Theses of Doctoral (PhD) Dissertation

Effects of bioactive plant extracts on immunological parameters, intestinal morphology, and microbiota in broiler chicken and common carp (Cyprinus carpio)

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1. Introduction and aim of the thesis

Several diseases can pose a risk to the health status and production of livestock species. Among these, bacterial infections are cured by antibiotics (VARGA et al., 2007). However, inappropriate usage of antibiotics has led to increasing concerns about multiresistant bacteria (HOELZER et al., 2017). Resistant pathogens can also reach the consumers through various routes (CHANG et al., 2015). To substitute or reduce the amount of antimicrobial agents, natural alternatives are often investigated these days to strengthen the defense system of poultry and fish (AHAD et al., 2017). These natural compounds can be derived from the yeast cell wall or plants and may have potential immunomodulant properties.

The avian immune system is divided into innate and acquired immunity, and teleost fish have non-specific and specific immunity, as well. Being part of both immune systems, cytokines are those proteins, which release after infection and have a role in signaling processes between cells. As part of humoral immunity, immunoglobulins are glycoproteins with antibody activity (DAVISON et al., 2008).

The gastrointestinal microbiota also plays an important role in immune homeostasis, forming a physical barrier and preventing the adhesion of pathogens and the production of toxic metabolites. The initial interaction between the gut microbiota and innate immunity enhances the development of the adaptive immune response (PAN and YU, 2014). In addition, morphological changes in the intestine also have a prominent role, which results in the absorption and utilization of nutrients.

Based on previous studies, active substances of plant or fungal origin may have a positive effect on the immunity of broiler chicken and carp, the composition of the microbiota, and the utilization of nutrients - and may therefore be able to enhance the host immunity.

AIM OF THE THESIS

The thesis aims to investigate the effects of the applied carotenoids, oligosaccharides, and anthocyanins, which are summarized below:

Broiler chicken experiment:

1. The impact of the applied carotenoids, oligosaccharides, and anthocyanins will be determined on the growth performance parameters (body weight, average daily gain, average daily feed intake) of broiler chickens

2. Effects of the applied carotenoids, oligosaccharides, and anthocyanins will be examined with gene expression analysis on chicken immunological parameters, such as *IL-1β*, *IL-6*, *IFN-α*, *IFN-γ*, *TLR-4*, and *TLR-5*.

3. The impacts of the applied bioactive compounds will be determined on the protein level of cytokines such as IL-1β and IL-6, and plasma IgG.

4. Effects of the natural plant extracts will be investigated on chicken intestinal microbiota, involving the following bacterial groups: *Lactobacillus*, *Bifidobacterium*, *Campylobacter*, *Clostridium*, *Salmonella*, and *Escherichia coli*.

5. Effects of the carotenoids, oligosaccharides, and anthocyanins will be determined on nutrient absorption and chicken intestinal morphological parameters, such as villus height, crypt depth, villus height to crypt depth ratio and total mucosa thickness will be measured.

6. The impacts of the mentioned bioactive compounds will be investigated on chicken behavior.

Fish experiment:

1. Potential immunomodulant effects of the carotenoids, oligosaccharides, and anthocyanins will be investigated on mRNA level of cytokines, such as *IL-1β*, *IL-8*, *TNF-α*, and *IRF-1* of the common carp

2. Impacts of the mentioned bioactive compounds will be determined on nutrient absorption of carp and intestinal morphological parameters, such as villus height, villus width, and intestinal mucosa layer will be examined.

2. Materials and methods

2.1 Preparation of the extracts

Carotenoids

Hungarian red sweet pepper powder was applied to extract carotenoids. The mixture was mixed in an ultrasonic water bath, then filtered and vaporized. Then the filtrate was solved in an HPLC pigment reagent. An HPLC separation was performed after DAD detection was applied. Based on the HPLC profile, carotenoid compounds with the greatest areas were identified and were the following: *capsanthin, cis-capsanthin, β-carotene, zeaxanthin*.

