CHAPTER III
MATERIALS AND METHODS

3.1 Ethics Approval

All experimental procedures were accepted by the Ethics Committee of Suranaree University of Technology, Animal Care and Use Committee (approval No. SUT-IACUC-0010/2023)

3.2 Centricon preparation duck blood protein hydrolysate

Duck blood protein hydrolysate (DBPH) was prepared following a previously reported method (Pérez-Gálvez et al., 2011) with some modifications. After that, DBPH was diluted with NaCl at a ratio of 2:1 and the DBPH mixture was boiled in a water bath at 95℃ for 15 minutes, followed by centrifugation at 12,500 rpm for 15 minutes at 4℃. The supernatant was transferred to a centrifugal filter tube with Centricon (10 kDa, Merck KGaA, Darmstadt, Germany) filter and centrifuged at 4000 × g for 50–60 minutes at 4℃. Using the filter, DBPH samples were separated by size while the debris was retained on the filter, as shown in Figure 3.1. The filtered solution was aliquoted and stored at –20℃ until use. Ultimately, the permeate fraction with a molecular weight below 10 kDa was collected and analyzed for its molecular weight via Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and size exclusion chromatography.

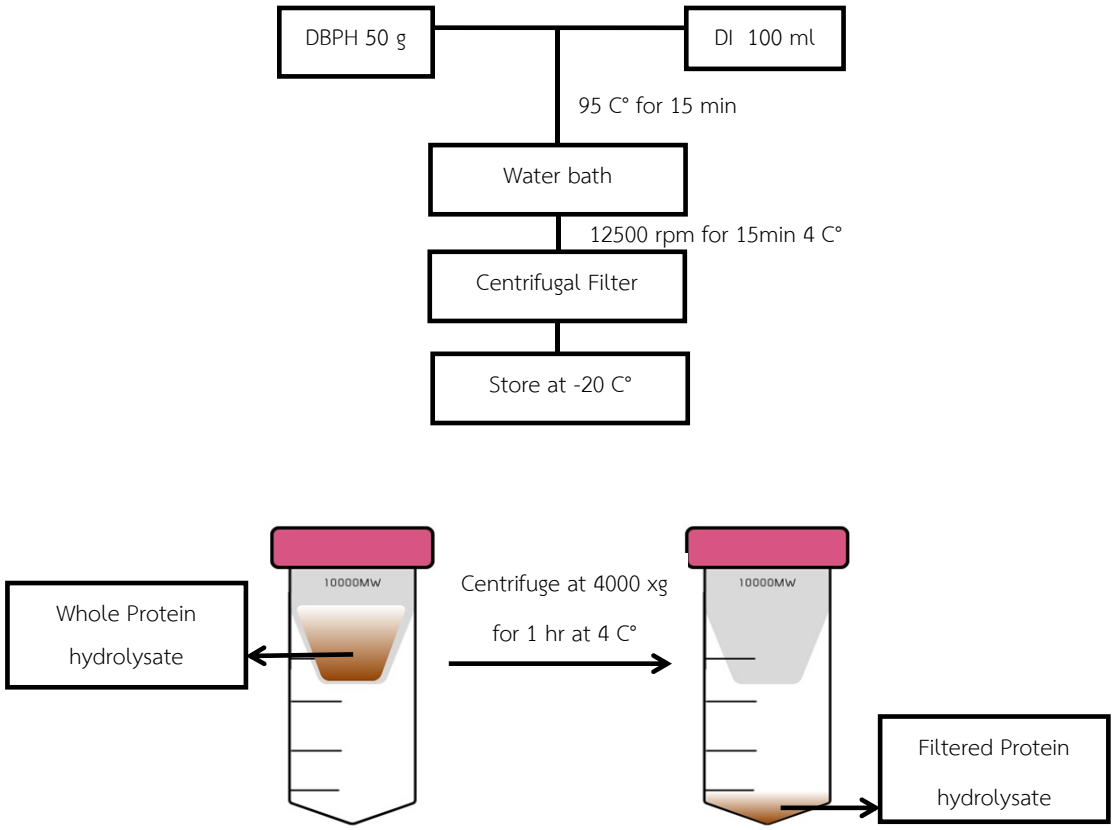

Figure 3.1 Filtration of Duck Blood Protein Hydrolysate.

