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Effect of Drone Brood Homogenate on Morphometry of Intestine, Production of MUC-2, sIgA, Expression of Cytokines, and Blood Values in Pigs

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Abstract: In this paper we describe how the administration of drone brood homogenate (DBH) influenced intestinal secretion of IgA, MUC-2, morphometric parameters, peripheral blood values, and expression of cytokines. A total of 12 pigs were divided into three groups, two experimental, first fed with 100 mg DBH.kg^{-1} (E1), second with 200 mg DBH.kg^{-1} (E2) of body weight, and a control (C) group without DBH in the diet. Animals didn't show significant changes in average weight gains and feed conversion among evaluated groups. Feeding a diet containing 200 mg DBH.kg^{-1} of body weight in the E2 group produced an increase ($p < 0.05$) in the red cell distribution width, the percentage of metabolic activity of blood phagocytes in the peripheral blood, as well as splenic cell subpopulations CD4+ and CD21+. The villi height and their cutting surface were increased in the E2 group ($p < 0.01$), together with a gradual decrease in the concentration of MUC-2 and sIgA ($p < 0.001$). Upregulation of IL-8 ($p < 0.0001$), IL-10 ($p < 0.05$), and IL-18 ($p < 0.001$) was found mainly in E2 group. Results suggest, that supplementation of DBH in the feed of pigs formed optimal intestinal homeostasis.

Keywords: drone brood homogenate; morphometry of intestine; blood values; MUC2; sIgA; cytokines; pigs

1. Introduction

Bee products have been used in natural medicine for centuries. Most scientific reports focus on the nutritive properties and therapeutic action of propolis, royal jelly, honey, bee venom, and pollen. Less information is available on drone brood, which is another product of beekeeping [1]. Honey drone larvae are male bees that develop from unfertilized eggs. Drones are responsible for the fertilization of a queen bee, thereby continuing the species. Apart from reproduction, drones have no others important function in the bee community, except draining food resources collected by worker bees. For this reason, the excess of the drone brood is removed from the hive by the beekeepers. Drone brood homogenate (DBH) made from drone larvae has a rich nutritional profile. Native DBH contains 70.3-73.6% of moisture, 3.5-3.8% lipids, 8.12-10% proteins, 38.81% amino acids, including free ones - 8,76%, out of 20 available amino acids, 9 are irreplaceable [2–5]. The nutritional value of honeybee drone larvae is a valuable food source for humans in some cultures [6]. The biological and therapeutic

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activities of drone larvae homogenate have been confirmed by performing laboratory and animal/human in vivo experiments [7]. DBH exhibits many healing and therapeutic properties. A positive effect of DBH administration on reproductive dysfunction, manifested by reducing the time to the first estrous cycle [8], some metabolic processes, and immune status, has been previously demonstrated in pigs, male broilers, and rats [9–11]. Several authors documented anabolic effects of DBH on animals [12–14].

Accumulating data indicate that gut microbiota is engaged in a dynamic interaction with the intestinal innate and adaptive immune system, affecting different aspects of its development and function [15,16]. The intestinal mucosal immune response is induced by the local mucosal tissue, where immunoreactive cells are continually exposed to antigen and immunomodulatory agents from the diet and the commensal microbiota.

Among the immunoreactive cells, the macrophages are critical players in host defence against infection, during inflammation, and in response to injury [17]. Macrophages are highly specialized for phagocytosis, and participate in tissue remodelling and removal of cellular debris [18]. According to their immunological function, macrophages can be divided into a pro-inflammatory M1 and an anti-inflammatory M2 subtype. In this context, pro-inflammatory stimuli like interferon (IFNγ) and lipopolysaccharide (LPS) promote M1 polarization, whereas stimuli like interleukin-4 (IL-4) promote anti-inflammatory M2 polarization [19,20].

Generally, mucosal immunity is characterized by the production of secretory immunoglobulin A [21,22]. Immunoglobulin A (IgA) is present in all mammals and birds in various isoforms (monomeric, dimeric, secretory). Monomeric IgA is present in serum, whereas secretory IgA (sIgA) predominates in mucosal secretion. It acts as the first line of defence against pathogens and, as such, facilitates mucus surface colonization by commensal microbiota and regulates immune homeostasis [23,24]. The majority of IgA producing plasma cells are located within mucosal membranes lining the intestines.

Mucins covering the intestinal epithelium serve as *gatekeepers of the intestinal mucosal barrier, and* are the major product of goblet cells (GCs). Cell secreting mucins are recognized by their apical accumulation of mucin containing granules. The main component of the mucus layer is MUC-2 [25], classified as one of the gel forming mucins providing viscoelastic properties for the mucus layer [26,27].

Data of drone brood homogenate (DBH) effect on the intestinal immune system in livestock animals are rare. To our knowledge, there is no experimental work focused on the secretion of sIgA, and MUC-2 in the intestine, including morphometric parameters, peripheral blood values, as well as immunophenotyping of blood and splenic cells in animals. In our trial, pigs were used as model animals for supplementation of DBH in the feed of animals. Moreover, we think that such information is a prerequisite for the commercial application of drone larvae homogenate to animals and is an ideal candidate for use as food or as a food ingredient in human population.

