker honey bees in all treatment groups from the start of the experiment until its conclusion (21 days). Any deceased honey bees were immediately removed from the cages during the course of the experiment.

Hypopharyngeal Gland Development

On day 7, five honey bee samples from each treatment were selected to assess hypopharyngeal gland (HPG) acini development. The HPG acini were carefully excised and placed in plastic petri dishes containing droplets of ice-cold normal saline solution (0.85%, isotonic to hemolymph). Micrographs of the HPG acini were captured using an Olympus BX53 digital upright microscope (Olympus Corporation, Tokyo, Japan) equipped with an IMTcam CCD5 PLUS camera (IMT i-Solution, Inc., Vancouver, BC, Canada) at 40x magnification. For each HPG acinus, the diameters of 20 randomly selected acini with clearly defined borders were measured in pixels and then converted to millimeters (mm), with three replicates per treatment. The average measurements of20 HPG acini per bee head were used for subsequent statistical analysis.

Diversity in the Gut Microbiome

Five samples of adult honey bees from each treatment on day 21 were individually surface-sterilized with a 90% ethanol solution for 30 seconds, followed by a wash in sterile water. The samples of the entire gut (midgut and hindgut) were carefully dissected using sterile scissors and forceps, with assistance from gently pulling on the sting shaft.[20-22] DNA extraction was performed using the QIAamp DNA Mini Kit (QIAGEN, Germany), and the quantity and quality of the DNA were assessed with a Micro UV-Vis Spectrophotometer (Life Sciences, China). DNA purification was carried out using the OneStep PCR Inhibitor Removal Kit. The extracted DNA samples were stored at -20 °C prior to library preparation.[19] The V4 region of the 16S rRNA gene was PCR-amplified using primers 515F (5'-GTGCC AGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVG GGTWTCTAAT-3') with a Thermal Cycler (Bioer – Gene Explorer, China). Sequencing was conducted using an Illumina MiSeq platform (Macrogen, Seoul, Korea). All sequences have been deposited in NCBI's Sequence Read Archive under the number PRJNA1211288. A metagenome amplicon sequencing approach was employed with paired-end reads of 301 bp length in FASTQ format. Sequence analysis was performed using the Qiime2 platform, employing the DADA2 algorithm to calculate alpha diversity. Taxonomic annotation was conducted using the SILVA v132 database.[19]

Statistical Analysis

The data with the normal distribution involved a one-way analysis of variance (ANOVA), followed by post-hoc Tukey's test. A significance level was set at $p<0.05$, conducted using SPSS version 27.0 (IBM Co., Armonk, NY, USA). Kaplan-Meier survival analysis was employed to evaluate the impact of diets on the lifespan of honey bees. The statistical significance of differences in time distributions between groups was assessed.

Results

SEM Analysis

The enzymatic treatment of animal meats with protease

induces significant structural changes in muscle fibers, enhancing their digestibility. This is evident from Scanning Electron Microscope (SEM) images (Figure 1), which show that protease treatment reduces the size of muscle fibers. Specifically, untreated meats retain intact and compact muscle structures, while treated meats exhibit protein aggregation into smaller clumps, a reduction in fiber diameter, and increased spacing between fibers. Especially, the structure of treated chicken meat appears to be the most digestible when compared to pork and beef.

Proximate Analysis and Amino Acid Profiles

The proximate analysis of animal meat-based diets formulated with hydrolyzed meat (pork, beef, and chicken) and maize pollen-based diets revealed significant (p<0.05) differences in moisture, carbohydrate, fat, ash, and energy content (Table 1). However, the protein content showed minimal variation (p≥0.05) across treatments. The hydrolyzed pork and chicken-based diet had significantly (p<0.05) higher moisture content (43.41% and 43.30%, respectively) compared to the maize pollen-based diet (30.48%). The carbohydrate content was lower in the hydrolyzed animal meat-based diets compared to the maize pollen-based diet, which had the highest carbohydrate level at 49.92%. Additionally, the carbohydrates in the hydrolyzed pork, beef, and chicken-based diets were 32.91%, 25.96%, and 35.40%, respectively (p<0.05). The next results showed that the fat content in the hydrolyzed beef and chicken-based artificial pollen diets was similarly high but significantly higher (p<0.05) compared to the hydrolyzed pork-based and maize pollen-based diets.