Oligosaccharides with high arabino-galactose content

As natural prebiotics, oligosaccharides with high arabino-galactose content were extracted from Hungarian red sweet pepper retained from industrial food waste. An HP 5890 Gas chromatograph with an SP-2380 capillary column was used to evaluate the composition of oligosaccharides. Samples were lyophilized and extracted with a solvent. After Flame Ionization Detection (FID), monomer units of oligosaccharides were identified and were the following: *glucose, arabinose, xylose, galactose, mannose.*

Anthocyanins

Anthocyanins were extracted from Hungarian sour cherry. After fruits were deseeded and homogenized, methanol:water:acetic acid solution was applied for extraction. Further processes involved mixing, filtering, and centrifuging, then a simple fractionation was carried out, then samples were vaporized and dried in a vacuum. Determination of anthocyanin profile was carried out with VWR-Hitachi ChromasterUltraRs UHPLC. Anthocyanin composition was quantified by comparison with the corresponding authentic standards and UV-VIS detection was performed (NEMES et al., 2018). The main anthocyanin compounds are identified as the following: *cyanidin-3-O-glucosyl-rutinoside, cyanidin-3-O-rutinoside, cyanidin-3-O-monoglucoside* (HOMOKI et al., 2016).

2.2 Broiler chicken experiment

2.2.1 Experimental design and sample collection

A total of 900, 1-day old Ross 308 mixed-sex broilers were used from a commercial hatchery. Chickens were kept in floor pens covered with wood shavings. The 1-day old Ross 308 hybrid chicken were randomly placed into 5 experimental groups (3 pens/treatment, 60 birds/pen). The experiment was started at 1 day of age and lasted until 42 days of age. The dietary treatments consisted of the control group (basal diet), the β-glucan considered as positive control and supplementation of carotenoids, oligosaccharides, or anthocyanins. β-glucan was added at 0.05% to the basal diet, additional treatments included 0.5% of bioactive extracts. Feed and water were available ad libitum during the entire experiment.

Broilers were weighed at 42 days of age. As growth performance parameters, average body weight, average daily gain, and average daily feed intake were calculated. Bodyweight and average daily gain were based on individual values (n = 18), average daily feed intakte was calculated for pens (n = 3).

On day 26, 6 male chickens per treatment were injected with 2 mg/kg live weight *Escherichia coli* O55:B5 lipopolysaccharide (LPS) with a concentration of 2 mg/ml, intraperitoneally. In the control group, another 6 male chickens were inoculated with isotonic saline solution in the same way. On day 27, 12 h after the challenge, the individual bodyweight of broilers was measured, then all of the injected birds (n = 6/treatment; control: n = 6/saline and n = 6/LPS-inoculated) were euthanized by cervical dislocation for the collection of tissue samples. Spleen and terminal ileum tissues were aseptically excised, the whole spleen was measured then samples were snap-frozen in liquid nitrogen and stored at -80°C for RNA isolation. Further 1 cm segments from the terminal sections of the ileum (n = 3/treatment) were collected and preserved in 10% formalin for tissue morphology (SALIM et al., 2013). For the determination of intestinal microbiota composition, excreta samples were collected on day 19.

2.2.2 RNA isolation and cDNA synthesis

Total RNA from spleen and ileum tissues was extracted using TRI Reagent and Direct-zol™ RNA MiniPrep according to the manufacturer's protocol, including the DNA digestion step. The concentration of the RNA in each sample was measured using a NanoDrop ND-1000 Spectrophotometer. RNA integrity was checked by 1% agarose gel electrophoresis. RNA was reverse-transcribed into cDNA using qPCR BIO cDNA Synthesis Kit. Each reaction involved

800 ng RNA, 4 µl 5x cDNA mix containing oligo (dT)s, and random hexamers, and 1 µl 20x MMLV type reverse transcriptase. Conditions consisted of reverse transcription at 42°C for 30 min and reverse transcriptase denaturation at 85°C for 10 min. cDNA samples were diluted 10-fold and stored at −20°C.