2. Materials and Methods

Production of Drone Brood Homogenate (DBH)

Six bee colonies were selected for the production of drone larvae in our trial. The bee colonies were placed in wooden hives of type B10, in an apiary at an altitude of 710 m. Production of drone larvae was carried out in May and June. Two frames for building activity and drone development were added to each brood chamber (consisting of 10 frames) on positions 3 and 8. The colonies were checked regularly, in 3-day intervals, in terms of egg laying by the queen and the larvae development. When the larvae reached the age of 6-7 days, drone frames were removed from the hive. Drone frames were inserted into the hives in 20-day intervals. Larvae were extracted from the drone frames by the wash-up method. Larvae were washed up into the wood sieve with fiberglass mesh by a weak stream of water at a temperature of 10-12 °C. Washed larvae were placed on filter paper for 5-10 minutes to remove the excess water, and then the larvae were frozen. The entire process took no more than 1 hour, from collecting the drone frames to freezing the drone larvae. The speed of the process created the prerequisite for obtaining high-quality larvae. Finally, the larvae were freeze-dried using laboratory lyophilizer (Heto PowerDry PL9000 –50 °C Shelf Freeze Dryer with HSC 500 Plus Temperature Controller, Thermo Scientific, Waltham, Massachusetts, USA) with chamber held at –49 °C steadily and vacuum held at 0.086 hPa, for 40-50 hours depending on the layer of the samples. Lyophilized larvae were then homogenized using a sample grinder with a high-performance drive, and electronic control with the possibility of cooling (RETSCH ZM 200 Ultra Centrifugal Mill, Retsch GmbH, Germany) and stored at –18 °C in the freezer until their administration in the feed.

2.1. Animals

Twelve 8-week-old hybrid Slovak White and Landrace pigs with an initial live weight of 19.5 ± 2.7 kg were included in the 18-day trial. The pigs were labeled with a permanent tattoo on their ear and randomly divided into 3 groups (n=4): E1 (experimental 1), E2 (experimental 2), and C (control). Each group contained one female and three males. The pig groups were separated by iron barriers. The pigs had free access to feed (Table 1) and water. Their diet corresponded to the commercial diet for pigs (Table 2) based on the feeding norms in Slovakia (TEKRO Nitra, Ltd). Animals of the E1 group were fed lyophilized DBH at a dose of 100 $mg.kg^{-1}$ added to the commercial diet, and pigs of the E2 group were administered with a dose of 200 $mg.kg^{-1}$. Lyophilized homogenate mixed with a commercial diet was applied twice daily at 7.00 a.m. and 4.00 p.m. for 18 days. Control animals were fed exclusively a commercial diet without DBH. On day 19, the pigs were euthanized in the experimental slaughterhouse by electrocution and subsequent bleeding by trained personnel. On this day, samples for hematology, flush protocol, histology and morphometry of ileum, and quantitative real-time PCR method were collected. Feed conversion was calculated as the ratio of feed intake to weight gain during the monitored period.

Table 1. Composition of basal diets.

Ingredient (% diet)	TEKRO starter
Wheat	41.60
Barley	25.00
Soybean meal extracted	9.00
Fish meal	10.00
Dried whey	5.00
Soybean oil	3.00
Monocalcium phosphate	0.65
Limestone	0.50
Salt	0.25
Vitamin-mineral premix[1]	5.00

[1]Supplied per kg of diet: vit. A 9 000 IU; vit. D3 1 500 IU; α– tocopherol 35.0 mg; vit. B1 1.7 mg; vit. B2 6.0 mg; vit. B6 2.5 mg; Ca-pantothenate 15.0 mg; niacin 38.0 mg; vit. K3 2.0 mg; biotin 0.12 mg; cyanocobalamin 0.03 mg; choline 156 mg; Fe 103.0 mg; Zn 116.5 mg; Mn 49.0 mg; Cu 40.0 mg; I 1.2 mg; Co 0.4 mg; Se 0.3 mg.

Table 2. Nutrient composition.

N-substances	$g.kg^{-1}$	195.0
Fat	$g.kg^{-1}$	55.0
Dietary fibre	$g.kg^{-1}$	35.0
Lysine	$g.kg^{-1}$	14.0
Methionine	$g.kg^{-1}$	5.3
Calcium	$g.kg^{-1}$	7.0
Phosphorus	$g.kg^{-1}$	5.5
Sodium	$g.kg^{-1}$	2.0

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Copper	$mg.kg^{-1}$	130
Zinc	$mg.kg^{-1}$	2500

2.2. Hematology

Peripheral blood samples were taken from the jugular vein into heparin (10-20 $U.mL^{-1}$; Zentiva, Czech Republic) and examined with automatic hemocytomer (BC-2800Vet, Germany). Red and white blood cell counts were determined. Differential blood count (DBC) was evaluated in blood smears stained with a commercial kit of Hemacolor (Hemacolor®Rapid staining of blood smear, Sigma-Aldrich 1.11674, Germany) by evaluation of 100 cells under a light microscope using immersion oil. Relative percentages of different types of leukocytes were converted to absolute values ($G.L^{-1}$) as follows: total leukocyte count x proportion of differential cells counted (%) / 100.