Protein is a vital component in honey bee diets, supporting

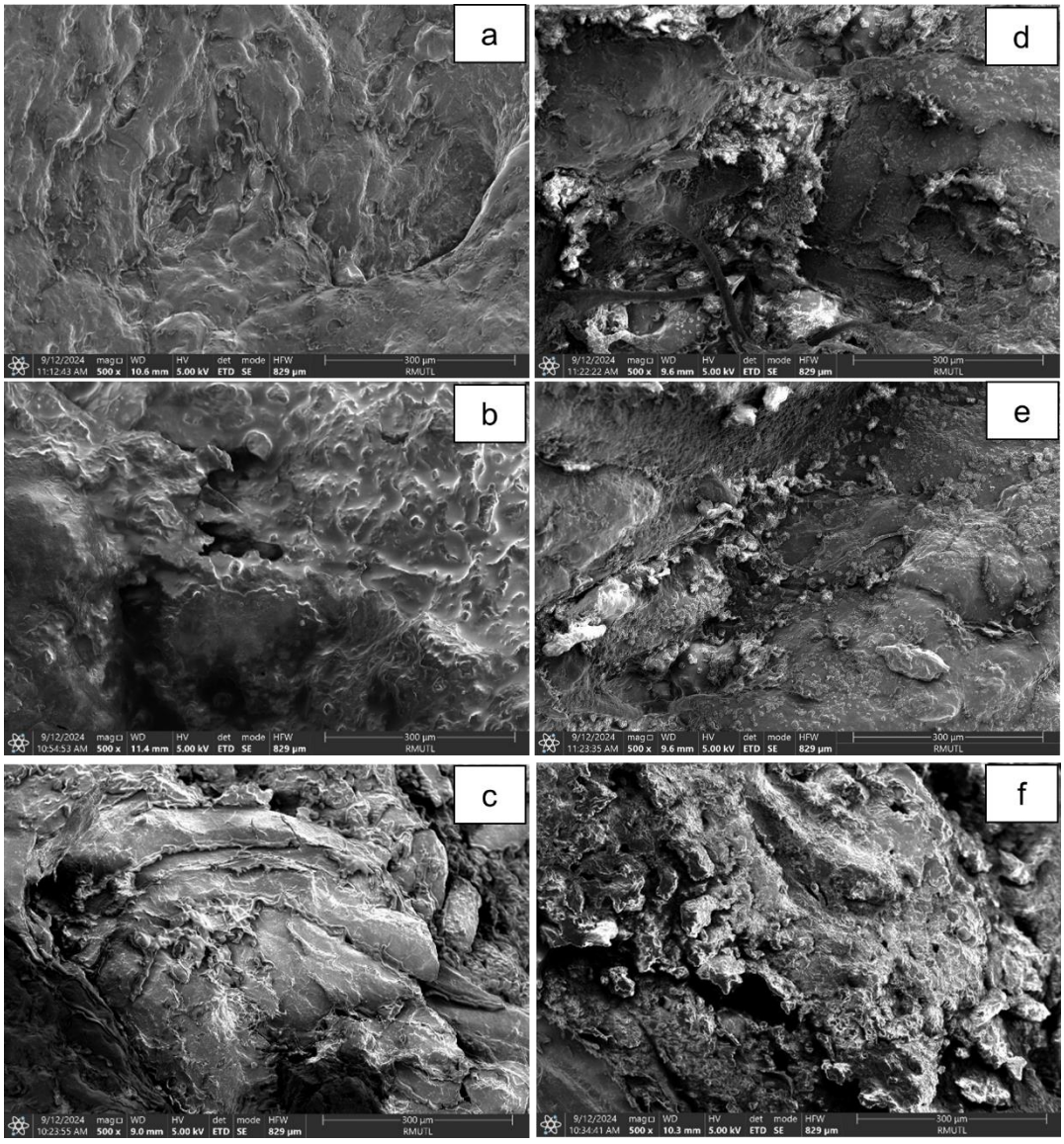

Figure 1. Scanning Electron Microscopy (SEM) Images at 500x Magnification of Untreated Meats: (a) beef, pork (b), and (c) chicken, and protease-treated meats: (d) beef, (e) pork, and (f) chicken, for 3 hours at 60 °C.

Table 1. Ingredients and Nutrition Compositions

Parameters	Animal meat-based diets				*p*-value
	Beef	Pork	Chicken	Pollen	
Ingredient (%)					
Maize pollen				67.67	
Hydrolyzed beef shank	60				
Hydrolyzed pork hock		60			
Hydrolyzed chicken breast			60		
Sugar syrup (50% sucrose)				33.33	
Sugar granule (sucrose)	35	35	35		
Plam oil	3	3	3		
Vitamin premix	0.5	0.5	0.5		
Mineral premix	0.5	0.5	0.5		
Xanthan gum	1	1	1		
Proximate analysis (%)					
Moisture	48.49 ± 3.48^{a}	43.41 ± 3.37^{a}	43.30 ± 3.99^{a}	30.48 ± 2.48^{b}	0.001
Protein	15.76 ± 1.45	14.28 ± 1.51	13.77 ± 1.32	14.37 ± 1.28	0.397
Carbohydrate	25.96 ± 2.37^{c}	32.91 ± 2.57^{b}	35.40 ± 2.72^{b}	49.92 ± 1.39^{a}	0.000
Fat	4.07 ± 0.44^{b}	5.03 ± 0.17^{a}	5.11 ± 0.26^{a}	3.87 ± 0.13^{b}	0.001
Ash	1.20 ± 0.17^{bc}	1.12 ± 0.09^{b}	1.35 ± 0.48^{ab}	1.81 ± 0.07^{a}	0.043
Energy (kcal/100g)	227.39 ± 6.94^{b}	229.11 ± 7.83^{b}	245.06 ± 33.51^{ab}	284.19 ± 12.64^{a}	0.019

The data were analyzed by ANOVA test. Mean values ± standard deviations. Means in the same row with different superscripts are significant at $p<0.05$ level as determined by Tukey's-b.

brood rearing, longevity, and overall colony health. In this study, protein content showed minimal variation across treatments ($p\geq0.05$), with protein levels in the hydrolyzed pork (14.28%), beef (15.76%), chicken (13.77%), and maize pollen-based (14.37%) diets remaining similar. However, the type of protein used may influence digestibility, the amino acid profile, and ultimately the overall health benefits of each artificial pollen diet for honey bees. The total amino acid content exhibited a significant difference ($p<0.05$), with the hydrolyzed chicken-based diet containing the highest total amino acids (26.92 ± 0.10 g/100g), followed by beef (24.60 ± 2.19 g/100g) and pork (24.15 ± 0.86 g/100g), respectively (Table 2). Leucine and lysine were the most abundant essential amino acids, each present at approximately 2.0 to 2.6 g/100 g while glutamic acid (a non-essential amino acid) had the highest concentration, ranging from 4.4 to 4.8 g/100 g across animal meat-based diets.