2.2.3 quantitative PCR (qPCR)

In the spleen *GAPDH*, in ileum *ACTB* were considered the most stable reference genes for normalization. Intron-spanning forward and reverse primers were designed and qPCR was performed by LightCycler 480 Instrument II. Reactions were run in triplicates using 384-well plates. Each reaction included a 1 µl (4 ng) cDNA template, 5 µl 2× Xceed qPCR SG Hi-ROX Mix, 0.2 µl 200 nM of each primer, and 13.6 µl distilled water. No template controls were included for each primer. Real-time PCR conditions were the following: initial denaturation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 5s, and annealing/extension at 60°C for 30s. Results were generated using the Pfaffl method by normalizing the expression of the target gene to a housekeeping gene (PFAFFL, 2001). Results were determined as fold changes of the expression of the target genes in the experimental groups compared with the LPS injected control group, using the following formula:

$$R = [(E_{\text{target gene}})^{\Delta Ct \text{ target gene (Control-Sample)}}] / [(E_{\text{reference gene}})^{\Delta Ct \text{ reference gene (Control-Sample)}}]$$

- R: relative expression,
- E: efficiency,
- Ct: threshold cycle.

2.2.4 ELISA assay

IL-1β, IL-6 cytokines, and IgG antibody were measured in chicken plasma using QnD Chicken ELISA Kit according to the manufacturer's protocol. Kits followed the double sandwich ELISA method. Concentrations for each sample were calculated using an HTX Synergy Multi-Mode Microplate Reader applying Gen5 3.03 software. The absorbance values of each sample were measured at 450 nm and were fitted to a linear standard calibration curve based on the standard dilutions to identify the concentrations. The x-axis involved the concentrations and the y-axis contained the absorbance values.

2.2.5 Bacterial DNA isolation and Polymerase Chain Reaction (PCR)

DNA was isolated from chicken excreta samples for intestinal microbiota determination. E.Z.N.A.® Stool DNA Kit was applied for the bacterial DNA isolation according to the manufacturer's protocol. DNA concentration was determined as described in chapter 2.2.2. DNA was stored at -20°C for further use.

Bacterial DNA was amplified with PCR technique using specific primer pairs to 16S rDNA sequences of bacterial groups, such as *Lactobacillus*, *Bifidobacterium*, *Campylobacter*, *Clostridium*, *Salmonella*, and *Escherichia coli*. Universal bacteria primer pairs were also used to amplify all the mentioned bacterial groups. Relative proportions contrasted to the universal PCR products were determined. PCR reactions with 20 µl final volume contained the following: 2 µl (10 ng) DNA template, 0.1 µl (0.5 U) DreamTaq polymerase, 0.2 µl 200 nM of each primer, 2 µl 10x DreamTaq Green Buffer, 0.4 µl 10 nM dNTP, 1.6 µl 25 nM $MgCl_2$, 13.5 µl distilled water. PCR conditions consisted of polymerase activation at 95°C 5 min, then 35 cycles of denaturation at 95°C 30s, annealing at 60°C 30s and elongation at 72°C 1 min, and a final elongation step at 72°C 5 min.

2.2.6 Intestine morphometric measurements

Formalin-preserved terminal ileal segments were used to determine villus height, crypt depth, villus height to crypt depth ratio, and total mucosa thickness. The hematoxylin-eosin stain was carried out on 18 samples (n = 3 birds per treatment) microscope paired with a camera was used to capture images of stained segments (FISCHER et al., 2008). Photos were evaluated by Adobe Photoshop CC version 19.1.6 software.

2.2.7 Behavioral monitoring

Chicken behavior was observed on the experimental days 25 and 32 in the morning (9 a.m. – 10 a.m.) and afternoon (2 p.m. – 3 p.m.). Chicken behavioral patterns, such as feed intake, drinking, resting, walking and wing stretching related to the comfort activities of the birds. The results were determined in frequencies (%) of the given activities.