3.3 Determination of Protein Molecular Weight and Protein Concentration

The DBPH samples was diluted with 20 µl of 2X Laemmli buffer at a 1:1 ratio and then the mixture was boiled in a heat block at 95℃ for 10 min. The sample was centrifuged at the maximal speed for 10 min at 25℃ and used to analyze the molecular weight of protein samples by SDS-PAGE technique.

The SDS-PAGE was prepared with the following chemicals.

15% separating gel	
40% Polyacrylamide	3.6 ml
1.5M Tris (pH8.8)	2.7 ml
10% SDS	100 µl
10% Ammonium persulfate	100 µl
TEMED	8 µl

ddH2O (DI)	3.2 ml
5% stacking gel (5%)	
40% Polyacrylamide	0.5 ml
1.5M Tris (pH6.8)	0.5 ml
10% SDS	40 µl
10% Ammonium persulfate	40 µl
TEMED (add it right before the gel is poured)	4 µl
ddH2O (DI)	3 ml

The DBPH samples was subjected to the gel as described above. Electrophoresis was performed at constant 50 V for 15 min for 5% stacking gel and 100 V for 50 min for resolving gel, respectively. Then, the gel was stained with staining solution (0.125% Coomassie brilliant blue R-250) on the shaker at room temperature overnight and destained twice in a solution containing 25% ethanol and 10% acetic acid on the shaker at room temperature for 2-3 hr or until the gel is clear. To visualize and analyze the images, we used the ChemiDoc MP Imaging SystemTM (Bio-Rad Laboratories, Hercules, CA, USA) determination of protein concentration.

The concentration of DBPH was determined using the Lowry protein assay with bovine serum albumin (BSA) as a standard protein. This method is based on the reaction of Cu^{+}, which is generated by the oxidation of peptide bonds from protein samples, with the Folin–Ciocalteu reagent. Briefly, the standard bovine serum was prepared at concentrations of 0, 0.1, 0.5, 1, 2, and 3 µg/ml to construct the standard curve. The Folin-Ciocalteu Reagent was diluted with ultrapure water at a ratio of 1:1 followed by transferring 10 µl of this diluent into 96 well plate. Then, 100 µl of copper sulfate solution was added to the same plate and incubated at room temperature for 30 m The protein concentration of the sample was measured by absorbance at 750 nm.

3.4 Molecular Weight Distribution Analysis

Molecular weight analysis is an essential parameter for evaluating peptide composition, estimating the extent of protein hydrolysis, and comparing the sizes of the peptides produced. In this study, the molecular weight distribution of gastrointestinal (GI) digests was analyzed

following the method described by Luasiri et al., 2024. A 100 µL aliquot of the peptide digest (10 mg/mL) was analyzed by chromatography using a Superdex Peptide 10/300 GL column (GE Healthcare, Piscataway, NJ, USA). The chromatography was performed with an AKTA Purifier (GE Healthcare, Piscataway, NJ, USA) using a mixture of 30% acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA) in deionized (DI) water. The system operated in isocratic mode at a flow rate of 0.7 mL/min, with a total elution volume of 30 mL. The peptide profile was determined by monitoring absorbance at 215 nm using UV detection. The study also utilized common substances such as cytochrome c, aprotinin, and synthetic peptides.

3.5 Diet Preparation

Preparation of DBPH and experimental media the filtered DBPH solution was aliquoted and stored at -20°C for further use. The protein concentration of DBPH was quantified using the Lowry protein assay using bovine serum albumin (BSA) as a standard. This colorimetric method is based on the reduction of Folin–Ciocalteu reagent with Cu^{+} ions, which are generated by the oxidation of peptide bonds within the protein sample. To generate a standard curve for calibration, BSA was prepared at concentrations of 0, 0.1, 0.5, 1, 2 and 3 µg/mL. The Folin–Ciocalteu reagent was diluted 1:1 with ultrapure water and 10 µL of this solution was pipetted into each well of a 96-well microplate. Then, 100 µL of copper sulfate solution was added to each well. The plates were incubated at room temperature for 30 min, the absorbance was measured at 750 nm using a microplate reader, and the protein concentration of the samples was calculated using a standard curve to control the same amount of protein for each preparation. Based on the quality and quantity of DBPH, five experimental formulas were formulated, Negative control: commercial diet mixed with 0.85% NaCl (saline), positive control: commercial diet supplemented with 0.1% Vitamin C, Treatment 1: commercial diet supplemented with 0.5% DBPH, Treatment 2: commercial diet supplemented with 1% DBPH and Treatment 3: commercial diet supplemented with 2% DBPH. The mixture of each diet was thoroughly homogenized with the respective supplement, then spread and allowed to dry for approximately 1 hour. After that, all diets were coated with squid oil at a ratio of 600 g of diet to 15 ml of squid oil (equivalent to 2.5% v/w) and then air-dried at room temperature for 2 hours. The prepared diets were then stored at 4°C used.