Honey Bee Lifespan

The section provides significant insights into the effects of different protein supplements on honey bee lifespan. A gradual increase in mortality was observed across all experimental groups throughout the study period, consistent with natural senescence. Over a 21-day observation period, no statistically significant ($p\geq0.05$) difference in survival rates was found between honey bees fed a maize-based pollen diet and those receiving a hydrolyzed chicken-based diet (Figure 2). These results suggest that the hydrolyzed chicken-based diet could serve as a viable artificial alternative to maize-based supplements, offering greater flexibility in nutritional management strategies for honey bees. Additionally, the treatment of a hydrolyzed chicken-based diet extended the median survival time to over 14 days, surpassing or equaling the performance of other meat protein treatments. Honey bees fed hydrolyzed chicken and

Table 2. Amino Acid Composition (g per 100 g on dry matter basis)

Amino acid	Animal meat-based diets			*p*-value
	Beef	Pork	Chicken	
Essential				
Histidine	1.02 ± 0.17	1.10 ± 0.03	0.92 ± 0.03	0.184
Isoleucine	1.10 ± 0.11^{b}	1.10 ± 0.01^{b}	1.30 ± 0.02^{a}	0.003
Leucine	2.02 ± 0.17^{b}	2.00 ± 0.01^{b}	2.22 ± 0.06^{a}	0.013
Lysine	2.30 ± 0.25	2.00 ± 0.68	2.62 ± 0.02	0.153
Methionine	0.47 ± 0.05^{b}	0.45 ± 0.03^{b}	0.67 ± 0.09^{a}	0.003
Phenylalanine	1.05 ± 0.08^{ab}	1.0 ± 0.02^{b}	1.10 ± 0.00^{a}	0.007
Threonine	1.27 ± 0.11^{b}	1.25 ± 0.02^{b}	1.40 ± 0.02^{a}	0.020
Valine	1.3 ± 0.13^{b}	1.3 ± 0.00^{b}	1.5 ± 0.01^{a}	0.003
Non-essential				
Alanine	1.62 ± 0.12^{ab}	1.55 ± 0.04^{b}	1.70 ± 0.01^{a}	0.029
Arginine	1.77 ± 0.12^{b}	1.72 ± 0.05^{b}	2.00 ± 0.06^{a}	0.002
Aspartic acid	1.80 ± 0.16^{ab}	1.77 ± 0.01^{b}	2.05 ± 0.02^{a}	0.004
Cysteine	0.12 ± 0.02	0.10 ± 0.00	0.12 ± 0.02	0.622
Glutamic acid	4.45 ± 0.39	4.42 ± 0.07	4.82 ± 0.08	0.089
Glycine	1.37 ± 0.06^{a}	1.30 ± 0.01^{b}	1.30 ± 0.02^{b}	0.007
Proline	1.05 ± 0.06	1.05 ± 0.11	1.07 ± 0.04	0.856
Serine	1.10 ± 0.09	1.10 ± 0.01	1.20 ± 0.01	0.100
Tyrosine	0.82 ± 0.18	0.80 ± 0.00	0.87 ± 0.07	0.689
Total	24.60±2.19^{ab}	24.15±0.86^{b}	26.92±0.10^{a}	0.040

The data were analyzed by ANOVA test. Mean values ± SD. Means in the same row with different superscripts are significant at $p<0.05$ level as determined by Tukey's-b.

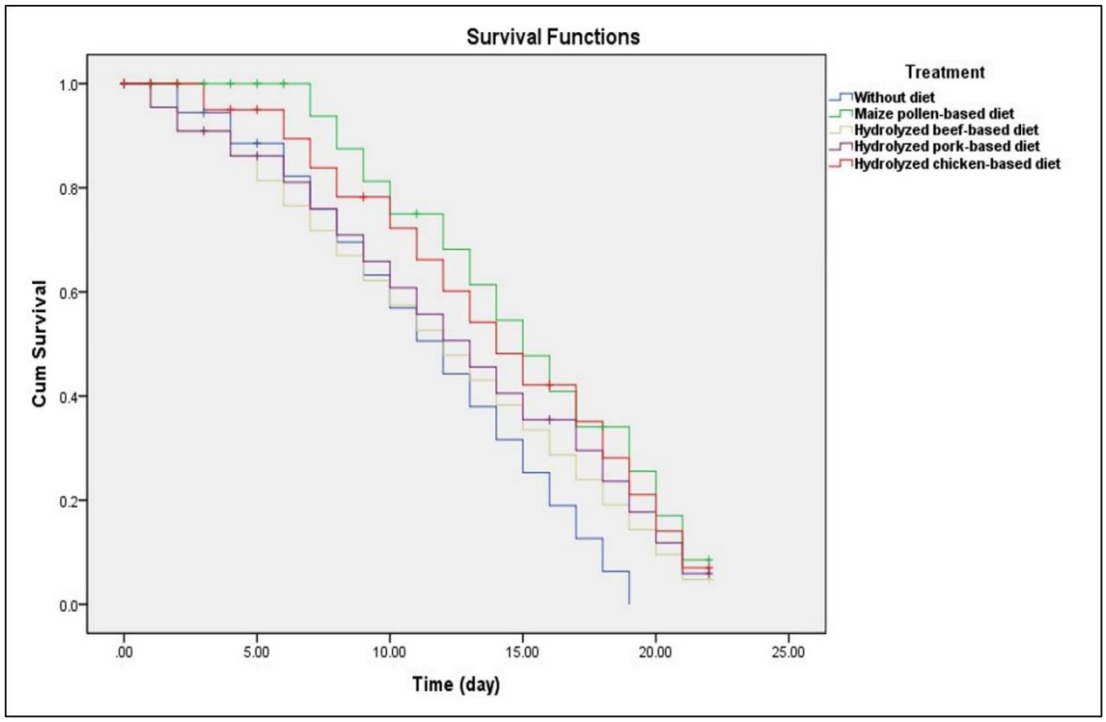

Figure 2. Kaplan–Meier Plot of Honey Bees Fed Diets with Different Protein Sources Over Time.