2.3 Fish experiment

2.3.1 Experimental design and sample collection

A total of 132 common carp (*Cyprinus carpio*) juveniles were used from artificial propagation and kept in a water recirculation system. Carp juveniles were randomly assigned to 3 experimental groups (3 tanks/treatment, 11 fish/tank), and a control group. The experiment was started at 6 months of age and lasted for 6 weeks. The feeding trial consisted of the control group (basal diet) and supplementation of carotenoids, oligosaccharides, or anthocyanins. Each treatment included 1% of the bioactive compounds. Eight carps were randomly selected from each group for tissue sampling at the end of the feeding trial (6th week). Fish were euthanized with clove oil solution and the whole spleen and 10-mm segments from the middle part of the mid intestine were collected and kept at -80 °C until analysis.

2.3.2 RNA isolation and cDNA synthesis

Total RNA was isolated as described in chapter 2.2.2. cDNA synthesis was performed using the Maxima H Minus First Strand cDNA Synthesis. Each mixture involved 400 ng RNA, 1 µl oligo d(T) primers, 2 µl dNTPs, 4 µl 5X reverse transcription buffer, 1 µl reverse transcriptase enzyme, 1 µl RNase inhibitor, and nuclease-free water. The conditions consisted of incubation at 65 °C for 30 min and termination at 85 °C for 5 minutes. cDNA samples were diluted 10 fold and kept at -20 °C.

2.3.3 quantitative PCR (qPCR)

Gene expression analysis was carried out as it is described in chapter 2.2.3, with the following differences: The *40S* for the spleen and *GAPDH* for the mid intestine was defined as the most stable reference gene for normalization. Each reaction contained: 2 µl (4 ng) cDNA template, 2 µl 5x HOT FIREPol® EvaGreen® qPCR Supermix, 0.2 µl 200 nM of each primer, and 5.6 µl distilled water. Conditions were the following: initial activation at 95 °C for 12 min, 40 cycles of denaturation at 95 °C for 15 sec, primer annealing at 60 °C for 20 sec and chain elongation at 72 °C for 20 sec.

2.3.4 Intestine morphometric measurements

Formalin-preserved segments from the mid intestine (n = 3/treatment) were used for hematoxylin-eosin staining then samples were evaluated with a microscope paired with a camera. Measurements were carried out with cellSens Entry software and morphological parameters, such as villus height, villus width, and intestinal mucosa layer were examined.

2.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 software. One-way analysis of variance (ANOVA) was used for the analysis. The equality of variance was checked by Bartlett's test. Tukey's test was used for the *post hoc* tests. In case of unequal variances, Tamhane's T2 test was applied. A two-way analysis of variance was used for behavioral observation. Differences among groups were considered significant at $p < 0.05$. Results were expressed as mean ± SEM (standard error of the mean). For the production parameters mean and RMSE (root mean square error) were given.

3. Results

3.1 Results of broiler chicken experiment

3.1.1 Effects of bioactive compounds on growth performance of broiler chicken

Dietary effects related to growth performance such as body weight (BW), average daily gain (ADG), and average daily feed intake (ADFI) were measured. BW and ADG were not impacted by dietary treatments through the experimental period. β-glucan (83 g/day/bird), oligosaccharide (82 g/day/bird), and anthocyanin (88 g/day/bird) supplementations increased ADFI through the experimental period (day 1–42) compared to the control (73 g/day/bird).

Table 1.

Effects of bioactive plant extracts on growth performance of broiler chickens

Growth performance	Treatment					RMSE*
	Cont.	β-gl.	Carot.	Oligos.	Anth.	
BW (g/bird) Day 42	2758	2727	2748	2618	2590	98
ADG (g/day/bird) Day 1-42	65	64	65	61	61	2
ADFI (g/day/bird) Day 1-42	73[a]	83[b]	81[a,b]	82[b]	88[b]	6

a, b = Different letters in a row indicate significant differences. Results are shown in average and RMSE. Cont. = control group, β-gl. = β-glucan treatment, Carot. = carotenoid treatment, Oligos. = oligosaccharide treatment, Anth. = anthocyanin treatment. *RMSE = Root mean square error.