2.3. Determination of Phagocytic Cell Functions by Flow Cytometry

The total phagocytic activity and index of phagocytic activity in granulocytes were determined in whole heparinized blood using the commercial PHAGOTEST kit (Lot 19 741, Glycotope Biotechnology, Germany), which contains fluorescein isothiocyanate-labeled opsonized *Escherichia coli*-FITC bacteria and other reagents required by manufacture protocol. The total phagocytic activity was expressed as the percentage of granulocytes participating in the ingestion of 1 or more bacteria. The index of phagocytic activity was expressed as the number of bacteria absorbed by one cell.

The oxidative burst of granulocytes was determined using a commercial PHAGOBURST kit (Lot 15 003, Glycotope Biotechnology, Germany). The kit detected the percentage of phagocytes producing reactive oxidative metabolites and their fluorescence intensity (oxidative activity). The individual oxidative activity of the cells was determined according to the mean fluorescence intensity (GeoMean, amount of Rhodamine 123/cell).

2.4. Determination of Lymphocyte Subpopulations

The method of flow cytometry (FACS) and direct and indirect immunofluorescence was used to detect lymphocyte subpopulations in peripheral blood and spleen. Mononuclear cells from peripheral blood and spleen, including lymphocytes and monocytes, were isolated on a Histopaque-1077 density gradient (Sigma, Germany) following Boyum (1974) [28]. Spleen samples were taken into PBS, homogenized in a Petri dish in PBS, and filtered through a nylon sieve (70 µm, BD, Germany), followed by lymphocyte isolation.

Isolated mononuclear cells were incubated with fluorescein isothiocyanate (FITC) or R-phycoerythrin (RPE) labeled and unlabeled mouse anti-pig monoclonal antibodies (Southern Biotechnology Associates, Inc. Birmingham, USA, Bio-Rad, Italy) diluted according to the manufacturers protocols (1 $\mu g/10^6$ cells – CD45, CD2, CD3, CD4, CD8, IgM, CD21, SWC3). Secondary polyclonal goat anti-mouse immunoglobulins IgG2a and IgG1 conjugated with FITC (DAKO, Denmark) were used to exclude cross-reactivity (Table 3).

Samples were measured using BD FACS CantoTM flow cytometer (Becton Dickinson, Biosciences, San Jose, CA, USA) by collecting 10,000 labelled lymphocytes and evaluated by two-parameter dot-plot histogram with BD FACS DivaTM software (Becton Dickinson, Germany). The results expressed as a relative percentage of individual lymphocyte subpopulations were converted to absolute values ($G.L^{-1}$) in the peripheral blood as follows: absolute count of lymphocytes x relative percentage of subpopulation's lymphocytes / 100. Isolated mononuclear cells showed positivity for the presence of a CD45 marker higher than 99.5%. Subsequently, proportions of lymphocyte subpopulations (CD2, CD3, CD4, CD8, CD21, and IgM) were analyzed by gating on lymphocytes and SWC3+ cells from the entire population obtained.

Table 3. List of monoclonal antibodies used for evaluation of mononuclear cell subpopulations.

Specificity	MoAbs	Isotype	Dilution	Cat. No.
CD45	K252.1B4	IgG1-FITC	1/10	MCA1222
CD2	MSA4	IgG2a	1/25	WS0590S-100
CD3	PPT3	IgG1-SPRD	1/50	0102-13
CD4	74-12-4	IgG2b-RPE	1/25	0104-09
CD8	76-2-11	IgG2a-FITC	1/25	0102-02
CD21	BB6-11C9.6	IgG1-R-PE	1/25	0102-09
IgM (μ chain)	K139.3EI	IgG2a	1/25	MCA633
SWC3	74-22-15	IgG1	1/25	donation
Mouse IgG1	15HG	IgG1-RPE	1/25	0102-09
Mouse IgG2a	HOPC-1	IgG2a-FITC	1/25	0103-02

2.5. Flush Protocol

During the necropsy, segments of the jejunum were collected from the intestine at the identical location in each pig. The length of intestinal segments was approximately 3 cm. The intestinal flush from the segment was then used for sIgA and also for MUC-2 production and secretion. The syringe with needle was filled with optimal volume (5 ml per sample) of warm flush solution (1 M Tris/glycine buffer with 0.25% Tween 20, pH 7.00, Sigma-Aldrich, USA). The needle was then injected into one end of the intestinal loop, and the entire intestinal content was expelled into a tube by repeated expiration. The intestinal flush samples were centrifuged for 5 min at 12,000 × g (Hettich Rotina 420R Centrifuge, DJB Labcare, UK), and the supernatants from each sample were frozen at –20 °C until the ELISA assay procedure.