To ensure consistent nutrient content the approximate composition of each diet was therefore analyzed according to the official methods of the Association of Official Analytical Chemists (AOAC, 1990). All diets contained approximately 38% crude protein, 10% crude moisture, 4% crude fiber and 4% crude fat, with no significant differences between the treatment groups. During the experiment, fish were fed *ad libitum* twice daily along with the individual fish diets.

3.6 Experimental Design, Fish Culture and Sampling

This study was conducted at the Aquaculture Laboratory, Suranaree University of Technology, Nakhon Ratchasima Province, Thailand. All experimental procedures were approved by the Animal Care and Use Committee, Suranaree University of Technology (Approval No. SUT-IACUC-0010/2023). Before the feeding experiment, 40 healthy flowerhorn cichlids (initial weight: 3.34±0.67 g; initial length: 3.40±0.61 cm) were acclimated for 14 days in 40 separate glass tanks (23″ × 13″ × 12″) under the same controlled environment. Due to the aggressive nature of flowerhorn cichlids, they were housed separately to prevent injuries from aggressive behavior. Each tank was equipped with aeration, water filtration, and temperature control. During the acclimation, the fish were fed ad libitum twice daily using ready-to-eat food. After acclimation, the fish were randomly assigned to 5 dietary treatment groups using a completely randomized design with 8 replications per group (n = 8). The dietary treatment groups were as follows: Negative control group: commercial diet mixed with 0.85% saline (NaCl), positive control group: commercial diet supplemented with 0.1% vitamin C, experimental groups supplemented with 0.5%, 1% and 2% DBPH, respectively. The feeding experiment lasted for 30 days. During this period, all fish were fed the experimental diets sequentially twice a day until they felt full. At the end of the feeding experiment, the growth performance was assessed by recording the weight gain rate (WGR), final body length and survival rate (SR). The growth parameters were calculated using the following formula:

Weight gain rate (WGR, g) = final body weight – initial body weight

Survival rate (SR, %) = 100 × final number of fish / initial number of fish

After the feeding experiment, fish from each diet group were divided into two subgroups: pre-challenge group (n = 4) and post-challenge group (n = 4). In the pre-challenge group, blood samples were collected from fish in each treatment for analysis of serum humoral immune parameters and assessment of antioxidant enzyme activities. The DBPH group showed the best performance in terms of growth, humoral immune response and antioxidant activities (see Section 3 Results) were identified as the most suitable DBPH level group for further analysis.

For microbiota analysis, gut samples were collected from the negative control and the most suitable DBPH groups. In addition, liver and spleen samples were harvested from the negative control, positive control, and the most suitable DBPH groups for basic immune gene expression analysis.

3.7 Bacterial Challenge Test

Assessment of the immune-enhancing effectiveness of duck blood protein hydrolysate (DBPH) in flowerhorn cichlids. The bacterial challenge test utilized *Streptococcus agalactiae*, a pathogen recognized for inducing considerable morbidity and mortality in multiple fish species. After a 30-day feeding trial, fish from the negative control, positive control (supplemented with 0.1% vitamin C), and 2% DBPH-supplemented groups were selected for the challenge. Each fish was injected intraperitoneally with 0.2 ml of *S. agalactiae*, bacteria at a concentration of 1×10^{8} CFU/mL. This dose is consistent with previous studies that have shown significant immune responses and mortality at similar bacterial doses in fish species. Abdel Rahman et al. (2023) 24 hours after infection, the fish were monitored for clinical signs of Streptococcus, including lethargy. The fish exhibited irregular swimming patterns and enlarged eyes. Tissue samples from the liver and spleen were collected for gene expression analysis to assess the activity of immune-related genes. In addition, serum samples were collected to assess the immune response of body fluids, including immunoglobulin M (IgM) levels, lysozyme activity, and complement C3 concentrations.