ZHANG et al. (2013) proved the same when β-1,3/1,6-glucan supplementations in 50 and 75 mg/kg could increase the BW of chicken. None of the compounds applied in this study could increase the ADG of chickens. Similarly, REZAEI et al. (2015) defined no significant differences in ADG of broilers, when the diet was supplemented using 0.5% and 1% oligosaccharides extract, which could have been due to the low concentrations of the used supplementations. Thus, the growth and activity of beneficial bacteria could not have been promoted.

3.1.2 Effects of bioactive compounds on immunological parameters of broiler chicken

The weight of the spleen compared to live weight did not show significant differences among treatments (*Figure 1*). A similar result was reported by SHANG et al. (2015) who discussed the healthy immune status of broiler chickens when relative weights of chickens' spleen and Bursa of Fabricius did not differ significantly on day 21 when the diet was supplemented with fructooligosaccharide and chickens challenged with *Salmonella enteritidis* lipopolysaccharide.

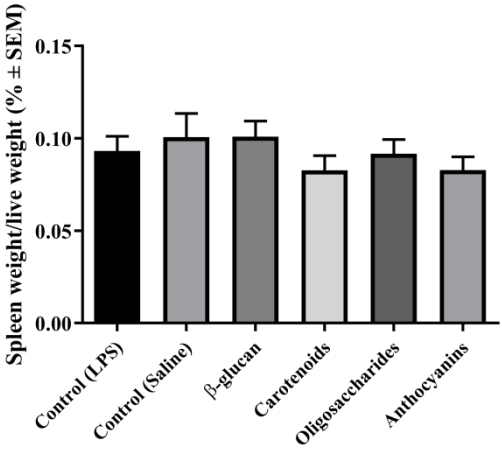
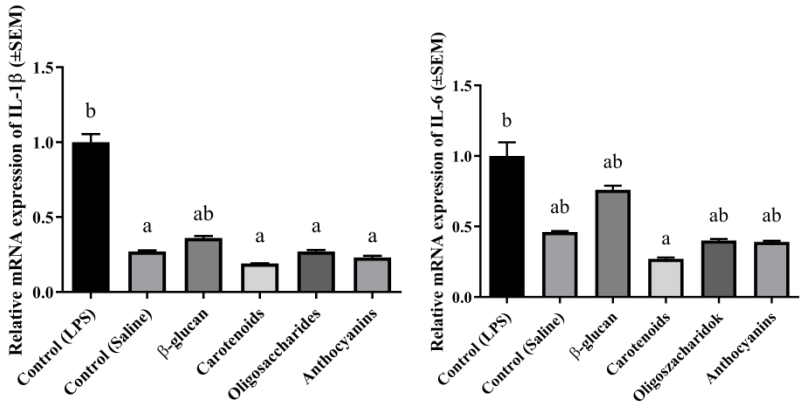


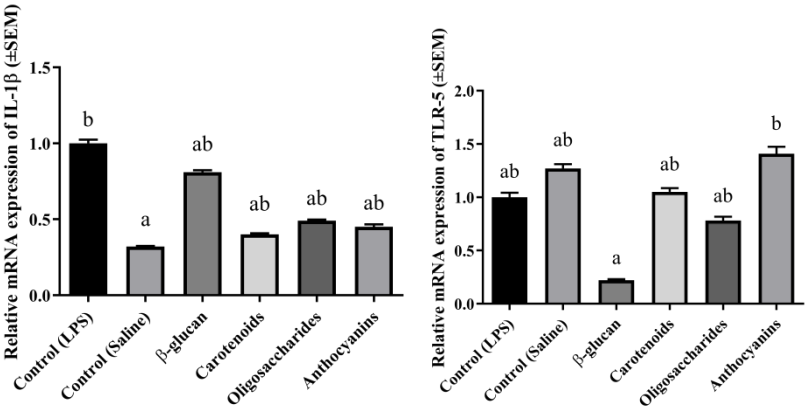
Figure 1. **Effects of bioactive compounds on spleen weight of broiler chicken**