2.6. Secretory IgA and MUC-2 Detection by ELISA

For detection of total sIgA in jejunal flushes, a commercial pig IgA ELISA kit (cat. No. KT-612, Kamiya Biomedical Company, USA) was used. Ninety-six-well microplates were coated with affinity purified anti-pig IgA antibody. After incubation of the microplates (22 °C, 20 min), the content of the wells was aspirated and washed 3 times with wash solution (ELISA kit component). The samples were diluted 1:5 in 1x diluent solution (kit component) and added in 100 μl doses into pre-designed wells in duplicates. Then 100 μl of diluted antibody conjugated with horseradish peroxidase in stabilizing buffer was applied into the plate wells, and incubated at 22 °C for 20 min. After incubation each plate was washed and 100 μl of 3.3.5.5′ tetramethylbenzidine substrate solution was added to each well. The reaction was stopped with 100 μl of stop solution and absorbance was measured spectroscopically at 450 nm on a microplate reader (Revelation Quicklink, Opsys MR, Dynex Technologies, USA).

Detection of total MUC-2 was performed using the commercial pig MUC-2 ELISA kit (cat. No. KT-94775, Kamiya Biomedical Company, USA). Ninety-six-well microplates were coated with affinity purified anti-pig MUC-2 antibody. After incubation, each plate was washed, and 50 μl of substrate solution was added to each well. The samples were diluted 1:5 in PBS (pH 7.0-7.2) and added in 100 μl doses into pre-designed well duplicates. Then, 10 μl of balance solution and 50 μl of horseradish peroxidase conjugate in stabilizing buffer was applied into the plate wells, and incubated at 37 °C for 1 h. The reaction was stopped with 50 μl of stop solution. The plates were incubated at 37 °C for 10 min. Finally, the absorbance was measured spectroscopically at 450 nm on a microplate reader (Revelation Quicklink, Opsys MR, Dynex Technologies, USA). Interpretation of total MUC-2 and sIgA was done using a calibration curve constructed according to the manufacturer´s protocol.

2.7. Histology and Morphometry of Ileum

A routine histological method with hematoxylin-eosin staining was used. The height and surface area of the villi along with the density of goblet cells in the *ileum* of four pigs from each group were analyzed. The *ileum* was chosen for the morphometry of intestinal mucus because in this period the height of the villi and the cutting surface of the *jejunum* are difficult to calculate. The histological samples were microphotographed (Nikon LABOPHOT 2 with a camera adapter DS Camera Control Unit DS-U2) and then the NIS-Elements version 3. Software (Laboratory Imaging, Nikon, Japan) was used. The height of the villi was measured on 100 well-aligned villi from the basal region segment, corresponding to the higher section of the crypts, to the apex (µm). The total cutting surface area of the same separate intestinal segments included the length and the breadth of villi (μm^2). The goblet cells were calculated within the scale in 100 villi of ileum per group and the total number was expressed as the average rate of goblet cells *per* group. The number of goblet cells *per* villus was counted in ten well-oriented villi. Finally the data were exported to MS Excel and statistically analyzed.

2.8. Homogenization of Samples and Isolation of Total RNA from Tissue Samples

Samples of the caudal part of jejunum (20 mg weighted pieces) were immediately placed in RNA Later solution (Qiagen, UK) and stored at –70 °C before the RNA purification and transcription as was described in Karaffová et al. [29].

2.9. Quantitative Real-Time PCR Method

The relative gene expression for selected interleukins (IL-8, IL-10, IL-18) was evaluated by the quantitative Real-Time-PCR method using the SsoAdvancedTM universal SYBR green supermix kit (Bio-Rad Laboratories, Hercules, California, USA) and specific primers (Table 4) on a LightCycler 480 II Instrument (Roche, Basel, Switzerland) according to a predefined temperature program. In addition, the mRNA relative expression of the reference gene encoding hypoxanthine guanine phosphoribosyltransferase (HPRT) was determined based on the stability of expression using geNorm software. All primer sets allowed DNA amplification efficiencies between 94% and 100%. The qRT-PCR reaction was initiated by denaturation at 95 °C for 30 s and followed by 39 cycles of amplification: denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and elongation step at 72 °C for 2 min. A melting curve from 55 °C to 95 °C with readings at every 0.5 °C was recorded for each individual RT-qPCR plate. Analysis was performed after every run to ensure a single amplified product for each reaction. Each real-time PCR reaction was performed in triplicate, and the mean values of the triplicate were used for further analysis.

The obtained Cq values of the genes were normalized to the average Cq value of the reference gene and the relative expression of each gene was calculated mathematically as $2^{-\Delta Cq}$.

Table 4. List of primer sequences for target genes.