pork-based diets exhibited significantly higher survival rates compared to those provided with beef (p<0.05). These findings indicate that both hydrolyzed chicken and pork-based diets may possess superior digestibility or greater alignment with the nutritional requirements of honey bees, while honey bees fed syrup alone had the shortest lifespan. Overall, these results underscore the potential of a hydrolyzed chicken-based diet as a cost-effective and nutritionally appropriate alternative for enhancing honey bee health and longevity, with important implications for sustainable apicultural practices

HPG Acini Size

This study investigated the average diameter of HPG acini in 7-day-old honey bees fed four different diets (Figure 3). The analysis of variance (ANOVA) revealed statistically significant differences in the mean HPG acini diameters among the diet groups (p<0.05). The between group variance was substantially greater than the within-group variance, indicating clear distinctions between the effects of diets. Tukey's post-hoc test results showed that the hydrolyzed beef-based and syrup alone resulted in the smallest mean HPG acini diameter of 12.86 ± 1.98 µm and 12.58 ± 1.49, while the maize pollen-based diet produced the largest mean diameter of 22.50 ± 2.38 µm. The hydrolyzed pork-based diet and hydrolyzed chicken-based diet produced mean diameters of 20.09 ± 2.60 µm and 21.80 ± 2.04 µm, respectively. In summary, the hydrolyzed beef-based diet showed a statistically significant (p<0.05) difference compared to all other groups. In contrast, the maize pollen-based diet, despite having the largest mean diameter of HPG acini, did not exhibit a statistically significant (p≥0.05) difference when compared to the hydrolyzed chicken-based diet, as their measured values were similar.

Gut Microbiome

The analysis of the gut microbiome of honey bees subjected to various dietary treatments identified the predominant bacterial taxa present in their digestive systems at both the genus and species levels. Supplementing the diet of honey bees with an artificial pollen diet resulted in an increase in overall microbial diversity compared to providing dietary sugars in the absence of protein (Figure 4). The identified microorganisms were categorized into two groups. First, the Gram-positive bacteria included seven genera of *Lactobacillus*, *Apilactobacillus*, *Bombilactobacillus*, *Fructobacillus*, *Bifido bacterium*, *Paenibacillus* and *Staphylococcus*. In contrast, the Gram-negative bacteria consisted of twelve genera, which included *Bartonella*, *Anaeropeptidivorans*, *Marilepto lyngbya*, *Potamosiphon*, *Bombella*, *Entomobacter*, *Rubrivivax*, *Snodgrassella*, *Enterobacter*, *Klebsiella*, *Serratia*, *Frischella,* and *Gilliamella*. Supplementing the diet of honey bees with an artificial pollen diet led to an increase in overall microbial diversity compared to the baseline, consistent with previous studies on the introduction of dietary sugars in the absence of protein.

Among the bacterial community, two dominant genera, *Bartonella* and *Lactobacillus*, accounted for more than 15% of the total composition, with proportions ranging from 18% to 50%. Both *Bartonella* and *Lactobacillus* exhibited stability