3.8 Immune Responses and Antioxidant Enzyme Activity

Immune Responses and Activity of Antioxidant Enzymes. Blood samples were obtained from the caudal vein of four fish in each treatment group following

anesthesia. The samples were permitted to remain at room temperature for two hours to enable serum separation and subsequently stored at -80℃ until further utilization.

3.9 Lysozyme Activity

Lysozyme activity was measured according to the method of Siwicki et al. Briefly, lysozyme at concentrations of 0, 2.5, 5, 10, 15, and 20 µg/ml in 6M PCB-NaCl (pH 6.0) was prepared as reference standards for the standard curve. After that, 10 µl of flowerhorn cichlid serum and each concentration of reference standard was added into wells of a 96-well flat bottom plate in triplicate. To determine serum lysozyme activity, a suspension of 190 µl of 0.3mg/ml dried Micrococcus lysodeikticus (ATCC 4698; Sigma-Aldrich, St. Louis, MO, USA) was quickly added into all wells of the plate. The reaction was carried out at 25℃, and the optical density (OD) was measured at 450 nm at the initial time (0 min), 30 min, and 60 min using a microplate spectrophotometer (BioTek™ EPOCH, Agilent Technologies, Santa Clara, CA, USA). The concentrations of lysozyme (µg/ml) were calculated from a standard curve of lysozyme concentrations.

3.10 Alternative Complement (ACH_{50}) Activity

ACH_{50} activity was determined following a previously described method [43]. Briefly, flowerhorn cichlid serum (50 µL) was two-fold serial diluted with EGTA-GVB buffer (gelatin veronal buffered saline, 10 mM ethyleneglycol-bis (beta-amino-ethyl ether) N-N'- tetraacetate) and then an equal volume of 5 × 107 cells/mL of GRBC suspension was added to the diluted plasma. After that, the mixture was incubated for 90 min at 25℃ and centrifuged at 3000× g for 10 min at 4℃. The optical density (OD) was measured at 415 nm using a microplate spectrophotometer (BioTek™ EPOCH). Flowerhorn fish diluted serum representing the volume of complement producing 50% hemolysis (ACH_{50}) of GRBCs was determined, and the number of ACH_{50} units/mL was calculated for each experimental group (in triplicate).

3.11 Activity of antioxidant enzymes

The activity of catalase (CAT), superoxide dismutase (SOD), malondialdehyde (MDA) was measured using commercial kit (Abbkine Corporation, Georgia, USA) according to the manufacturer's recommended protocol.

3.12 Total Immunoglobulin (Ig)

The total immunoglobulin (Ig) concentration (mg/mL) in flowerhorn cichlid serum was determined using the total protein kit (Biuret method; Erba, Mannheim, Germany) according to the method of Siwicki et al. Briefly, flowerhorn cichlid serum (10 µl) was added in a 1.5 ml microcentrifuge tube, followed by the addition of an equal volume of 12% PEG solution (polyethylene glycol; Sigma-Aldrich, St. Louis, MO, USA). The mixing samples will incubate at 25°C for 30 min and centrifuged at 12,500 rpm at 4°C for 10 min. Following centrifugation, the mixture was separated into supernatant (non-Igs) and pellet (total Igs). After that, 4 µl of the supernatant, serum (total protein), and standard bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) was added into a 96-well flat bottom plate in triplicate. The protein concentration was determined according to the manufacturer's recommended protocol, and the optical density (OD) was measured at 546 nm using a microplate spectrophotometer (BioTek™ EPOCH). After precipitation, total Igs was calculated by subtracting total protein from non-Igs. The concentrations of proteins were derived from the standard curve constructed with bovine serum albumin.