Primer	Sequence 5 ‘–3’	References
IL-8 Fw	TTATCGGAGGCCACAATAAG	[30]
IL-8 Rev	TGGAATAGTAGATGGAGCCA	
IL-10 Fw	CGGCGCTGTCATCAATTTCTG	[31]
IL-10 Rev	CCCCTCTCTTGGAGCTTGCTA	
IL-18 Fw	CTGCTGAACCGGAAGACAAT	[32]
IL-18 Rev	TCCGATTCCAGGTCTTCATC	
HPRT Fw	AACCTTGCTTTCCTTGGTCA	[33]
HPRT Rev	TCAAGGGCATAGCCTACCAC	

2.10. Statistical Analysis

Statistical analysis of data was performed using one-way ANOVA with Tukey´s post-test in GraphPad Prism Software, statistical version 4.0 (San Diego, CA, USA). Differences between the mean values for different treatment groups were considered statistically significant at $^{ab}p < 0.05$, $^{ac}p < 0.01$, $^{ad}p < 0.001$, and $^{ae}p < 0.0001$. Values in tables are expressed as means with standard deviation (±SD).

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3. Results

3.1. Growth Performance of Weaning Pigs

The body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) were determined daily during the experiment. The body weight gain of experimental and control pigs at the end of the trial was not significantly changed. Similarly, the average daily weight gains and feed conversion of experimental animals were not statistically significant to control pigs (Table 5).

Table 5. Productive parameters of the weaned pigs, fed a diet supplemented with drone brood homogenate (mean ± SD).

Groups (n = 4)	BW beginning of trial/kg	BW end of trial/kg	Daily weight gain/g	% to Control body mass	Feed conversion ratio	% to Control feed conversion
C	21.37 ± 1.60	36.77 ± 0.95	860 ± 0.11	100.0	1.66 ± 0.22	100.0
E1	21.39 ± 3.14	37.72 ± 4.88	910 ± 0.01	106.0	1.57 ± 0.17	94.3
E2	21.38 ± 2.70	38.14 ± 3.88	930 ± 0.08	108.8	1.56 ± 0.13	93.9

Note: E1-100 $mg.kg^{-1}$DBH; E2-200 $mg.kg^{-1}$DBH; BW – Body weight.

3.2. Peripheral Blood Values

Peripheral white blood cells (Table 6) showed no significant differences between evaluated groups. There was only a trend toward an increase in the number of leukocytes, lymphocytes, and monocytes in experimental groups compared to control pigs. Most of the determined parameters of red blood cell values and platelets (Table 7) were not statistically significant. Only a percentage of RDW showed increased values in group E2 ($p < 0.05$) compared to other groups. Gradually increasing percentage of platelets was found in both experimental groups in comparison to controls.

Table 6. Absolute count of white blood cells (WBC: $G.L^{-1} = 10^9.L^{-1}$) in the peripheral blood of pigs (means ± SD).

WBC	Groups			P value
	Control	E1	E2	
Leukocytes	15.98 ± 2.65	16.98 ± 2.29	18.95 ± 4.02	0.4165
Lymphocytes	9.04 ± 1.23	10.16 ± 0.74	11.35 ± 2.44	0.1917
Neutrophils Seg.	3.49 ± 1.48	2.48 ± 0.72	3.76 ± 1.57	0.3864
Neutrophils Band	2.53 ± 0.33	3.42 ± 1.26	2.60 ± 0.96	0.3637
Eosinophils	0.41 ± 0.15	0.35 ± 0.18	0.60 ± 0.08	0.7703
Basophils	0.16 ± 0.03	0.17 ± 0.02	0.29 ± 0.15	0.1043
Monocytes	0.36 ± 0.08	0.39 ± 0.14	0.49 ± 0.08	0.1956

Note: E1-100 $mg.kg^{-1}$DBH; E2-200 $mg.kg^{-1}$DBH; Seg-segments.

Table 7. Determination of red blood cells and platelet values in the peripheral blood of pigs (means ± SD).

Parameters	Groups			P values
	Control	E1	E2	
RBC $10^{12}.L^{-1}$	6.64 ± 0.36	6.96 ± 0.38	6.68 ± 0.80	0.6898
HGB g/dl	14.33 ± 0.50	14.03 ± 0.27	13.68 ± 0.66	0.2469
HCT %	0.44 ± 0.02	0.44 ± 0.01	0.43 ± 0.04	0.8086
MCV fL	66.80 ± 0.93	62.85 ± 2.13	64.45 ± 2.84	0.0749
MCH pg	21.53 ± 0.43	20.13 ± 1.24	20.60 ± 1.71	0.3168
MCHC g/dl	32.30 ± 0.35	29.90 ± 4.61	31.95 ± 1.55	0.4595
RDW %	15.48 ± 0.82[a]	15.30 ± 0.22[a]	16.53 ± 0.35[b]	0.0196
PLT $10^9.L^{-1}$	399.80 ± 48.36	419.80 ± 135.90	563.80 ± 106.20	0.1007
MPV fL	8.78 ± 0.50	9.12 ± 0.49	9.17 ± 0.34	0.4265
PDW	16.13 ± 0.25	16.13 ± 0.17	16.08 ± 0.05	0.9004
PCT %	0.35 ± 0.05	0.38 ± 0.11	0.52 ± 0.10	0.0703

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Note: E1-100 mg.kg^{-1}DBH; E2-200 mg.kg^{-1}DBH; RBC-Red blood cells; HGB-Hemoglobin; HCT-Hematocrit; MCV-Mean corpuscular volume; MCH-Mean corpuscular hemoglobin; MCHC-Mean corpuscular hemoglobin concentration; RDW-Red cell distribution width; PLT-Platelets; MPV-Mean platelet volume; PDW-platelet distribution width; PCT-Plateletcrit; Means with different superscript letters in the same line differ significantly at $^{ab}p < 0.05$.