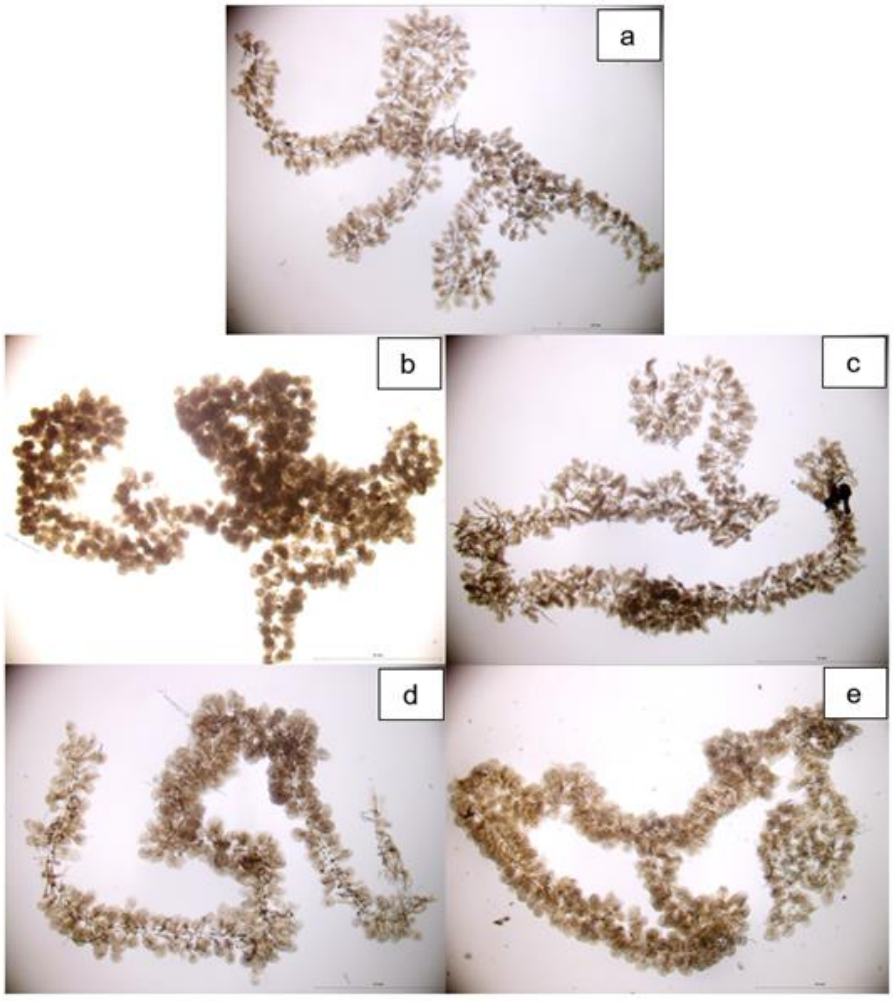

Figure 3. HPG Acini in Honey Bees Fed with Different Diets: without diet (A), maize pollen-based diet (b), hydrolyzed beef-based diet (c), hydrolyzed pork-based diet (d) and hydrolyzed chicken-based diet (e).

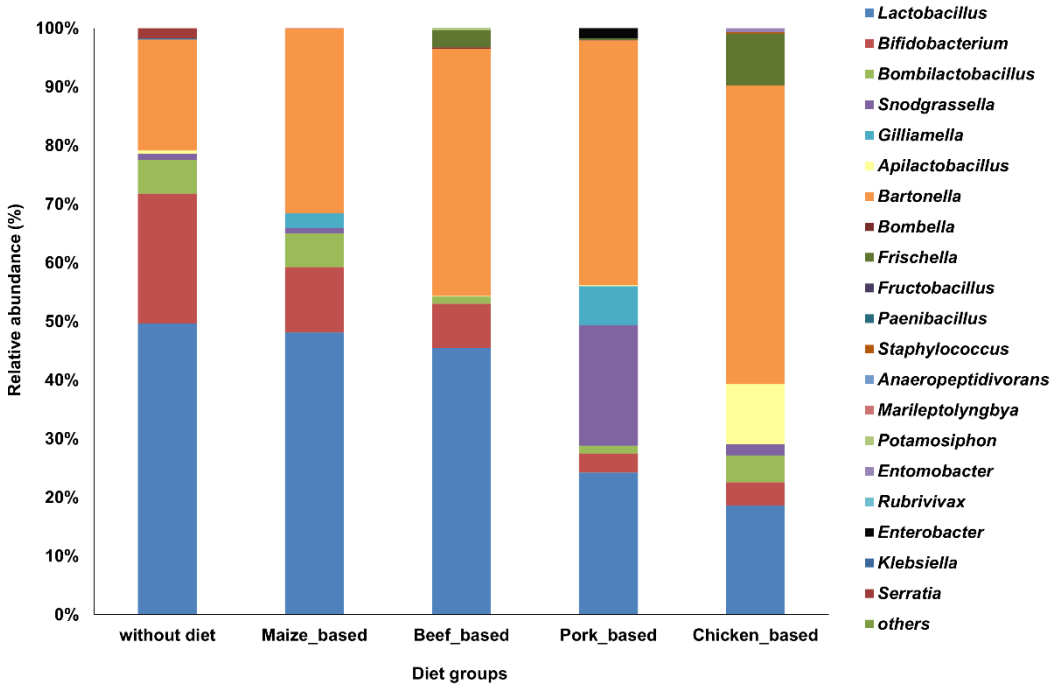

Figure 4. Changes of Gut Microbial Community at Genus Level of Honey Bees between Five Experimental Diets.