3.13 DNA extraction of bacterial 16S rRNA genes

The gut specimens were squeezed out with sterile instruments to collect the luminal content. The gut samples were cut laterally to remove the mucus layer of the fish gut by visual inspection according to the method of NucleoSpin® Tissue kit. A cover glass was used to separate the mucus layer from the gut samples. The luminal content and mucus layer were pooled and transferred to a sterile conical tube containing 6.5 mM dithiothreitol for mucus degradation. After incubation for 3 hours at 56°C, the lysate sample Vortex the samples. Add 200 µL Buffer B3, vortex vigorously and incubate at 70°C for 10 min. Vortex briefly, Adjust DNA binding conditions Add 210 µL ethanol (96–100 %) to the sample and vortex vigorously, Bind DNA For each sample, place one NucleoSpin® Tissue Column into a Collection Tube. Apply the sample to the column. Centrifuge for 1 min at 11,000 x g. Discard the flow-through and place the column back into the Collection Tube. And then Wash silica membrane Add 500 µL Buffer BW. Centrifuge for 1 min at 11,000 x g. 2nd wash Add 600 µL Buffer B5 to the column and centrifuge for 1 min at 11,000 x g. Dry silica membrane Centrifuge the column for 1 min at 11,000 x g. Elute, highly pure DNA Place the NucleoSpin®

Tissue Column into a 1.5 mL microcentrifuge tube (not provided) and add 100 µL prewarmed Buffer BE (70°C). Incubate at room temperature for 1 min. Centrifuge 1 min at 11,000 x g. The eluted DNA was stored at -80°C until use. To determine the quantity and quality of genomic DNA, 2 µL of each gDNA sample was analyzed using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the integrity of the gDNA was assessed through agarose gel electrophoresis. Subsequently, three DNA libraries from the control group and another three from the DBPH group were amplified using the V3-V4 region of the bacterial 16S ribosomal RNA gene. The libraries were prepared and sequenced on Illumina Novaseq 6000, and data were then analyzed by bioinformatics (Biomarker Technologies BMKGENE; Münster, Germany).

To generate high-quality reads, the raw reads were filtered by trimmomatic v0.33, and the primer sequences were removed by cutadapt 1.9.1. Next, Dada2 in QIIME2 was performed to de-noise and remove chimeric sequences. Taxonomic annotation was conducted on operational taxonomic units (OTUs) using the SILVA reference database. The abundance of each species in the samples was calculated at the phylum, class, order, family, genus, and species levels using statistical analysis based on the composition of each sample. The abundance of data was generated by the R package for the characterization of microbial communities present in the samples. To measure species diversity within individual samples, alpha diversity analysis was assessed using various metrics, including the Chao1, Shannon, and Simpson indices. Conversely, beta diversity analysis was assessed to compare species diversity between different samples using principal component analysis (PCA) generated by the R package.

3.14 Challenge Trial

Preparation of *Streptococcus agalactiae*. The *S. agalactiae* virulent strain was isolated from the infected fish from an earthen culture pond in the northeast part of Thailand and maintained in the Laboratory of Biotechnology for Aquaculture, Suranaree University of Technology. A single colony was resuspended in tryptic soy broth (Merck KGaA, Darmstadt, Germany) and cultured at 37°C, 150 rpm for 16-18 h. The bacterial suspension was adjusted to a final concentration of $1x10^8$ CFU/mL with an optical density at 600 nm of 1.0.

3.15 Expression of DBPH on mRNA expression levels of inflammatory and antioxidant genes via qRT-PCR

3.15.1 Total RNA extraction

Experimental fish were dissected and liver, spleen, head kidney, and intestine were collected. Total RNA was extracted using TRIzol reagent (Gibco BRL, USA) according to the manufacturer's instructions. One ml of TRIzol reagent (Gibco BRL, USA) was added to a homogenized tube containing 100 mg of collected tissue. All samples were homogenized in a tissue homogenizing machine (MP automatic tissue extractor; Fast Prep®-24). After that, the homogenized sample was incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes, and 0.3 ml of chloroform per 1 ml TRIzol reagent was added to the sample. Sample tubes were shaken vigorously by hand for 15 sec and incubated at room temperature for 3 min. Then, the sample was centrifuged at 12,500 rpm, 4°C for 15 min. Following centrifugation, the mixture was separated into a lower red (phenol-chloroform phase), interphase, and a colorless upper aqueous phase. The aqueous phase was transferred to a new tube and RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol in an equal volume to the aqueous phase transferred. The sample was incubated on ice for 30 min and centrifuged at 12,000 rpm, 4°C for 30 min. The supernatant was removed, and 1 ml of 80% ethanol was added to the sample. After that, the sample was mixed with a vortex mixer and centrifuged at 8,000 rpm, 4°C for 5 min. The ethanol wash was removed, and the RNA pellet was air-dried for 5–10 min on ice. Total RNA was dissolved in DEPC. Total RNA samples were treated with DNase I (Fermentas, USA) to remove contaminating genomic DNA from RNA samples.