The mRNA expressions of splenic cytokines (*IL-1β, IL-6*) in broiler chicken are shown in *Figure 2*. The β-glucan treatment did not affect the gene expression levels. Carotenoid treatment resulted in more than 80% lower (p = 0.0114) *IL-1β* gene expression in the spleen compared to the LPS-injected control group. Carotenoids reduced (p = 0.0325) *IL-6* gene expression in the spleen compared to the control (LPS) group and resulted in more than 70% lower mRNA levels of the mentioned cytokine. Splenic *IL-1β* gene expression was also lower by 70% (p = 0.0497) after oligosaccharide supplementation, but additional genes were not affected by the treatment. Anthocyanins reduced *IL-1β* (p = 0.0303) mRNA level in the spleen by 80%.



a, b: $p < 0.05$

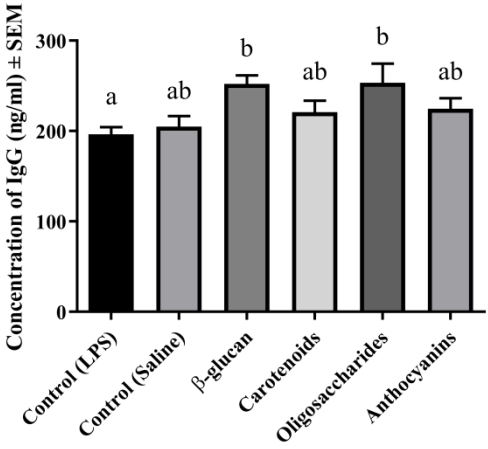
Figure 2. **Effects of bioactive compounds on splenic gene expression levels of chicken cytokines**



a, b: $p < 0.05$

Figure 3. **Effects of bioactive compounds on ileal gene expression levels of chicken cytokine and receptor**

The mRNA expressions of ileal cytokines and receptors of broiler chickens are shown in *Figure 3*. Lower *TLR-5* gene expression was measured in the β-glucan treatment (p = 0.0387) compared to anthocyanin treatment and *TLR-5* mRNA level decreased by 22%. In our study, β-glucan neither has any effect on chicken splenic or ileal cytokines and receptors (*IL-1β, IL-6*, *IFN-α, IFN-γ, TLR-4*, *TLR-5*) compared to LPS-injected control birds. SHEORAN et al. (2017) explained reduced *TLR-5* mRNA expression with the decreasing colonization of pathogens. The effect of carotenoid supplementation was also investigated on chicken immune cytokines. As we predicted, both splenic and ileal *IL-1β* gene expression levels were high in LPS-injected birds compared to saline-inoculated controls, whereby *Escherichia coli* LPS could induce an acute immune response and a bacterial illness (WU et al., 2017). MUNYAKA et al. (2012) found the same and reported higher *IL-1β* gene expression levels in the spleen of lipopolysaccharide-injected chickens compared to the saline-inoculated ones. In our study, carotenoids could inhibit splenic IL-1β gene expression levels compared to lipopolysaccharide-treated control birds. Similar to IL-1β, up-regulation of pro-inflammatory *IL-6* can be explained as an acute-phase reaction (HONG et al., 2006). This study showed a high expression of interleukin-6, and carotenoids could reach a lower gene expression level of the mentioned cytokine in the spleen. These results suggested carotenoids are useful in reducing the effect of inflammation by decreasing inflammatory parameters. The impact of oligosaccharides with high arabino-galactose content was also investigated in this study. Applied oligosaccharides could reach a low gene expression level of pro-inflammatory *IL-1β* in the spleen, which results showed oligosaccharides can be also effective in mitigating inflammation. It could not influence the mRNA expression level of *IL-1β* in the ileum. The effects of anthocyanins were also examined on the chicken immune response. Our research showed anthocyanins could reduce the amount of *IL-1β* mRNA in the chicken spleen. CHANGXING et al. (2018) also studied the effect of anthocyanins and described that anthocyanin supplementation could reduce cyclo-oxygenase-1 (COX-1) and cyclo-oxygenase (COX-2) inflammatory enzymes, which could inhibit the expressions of pro-inflammatory interleukins.