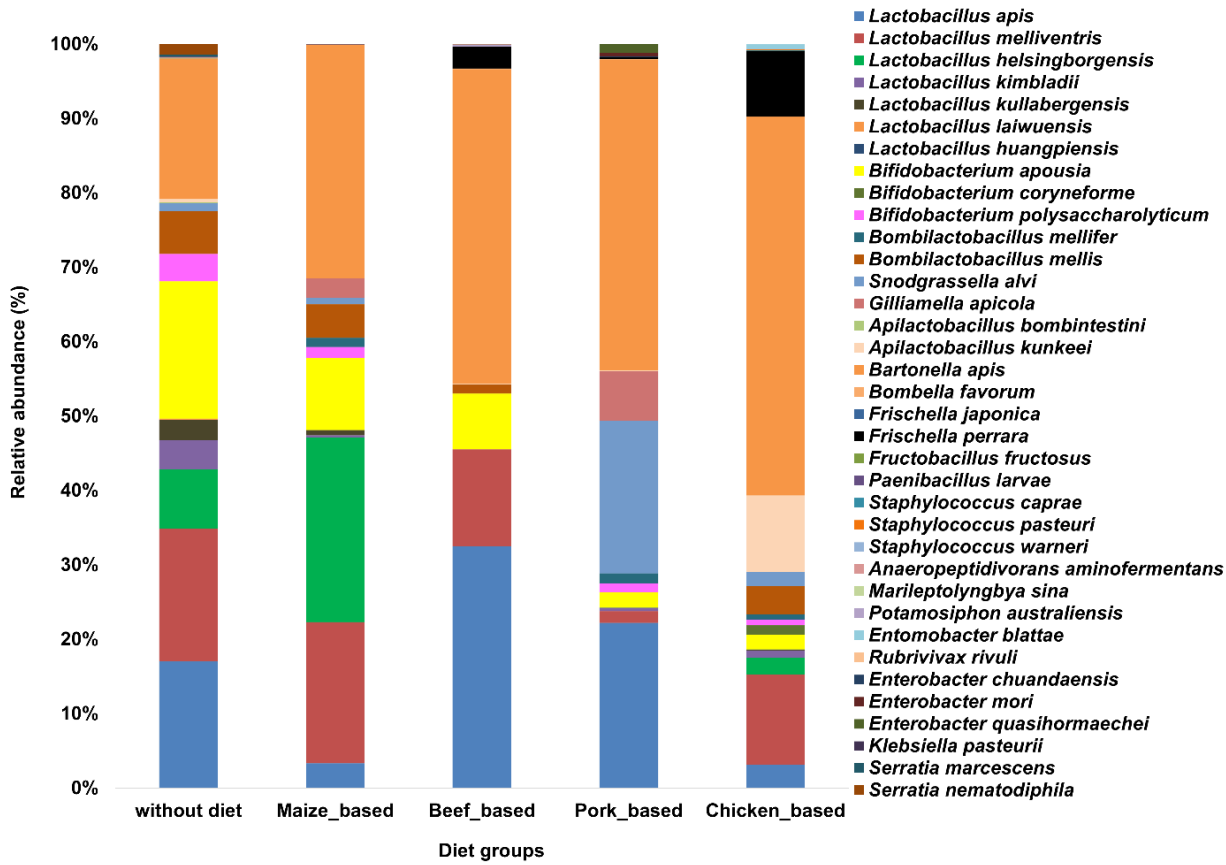

Figure 5. Changes of Gut Microbial Community at Species Level of Honey Bees between Five Experimental Diets.

across all five feeding treatments, demonstrating their consistent presence regardless of dietary variations involving hydrolyzed animal protein sources, similar to those observed when honey bees were fed a maize-based pollen diet. The bacterial genera *Bombilactobacillus* and *Bifidobacterium* have been previously identified as core members of the honey bee gut microbiota. These genera consistently represented significant proportions (exceeding 1.0%) of the gut microbiome across all experimental dietary treatments. In the case of *Snodgrassella*, *Frischella*, and *Gilliamella*, which were observed to exhibit higher abundance in honey bees fed artificial pollen diets compared to those receiving syrup without additional protein supplementation. This observation indicates that artificial pollen diets may play a critical role in shaping the composition of these specific microbial communities within the honey bee gut.

Supplementing diets with hydrolyzed animal meat proteins can enhance the growth of beneficial microbes in honey bees without the need for exogenous bacterial species via probiotic supplementation. In comparison, honey bees that were exclusively fed syrup without the addition of any other proteins showed a significantly lower proportion of *B. apis* at 18%. On the other hand, honey bees fed with a hydrolyzed chicken-based diet, as well as other protein-based diets, displayed a higher abundance of *B. apis*, ranging from 30-50%. Furthermore, the core bacterial genera in the honey bee gut, including *Lactobacillus*, *Apilactobacillus*, *Bombilactobacillus*, *Bifidobacterium*, *Gilliamella*, and *Snodgrassella*, were found in relatively lower proportions (Figure 5). The findings indicate that honey bees provided with artificial pollen diets exhibit a distinct gut microbial composition, characterized by the dominance of *Bartonella* (*B. apis*) as a key member of the core microbiota, while other core bacteria, such as *Lactobacillus* and *Bifidobacterium*, demonstrate a reduced prevalence. The study findings indicate that *Bartonella* (*B. apis*) is a significant and dominant microorganism within the core microbiota of honey bees during the study period. Although variations in artificial pollen diet may influence the distribution of certain microbial species, the core microbiota, which plays a critical role in the gut function of bees, remains largely stable. However, the limitations of this study include the relatively small sample size and the absence of field experiments. Conducting field experiments would offer more comprehensive insights, as honey bees would be exposed to their natural environment and foraging behaviors.

Discussion

Honey bees are unable to efficiently digest large molecular proteins due to the lack of specialized acidic digestive systems, such as those found in vulture bees (*Trigona necrophaga*), which are adapted for breaking down animal

proteins.[23] Protease treatment addresses this limitation by reducing the molecular size of animal-derived proteins, enhancing their digestibility for honey bees. Enzymatic treatment alters the molecular structure of proteins, making them more suitable for ingestion and processing. SEM analysis corroborates previous findings, which observed reductions in meat fiber size and increased spacing between fibers following enzymatic treatment.[24,25] These processes have been effectively applied to produce hydrolyzed meat, which serves as a primary component in artificial pollen diet formulations. Animal meat-based diets formulated with hydrolyzed meats exhibit elevated moisture content, which is consistent with the naturally high moisture levels present in animal-derived proteins such as chicken.[26] Furthermore, hydrolyzed chicken meat has an average moisture content ranging from 79% to 86%.[27] These findings support the experimental conclusion that the inclusion of hydrolyzed meat in diets leads to an increase in their moisture content. In contrast, the carbohydrate content in the hydrolyzed meat-based diets (25-35%) was lower compared to the maize pollen-based diet (49.92%). This is consistent with findings that identified maize pollen as one of the pollen types with the highest carbohydrate content (78.1%) based on a study of 11 maize pollen samples.[28]

The high fat content in animal meat-based diets may provide benefits for honey bee growth and development.[29] This is because fats and lipids are essential components of the fat body, an organ in insects responsible for energy storage and exhibiting high biosynthetic and metabolic activity.[30] Additionally, although the ash (mineral content) in all the tested diets was relatively low, it may still fulfill the nutritional requirements of honey bees when considered as part of a complete diet.[16,31] Overall, animal meat-based diets provided protein levels comparable to those of the maize pollen-based diet, suggesting that each developed formulation has the potential to serve as a supplementary feed for honey bees. The protein content in all four diets averaged between 13% and 15%, with no statistically significant ($p \geq 0.05$) differences. These findings align with several reports that highlight the nutritional composition of honey bee feed, with protein levels typically averaging between 13% and 30%.[5,6,16,32-35] and sugar content ranging between 20 and 40%,[5,16,36,37] can effectively support honey bee colony management.