3.3. Functions of Phagocytes

Functional tests of phagocytes (Table 8) showed significant changes only in metabolic activity with a higher percentage in the E2 group compared to control pigs ($p < 0.05$). While the index of metabolic activity tended to decrease in E1 and E2 groups, the index of phagocytic activity in animals administered both doses exhibited values exceeding those of the control group.

Table 8. Functional parameters of phagocytes (means ± SD).

Parameters	Groups			P values
	Control	E1	E2	
Phagocytic activity (PA %)	93.03 ± 1.52	93.53 ± 2.46	89.78 ± 7.78	0.5120
Index of PA	24 344 ± 5 120	25 936 ± 9 178	25 803 ± 8 544	0.9550
Metabolic activity (MA %)	67.78 ± 14.29^{a}	72.30 ± 4.54	88.30 ± 6.16^{b}	0.0300
Index of MA	2 147 ± 841	1 730 ± 249	1 530 ± 401.3	0.3250

Note: E1-100 mg.kg^{-1}DBH; E2-200 mg.kg^{-1}DBH; Means with different superscript letters in the same line differ significantly at $^{ab}p < 0.05$.

3.4. Phenotyping of Lymphocytes in Blood and Spleen

The determination of cell subpopulations in the peripheral blood (Table 9) showed only an insignificant increase in the absolute values of T (CD2, CD3, CD4, CD8), and B lymphocytes (CD21, IgM), as well as monocytes (SWC3) mainly in the E2 group with 200 mg dose in the diet when compared to the control.

On the other hand, splenic subpopulations (Table 10) presented CD4+ T lymphocyte improvement in the E2 group against E1 and control groups ($p < 0.05$). Similarly, CD21+ cells representing B lymphocytes showed higher values in the E2 group than in the control and E1 group ($p < 0.05$).

Table 9. Absolute count of porcine blood lymphocyte subpopulations expressed in. G.L-1=109.L-1 (means ± SD).

Parameters	Groups			P values
	Control	E1	E2	
CD2	3.77 ± 1.14	3.68 ± 1.36	4.30 ± 1.32	0.77
CD3	3.47 ± 1.13	4.69 ± 0.66	5.31 ± 1.21	0.09
CD4	2.20 ± 0.45	2.60 ± 0.25	2.87 ± 0.48	0.11
CD8	1.45 ± 0.35	1.24 ± 0.26	1.74 ± 0.76	0.41
CD21	1.32 ± 0.33	1.93 ± 0.34	1.97 ± 0.72	0.18
IgM	1.53 ± 0.27	1.74 ± 0.32	1.68 ± 0.32	0.61
SWC3	0.04 ± 0.02	0.04 ± 0.01	0.16 ± 0.15	0.12

Note: E1-100 mg.kg^{-1}DBH; E2-200 mg.kg^{-1}DBH.

Table 10. Subpopulations of porcine splenic lymphocytes expressed in relative percentage (means ± SD).

Parameters	Groups			P values
	Control	E1	E2	
CD2	28.48 ± 1.72	26.70 ± 10.91	23.13 ± 4.09	0.5701
CD3	17.73 ± 2.39	17.20 ± 3.62	20.33 ± 5.04	0.5290
CD4	23.63 ± 1.73^{b}	22.60 ± 2.65^{b}	29.60 ± 2.55^{a}	0.0097

CD8	5.85 ± 0.95	5.38 ± 1.63	4.23 ± 0.15	0.2403
CD21	15.43 ± 8.47^{b}	16.60 ± 6.11^{b}	35.87 ± 11.72^{a}	0.0286
IgM	16.48 ± 3.84	19.10 ± 5.81	20.38 ± 6.84	0.6235
SWC3	2.03 ± 1.28	1.98 ± 0.83	3.15 ± 1.49	0.3545

Note: E1-100 mg.kg^{-1}DBH; E2-200 mg.kg^{-1}DBH; Means with different superscript letters in the same line differ significantly at $^{ab}p < 0.05$.

3.5. Morphometry of Intestine

Morphometry was performed in the *ileum* and the data are summarized in Table 11. The height of the ileal villi was higher in the E2 group ($p < 0.01$) than in the control and the E1 group. The cutting surface of ileal villi was increased in group E2 ($p < 0.01$) in comparison with control pigs. The density of goblet cells was not statistically influenced by 100 or 200 mg doses of DBH between groups in the trial.

Table 11. Morphometric parameters of ileal villi (mean ± SD).