3.15.2 First strand cDNA synthesis

The first-strand cDNA was synthesized using the ImProm-II™ Reverse Transcription System kit (Promega Corporation, Madison, WI, USA). Oligo (dT) and 1 µg of total RNA from each tissue were incubated at 70°C for 5 min. Fifteen microliters of master mix consisting of 4 µl of 5X reaction buffer, 4.8 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP, 0.5 µl of RNase inhibitor, 1 µl of RNase transcriptase, and 3.7 µl of nuclease-free water were prepared on ice. The total volume of 20 µl was mixed well and then incubated at 25°C for 10 min, 42°C for 90 min, and 72°C for 15 min, respectively. The first-strand cDNAs were kept at -20°C until used.

3.15.3 Real-time PCR analysis Inflammatory and Antioxidant Gene Expression

To assess the influence of DBPH on mRNA expression levels of inflammatory and antioxidant genes following the feeding trial, liver and spleen tissues were obtained from each experiment (n = 4), both before and after the challenge. The total RNA, in 3.15.1 was then converted into complementary DNAs (cDNAs) in 3.15.2 Subsequently, partial fragments of these genes and the β-actin gene were amplified from the liver and spleen cDNA using specific primer sets, as illustrated in Table 3.1 The β-actin gene was used as an internal reference gene for normalization. The cleaned-up PCR products were inserted into a pGEM® T-Easy plasmid (Promega Corporation, Madison, WI, USA), and the plasmid copies were sequenced by Macrogen, Inc. (Seoul, Korea) to check that the standard plasmids for qRT-PCR analysis were correct. The sequence data for each gene are shown in Table 3.1 We used the CFX Opus Real-Time PCR System (Bio-Rad, Hercules, CA, USA) and THUNDERBIRD® SYBR®qPCR Master Mix (TOYOBO, Osaka, Japan) to check the mRNA levels of antioxidant genes three times, following our earlier research One microliter of first-strand cDNAs from First strand cDNA synthesis was analyzed by quantitative real-time PCR analysis using an the CFX Opus Real-Time PCR System machine (Bio-Rad, Hercuies, CA, USA). Ten microliters of the PCR mixture containing 1 µl first strand cDNA, 5 µl SYBR Green qPCR Master Mix (Toyobo), 2 µl dH2O, and 1 µl each specific primer was illustrated in Table 3.1. The PCR conditions was performed at 95°C for 3 min, followed by 40 cycles of 95°C for 30 s and 55-63°C for 30 s. DNA melting curve analysis was used to verify the specificity of the primers. The melting curve analysis was used to confirm the specificity of the primers by evaluating the dissociation characteristics of double-stranded DNA during heating.

Table 3.1 Primers used for qRT-PCR analysis.