Although the hydrolyzed chicken-based diet exhibited higher levels of certain amino acids compared to the pork and beef-based diets, the overall amino acid profiles displayed a similar trend. This profiling is also consistent with the amino acid composition of maize pollen, which was previously reported by our collaborators.[28] Since honey bees are unable to synthesize essential amino acids such as arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine, and valine, it is crucial that these amino acids are included in their diet.[14,38] Therefore, the animal meat-based diets, which provide a balanced amino acid profile, are considered to meet the basic nutritional requirements of honey bees as previously reported in the literatures.[14,39,40]

Animal-meat diets have been confirmed as a viable option for feeding honey bees due to their appropriate nutritional value. It has been demonstrated that honey bees fed such diets exhibit survival rates comparable to those fed maize-based pollen. This is because it provides essential nutrients that align with honey bee nutritional requirements, which is a key factor in honey bee nutritional research.[41] Previous studies have consistently emphasized that providing nutritionally appropriate feed sources, even in synthetic forms, can significantly enhance honey bee health and longevity.[42,43] However, honey bees fed a hydrolyzed beef-based diet had the lowest survival rates and the shortest lifespans compared to those receiving other animal-derived proteins. Based on these results, we recommend using chicken meat as a protein source, as it offers excellent nutritional value and is non-toxic in various animal species, including humans.[13] Additionally, chicken meat is the most cost-effective protein source when compared to alternatives such as pork and beef. Moreover, the findings from this study suggest that a hydrolyzed chicken-based diet, which contains approximately 15% protein, is likely to promote honey bee growth and longevity similarly to natural pollen. Overall, our results indicate that a hydrolyzed chicken-based diet has significant potential as a pollen substitute or pollen replacement based on its nutritional content and effects on worker honey bee physiology. However, the production of supplementary feed for honey bees should carefully consider various factors, as experimental results may not always align with previous studies. For instance, caged honey bees fed a high-protein diet (50%) exhibited higher mortality rates compared to those fed diets with lower protein levels (5% and 10%).[44] In addition, honey bees had the lowest mortality rates when provided with a protein-free diet.[45] These observations align with findings from laboratory-scale cage experiments, where bees with low activity levels that were fed only syrup survived for up to 18 days.

The expansion of HPG acini serves as a reliable indicator of the physiological health and preparedness of worker bees for brood care and royal jelly secretion. [46,47] The observed differences in HPG acini diameters among the diets underline the critical role of dietary protein in supporting HPG acini development. Honey bees fed a hydrolyzed beef-based diet produced the smallest HPG acini, which may be attributed to the difficulty in consuming this diet due to its firm texture and the dense muscle fiber structure, as revealed by the SEM analysis. In contrast, honey bees fed a maize pollen-based diet produced the largest HPG acini, which is consistent with the known nutritional composition of maize

pollen containing an optimal protein level of around 17%.[28] However, the lack of a significant difference between honey bees fed the maize pollen-based diet and those fed a hydrolyzed chicken-based diet suggests that the latter may serve as a suitable alternative for supplementation in place of pollen. The findings of this study support the benefits of nutritionally appropriate protein-based diets in promoting glandular development.[4,48] Subsequently, adequate protein intake, particularly from sources with a well-balanced amino acid profile, was further emphasized as a critical factor in promoting HPG acini size and improving honey bee physiology.[49] In particular, the importance of essential amino acids for honey bees,[14] remains evident in this context. The findings of this study show that honey bees fed a hydrolyzed chicken-based diet with a complete amino acid profile demonstrate improved HPG acini development and higher survival rates.

The honey bee worker gut microbiota is composed of five core bacterial genera: *Lactobacillus*, *Bombilactobacillus*, *Gilliamella*, *Snodgrassella*, and *Bifidobacterium*. Additionally, three non-core bacteria of *Frischella*, *Bartonella*, and *Commensalibacter* are frequently detected in workers.[50] This study revealed that *Bartonella*, a bacterium classified as a non-core member of the honey bee microbiome, played a prominent role in microbial composition. It was observed to be consistently prevalent across all diet types tested, regardless of variations in nutritional content or source. The abundance of *Bartonella* notably exceeded that of other microbial groups, highlighting its potential resilience or adaptability within the honey bee gut environment.[51] Moreover, *Bartonella* may have been present in the honey bees analyzed from the onset of the study, indicating its consistent association with the honey bee microbiome irrespective of external factors.[52] This study demonstrates that the microbiome profile of honey bees exhibits a high degree of stability, even when their diets are supplemented with proteins derived from animal meat-based sources or modified from alternative origins. Therefore, the study demonstrates that the honey bee microbiome is highly resilient to dietary changes while maintaining the stability of essential microorganisms involved in digestion and overall health maintenance of the honey bees.[53]