Parameters	Groups			P values
	Control	E1	E2	
Height of villi (µm)	810.17 ± 178.79^{c}	806.56 ± 197.16^{c}	893.52 ± 205.95^{a}	0.0020
Cutting surface (mm^{2})	0.304 ± 0.109^{c}	0.330±0.109	0.360 ±0.133^{a}	0.0043
Density of goblet cells (µm^{2})	6.22 ± 2.05	6.42 ± 1.78	6.26 ± 2.0	0.6819

Note: E1-100 mg.kg^{-1}DBH; E2-200 mg.kg^{-1}DBH; Means with different superscript letters in the same line differ significantly at $^{ac}p < 0.01$.

3.6. Mucin and sIgA Quantitation

The concentration of MUC-2 (ng/mL) in the intestinal flush from the *jejunum* (Table 12) was decreased in the E2 group ($p < 0.001$) compared to the control as well as E1 group. In like manner, the concentration of jejunal sIgA (ng/ml) in the intestinal flush (Table 12) showed the gradual decrease ($p < 0.001$) between both experimental (E1 to E2) groups and control animals.

Table 12. Concentration of MUC-2 (ng/ml) and sIgA (ng/ml) in *jejunum* of pigs administered with drone larvae homogenate (means ± SD).

Parameters	Groups			P values
	Control	E1	E2	
MUC-2	0.471 ± 0.015^{d}	0.468 ± 0.014^{d}	0.449 ± 0.015^{a}	0.0002
sIgA	0.0685 ± 0.0002^{a}	0.0679 ± 0.0002d^{da}	0.0672 ± 0.0001^{dd}	0.0002

Note: E1-100 mg.kg^{-1}DBH; E2-200 mg.kg^{-1}DBH; Means with different superscript letters in the same line differ significantly at $^{ad}p < 0.001$.

3.7. Relative Expression of Genes in qRT-PCR

Relative mRNA expression of IL-8 was highly upregulated ($p < 0.0001$) in the E2 group compared to the E1 group and control animals (Table 13). Lower relative mRNA upregulation ($p < 0.05$) in the E2 group was recorded in the expression of IL-10. On the other hand, a gradual increase in upregulation of IL-18 was observed in the E1 ($p < 0.01$) and in control group ($p < 0.001$) in relation to the E2 group.

Table 13. Relative gene expression interleukins in jejunum of pig ($2^{-\Delta Cq}$/ SD).

Groups	Control	E1	E2
IL-8	0.018 ± 0.0042^{e}	0.038 ± 0.0035^{e}	0.510 ± 0.1296^{a}
IL-10	0.003 ± 0.0003^{b}	0.003 ± 0.0003^{b}	0.004 ± 0.0003^{a}
IL-18	0.002 ± 0.0008^{d}	0.004 ± 0.0015^{c}	0.008 ± 0.0010^{a}

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Note: E1-100 mg.kg^{-1}DBH; E2-200 mg.kg^{-1}DBH; Means with different superscript letters in the same line differ significantly at $^{ab}p < 0.05$; $^{ac}p < 0.01$; $^{ad}p < 0.001$ $^{ae}p < 0.0001$.