Primer name	5′ to 3′ Nucleotide Sequences	Product size (bp)	Annealing temperature (°C)	Sequence data	Product size (bp)
SODF	GGAGACCTGGGAAATGTGAC	171	55	**GGAGACCTGGGAAATGTGAC**TGCAGGAGCAGA	171
				TAATGTTGCCAAGATAGACATCACTGACAGTGTG	
				ATCAAGCTCACAGGTGCCGACTCCATCATTGGAA	
SODR	ACCAGTCTTCAGGCTCTCTT			GAACCATGGTGATCCATGAGAAGGCTGATGACCT	
				GGGTAAAGGAGGAGAT**GAAGAGAGCCTGAAGA**	
				CTGGT	
CATF	TCTCAACAGGAACCCAGTCA	174	55	**TCTCAACAGGAACCCAGTCA**ATTACTTTGCAG	174
				AGGTGGAGCAGCTGGCCTTCGACCCCAGCAACAT	
				GCCACCGGGCATTGAGCCCAGCCCTGACAAGATG	
CATR	ACAGGGATCTGCAGGTAGTT			CTGCAGGGTCGACTCTTCTCCTACCCAGACACAC	
				ATCGTCACCGGCTTGGGGCA**AACTACCTGCAGA**	
				TCCCTGT	
IL-1βF	GTGACCACTGGCAGAAAGAT	150	55	**GTGACCACTGGCAGAAAGATCTCGTCCTGTC**	150
				AGGAGACTTACAGCTGCAGGCCATCACTCTGAA	
IL-1βR	GACAGGACAACAGTCAGACC			AGGAGGAAACTACCAACACAAAGTGAATTTTAAA	
				ATGTCGCGGTACAACTCTTCCTCCGTCACTCCTG	
				GTGAT**GGTCTGACTGTTGTCCTGTC**	

Table 3.1 (Continue).

Primer name	5′ to 3′ Nucleotide Sequences	Product size (bp)	Annealing temperature (°C)	Sequence data	Product size (bp)
IL-6F	CCAACCAGCAGTGAGGAG	191	55	**CCAACCAGCAGTGAGGAG**CAGATGCTCAAGGT	191
				CAACAGTCCGTATGCTTTCCATAGAAAGATGAAA	
				GCGCACAACATCCTGAATCATCTCTTTGATTTCC	
IL-6R	GCTGCCATATTTCTCCTGGT			TCAAAGAAGTGAAAAGATCTATCTGTAGAATGGA	
				GATGAAG**ACCAGGAGAAATATGGCAGC**	
CCF	ACAGAGCCGATCTTGGGTTACTTG	229	55	**ACAGAGCCGATCTTGGGTTACTTG**GTCCAGAG	229
				AGCAAGGCGTCCATGTGTCAATGCAGTCATCTTT	
				CAGACACAGTCCGGTCTTTTCTGCATCAATGGGA	
				GAGCTCCCTGGGTTCGTGCCACGATTGTTGCATT	
CCR	TGAAGGAGAGGCGGTGGATGTTAT			CGAGAAAGCTAAAGCCCAGTCCACTACACCATCT	
				GTGGTCACTACATCTCCAGTCTCCCTTCTCTCCA	
				TC**ATAACATCCACCGCCTCTCCTTCA**	
CXCF	TGTCTGTGTCACCGTGTCAGGAAT	151	55	**TGTCTGTGTCACCGTGTCAGGAAT**CGTGTTGG	
				CCTGAAGTCGGAAATAAAGGACATTCAGATCTAC	
				CCAGCAACCATCTTCTGCAACAAAGTGGAGATTG	
CXCR	CCTTCAGCTCAGGGTTCAAGCAAT			TTGTCACCTTGAACAACAGCTATCGCT**ATTGCTT**	
				GAACCCTGAGCTGAAGG	

Table 3.1 (Continue).

Primer name	5′ to 3′ Nucleotide Sequences	Product size (bp)	Annealing temperature (°C)	Sequence data	Product size (bp)
β-actinF	ACAGGATGCAGAAGGAGATCACAG	155	55	**ACAGGATGCAGAAGGAGATCACAG**CCCTGGC CCCATCCACCATGAAGATCAAGATCATTGCCCCA CCTGAGCGTAAATACTCCGTCTGGATCGGAGGCT CCATCCTGGCCTCCCTGTCCACCTTCCAGCAG**AT** **GTGGATCAGCAAGCAGGAGTAC**	
β-actinR	GTACTCCTGCTTGCTGATCCACAT				

3.16 Statistical analysis

SPSS 25.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. The results are reported as least squares mean values. The data was analyzed using a one-way analysis of variance, according to the complete randomized design (CRD) experimental plan to compare the differences between the means in each experimental group, using Tukey's multiple tests at the accepted significance level of $P < 0.05$. In addition, an independent sample T-test was conducted to evaluate the difference between the negative control group and the optimal percentage DBPH group for the microbiome analysis, as well as between the pre-and post-challenge group for gene expression analysis ($P < 0.05$).