Additional genera of core bacteria have been detected, including *Lactobacillus*, *Bifidobacterium* and other *Bombil actobacillus*, *Gilliamella*, *Snodgrassella, Enterobacter,* and *Apilactobacillus* but they typically account for a relatively small proportion of honey bee gut microbiota. The composition of these microbial groups may fluctuate based on environmental factors and other variables.[52] The composition of these microbial groups may fluctuate based on environmental factors and other variables, particularly the type and amount of nutrients (i.e., pollen and nectar) available during the foraging season, which can have profound effects on the composition of the gut microbiota.[54,55]

Similar to other animals, LAB plays a critical role in the microbiota of honey bees. The presence of LAB in honey bees has been extensively studied over the years. A study identified the presence of *Lactobacillus* and *Bifidobacterium* in the honey bee crop, with *L. apis* and *L. melliventris* showing notable abundance across all experimental treatments, further supporting their role in maintaining a balanced gut microbiota.[55,56] Both recognized for their probiotic properties, likely facilitate the fermentation of dietary sugars, producing beneficial metabolites such as lactic acid.[56] This process may contribute to the maintenance of a low pH environment in the gut, which in turn may inhibit the growth of pathogenic microorganisms.[57] The *Bifidobacterium* and *Bombilactobacillus* also emerged as key players in the microbial community, consistently accounting for greater than 1.0% of the bacterial composition across all diets. These genera have been previously associated with beneficial gut microbiota in other species, suggesting that they might play complementary roles in maintaining the gut ecological balance, enhancing digestion, and supporting the immune system.[58] The study shows that artificial pollen supplemented with animal-derived protein does not affect the composition of core bacteria in the honey bee gut. These bacteria continue to play a crucial role in digesting sugars, producing beneficial metabolites, and maintaining gut microbiome balance, as well as supporting the immune system.

At the species level, the primary bacterium in honey bees was identified as *B. apis*, while *Lactobacillus* spp. and *Bifidobacterium* spp. were detected as secondary bacteria. This finding contrasts with previous studies on the honey bee gut microbiome, which commonly identified bacteria from *Lactobacillus* and *Bifidobacterium* as the dominant microbial groups.[50] This suggests that the composition of the dominant bacterial communities in the honey bee gut may be influenced by external factors such as temperature, seasonal variations, toxins, and the types of nutrients available to the honey bees.[54,55,59] Higher proportions (40-50%) of *B. apis* were observed when honey bees were fed hydrolyzed meat protein, whereas a lower abundance (18%) was found in honey bees fed with syrup alone. This phenomenon is similar to previous findings showing that winter worker honey bees exhibit a prominent presence of Bartonella, likely due to their consumption of stored feed within the hive, which is rich in proteins and carbohydrates, such as pollen and bee bread.[52] In contrast, summer worker honey bees which forage for external feed sources primarily composed of carbohydrates from flower nectar, exhibit a reduced presence of *Bartonella*. Additionally, there is a notable presence of *Bartonella* in honey bees during periods of feed scarcity in natural environments.[51] Therefore, *B. apis* may play a role in protein digestion and thrive in protein-

rich diets, emphasizing the impact of diet on the composition and function of honey bee gut microbiome.

Our study also found higher levels of *Lactobacillus* spp. and *Bifidobacterium* spp. in honey bees fed sucrose without a protein supplement compared to those receiving a diet containing both protein and sugar. This is consistent with previous research highlighting these two bacteria as dominant species with the ability to metabolize sucrose.[60] and their roles in nectar processing and carbohydrate metabolism.[61] However, feeding honey bees only sucrose may reduce the diversity of their gut bacterial communities. Therefore, providing honey bees with a nutritionally balanced diet plays a critical role in enhancing the diversity of their gut microbiome.[22] Although this study relies on data from a controlled laboratory setting, which may limit conclusions about the honey bee gut microbiome, our findings suggest that diet type influences the variation in gut bacterial communities. Specifically, artificial pollen diets supplemented with chicken-derived proteins appear to be a viable option when considering factors such as the ease of sourcing ingredients, cost value, and the impact of the diet on honey bee growth, health, and microbiome composition.

Conclusion

This study demonstrates the impact of various animal-based protein diets, particularly hydrolyzed chicken-based diets, on honey bee health and the subsequent effects on microbiomes. The results reveal that the group fed the hydrolyzed chicken-based diet exhibited comparable health outcomes and microbiome diversity to those fed with the maize pollen-based diet. Specifically, the abundance of *Bartonella*, *Lactobacillus*, and *Bifidobacterium*, microbial genera linked to improved honey bee health when present in appropriate proportions. Based on these findings, we propose that a hydrolyzed chicken-based diet can be used as honey bee feed. However, it is essential to verify the effects of these diets at the colony level under real-world environmental conditions. Additionally, further colony-level experiments are necessary to investigate the relationship between diet, microbiome, and honey bee health, as environmental factors can influence microbiome composition and, consequently, the health of honey bees.

Authors' Contributions

Conceptualization: KD, CJ, and BC. Data curation: KD, CJ, SG, MCW, PP SS, HJ, and BC. Formal analysis: KD, CJ, SG, PP, SS, and HJ. Funding acquisition: KD, CJ, and BC. Methodology: KD, CJ, SG, SS, HJ, and BC. Project administration: KD, CJ, and BC. Supervision: CJ and BC. Validation: KD, CJ, SG, MCW, and BC. Visualization: KD, SG, SS, and HJ Writing original draft: KD, CJ, SG, MCW, and BC. Writing – review and editing: KD, CJ, SG, MCW, and BC. All authors have accepted responsibility for the entire content of this manuscript and consented to its submission to the journal, reviewed all the results and approved the final version of the manuscript.

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Ethical Approval

Ethics approval for the experiment was obtained through the Laboratory Animal Research Center, University of Phayao under approval number 1-003-68.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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