4. Discussion

Data obtained during the experimental administration of drone brood homogenate (DBH) in the diet of pigs revealed only tendency to increase growth performance between the groups evaluated in our trial. The body weight gain of experimental and control pigs at the end of the trial showed an increase of 6% in E1 and 8.8% in E2 compared to the control, and the and average daily weight gains were increased in experimental groups by 5.8 % resp. 8.1%. Promising results revealed also feed conversion of experimental animals (5.8 % and 8.1% respectively) better than in controls. Increased of weight gain found Kistanova et al. [14] after daily supplementation of 25 mg.kg^{-1} forage of DBH for 180 days. The authors found higher average daily gain in treated pigs in the earlier stages of development. Seres [13] found a significant increase in the weight of the *levator ani muscle* in castrated rats treated with DBH. Non-significant changes in growth performance of pigs found in our trial could be related to low number of pigs, low doses administration of DBH or short experimental period for feeding of DBH. Effect of DBH on growth performance of animals will be our future research activity. The results obtained in our study provide indication that improved hematological parameters, such as percentage of red cell distribution width and platelet count, correlated with growing of pigs after daily supplementation of DBH in feed. Our results are consistent with the findings of Bhattarai and Nielsen [34] who found in their trial that improved hematological status, especially the mature erythrocyte indices at weaning, is positively associated with the growth rate of growing piglets. Some authors suggest [35,36] that growth in piglets may be associated with leucocyte and platelet levels, as studies reported that these parameters are related to iron deficiency anemia in human patients. In addition to iron deficiency states or a certain degree of inflammation causing membrane deformability [37,38], erythrocyte size variations may also be associated with intensive metabolic and physiological processes in growing piglets. These variations may also reflect higher weight gain and improved feed conversion ratio in the experimental animals supplemented with drone larvae homogenate [34,39]. The increase**e** of phagocytic metabolic activity in pigs following daily DBH supplementation at a dose of 200 mg.kg^{-1} of body weight in our trial demonstrates its effect on the modulation of macrophage metabolism. Metabolism plays a crucial role in supporting and regulating macrophage functions by producing bioactive and signaling molecules, communicating with the inflammasome, and regulating as well as epigenetic remodelling **[18].** The nutritional value of honeybee drone homogenate is due to its high content of proteins, lipids and other nutrients. It is supposed that DBH rich in lipid extracts can stimulate macrophages and activate anti-inflammatory macrophages [40]. Finally, lipid extracts from honeybee drone larvae significantly decreased nitric oxide (NO), reactive oxygen (ROS) production, and mRNA expression of IL-6, IL-10, COX-2, and iNOS [41]. On the other hand, DBH reduced the high level of proinflammatory cytokines induced by sepsis in adult male rats [11,42,43]. Sidor and Džugan [44] suggested that the flavonoids found in DBH influence the level of proinflammatory cytokines. In our trial IL-8, IL-10, and IL-18 showed increased upregulation mainly in animals fed a diet supplemented with DBH at a dose of 200 mg.kg^{-1} of body weight. Interleukin-8 (IL-8) and IL-18 are produced mainly by macrophages and are able to induce severe inflammatory processes [45,46]. On the other hand, IL-10 is a pleiotropic cytokine that has a fundamental role in modulating inflammation and in maintaining cell homeostasis [47]. Results obtained in our trial demonstrate that DBH rich nutritional content influences gene regulation of cytokines produced by immunocompetent cells. Immune cells play a key role in controlling tissue homeostasis, infection, and excessive inflammation [18]. Porcine B cell differentiation is determined by the expression and labeling of the CD21 marker. It is also known as a complement receptor, expressed on all mature circulating B lymphocytes and low density has also been reported on granulocytes and monocytes [48]. Our data indicate that modulation of CD21 cells in porcine spleen supplemented by the diet enriched with 200 mg of DBH/kg^{-1} of body weight may be related to the modulation of metabolic activity in these pigs. The CD4 molecule is a cell-surface glycoprotein receptor expressed by helper T cells, monocytes, macrophages, and dendritic cells [49]. Similarly, the enhanced expression of CD4 molecule may be related to the increased percentage of metabolic activity of macrophages in DBH groups with significance in the E2 group.

Intestinal bacteria can stimulate mucus secretion and its modification to become thicker and less penetrable [25,50]. Non-attached outer mucus layer allows bacteria to enter and use the mucins as a nutritional source and bacterial habitat. Our results demonstrated a decreased concentration of jejunal MUC-2 in pigs, after DBH dietary administration in both experimental groups in our trial [51] showed that decanoic acids contained in DBH have an antibacterial and anti-inflammatory effect against a number of pathogenic microorganisms in the gastrointestinal tract. These facts could correspond with a lower quantity of surface intestinal MUC-2 in our trial. The density of goblet cells remained unchanged in all groups of pigs, suggesting only a lower secretion of mucins in the group of pigs fed a diet supplemented with 200 mg of DBH/kg^{-1} of body weight. In contrast, the height of the villi and the cutting surface was increased in animals fed diet supplemented with 200 mg of DBH/kg^{-1} of body weight. These data suggest that 200 mg of DBH/kg^{-1} in the diet is effective for increasing body weight. Similarly, reduction of intestinal sIgA was found in pigs fed a diet supplemented with DBH in our trial. We propose, that the lower concentration of sIgA and MUC-2 in intestinal mucus in our experiment can be explained by the formation of optimal intestinal homeostasis in the gut after permanent dotation of DBH to pigs and the creation of antibacterial effect of drone larvae homogenate on intestinal microbiota. Reduction of IgA was also found by Selecká et al. [52] after prolonged supplementation of Lacto-Immunovital in drinking water to chickens. Secretory IgA protects the intestine against the adhesion of pathogens and their penetration into the intestinal barrier. Moreover, sIgA regulates gut microbiota composition and ensures intestinal homeostasis [24,53]. In addition, the production of IgA occurs in response to bacterial colonization of the intestine [23] and its generation is T-cell dependent. On the other hand, Fagarasan et al. [54] found a microbiota depletion and a significant decrease in the number and size of Peyer´s patches caused by antibiotic therapy.

5. Conclusions

Eighteen day administration of DBH indicate an increased percentage of metabolic activity of macrophages modulated splenic CD4+ and CD21+ in the group of pigs with administration of 200 mg of DBH/kg^{-1} of body weight. Higher doses of supplemented DBH influenced the height and cutting surface of intestinal villi. Obtained results suggest possibilities for the formation of proinflammatory and anti-inflammatory conditions and optimal intestinal homeostasis in pigs after prolonged supplementation of DBH in feed. Administration of DBH as a preventive immunomodulator against pathogens could be a good option to achieve reliable positive effects on pig health and performance. Nevertheless, further studies should be conducted to yield satisfactory results and a complete picture of red blood cell distribution in animals after drone brood homogenate administration.

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Data Availability Statement: The raw data were generated at the University of Veterinary Medicine and Pharmacy, Košice (Slovakia), the Research Institute for Animal Production, Nitra (Slovakia). Derived data supporting the findings of this study are available from the corresponding author on request.

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