a, b: $p < 0.05$

Figure 4. **Effects of bioactive compounds on plasma IgG level of chicken**

The concentrations of plasma IgG levels are shown in *Figure 4*. Immunoglobulin G antibody is involved in humoral immunity. Broiler chicken plasma IgG levels were increased in the β-glucan (positive control) group (p = 0.0382) and in the oligosaccharide treatment (p = 0.0449), while additional treatments did not affect plasma IgG levels. CAI et al. (2012) also experienced that feed supplementation increased IgG levels in broilers, and the authors explained that by the improvement of the humoral immune response. Plasma IgG levels were also increased in 21-day-old chickens fed a diet supplemented with 2 mg/kg xylooligosaccharides and the authors discussed xylooligosaccharides could improve immune function (YUAN et al., 2018).

3.1.2 Effects of bioactive compounds on intestinal morphology of broiler chickens

Intestinal morphological measurements (*Table 2*.) were also carried out to define the changes in terminal ileum tissues which can refer to digestive functions. Increased villus heights and decreased depth of the crypt can provide a larger surface for digestion and absorption of nutrients (MUNYAKA et al., 2012). Higher villus height to crypt depth ratios can ensure more effective nutrient absorption as well (SONG et al., 2019).

In our study, β-glucan, carotenoid, oligosaccharide, and anthocyanin supplementations influenced positively the length of villus in terminal ileum segments which can point to a beneficial effect in absorption functions. Among treatments, oligosaccharides increased the depth of the crypt in the ileum. No other alterations were observed in crypt depth except in the ileum of saline-injected birds where shorter crypt depth was measured, in contrast to the intestinal segments of LPS-inoculated birds. Higher villus height to crypt depth ratios (VH:CD) were shown only in the β-glucan and anthocyanin treatment. Diets supplemented with β-glucan, oligosaccharides and anthocyanin thickened the mucosa. These findings indicate an increased absorption area in the ileum of treated birds.

Similar to our results, villus height, crypt depth, and total mucosa thickness were significantly higher when 0.5% fructooligosaccharide supplementation was applied in the diet of chickens challenged with *Escherichia coli* LPS (SHANG et al., 2015). XU et al. (2003) reported the same when 0.4% fructooligosaccharide supplementation resulted in higher villus length and VH:CD ratio in the ileum in broilers. SHANMUGASUNDARAM et al. (2013) also reported higher VH:CD ratios in chicken fed yeast cell wall supplemented diet.

Table 2.

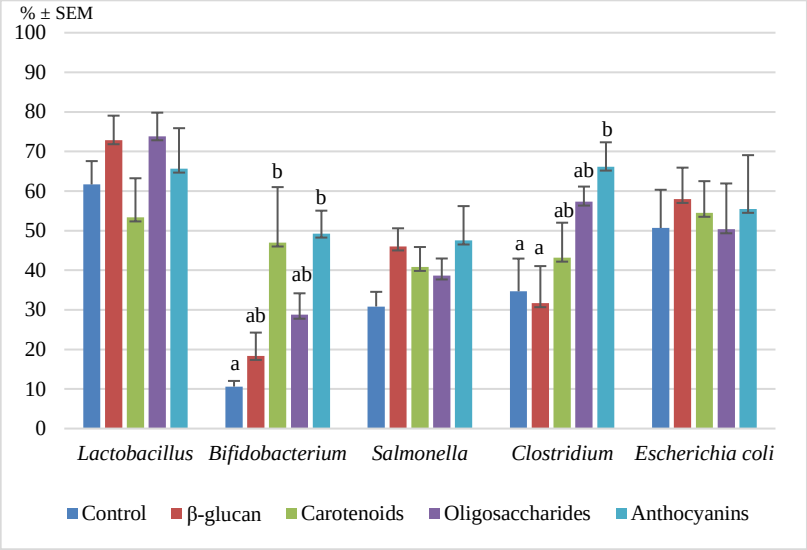
Effects of natural compounds on ileum morphology of broiler chickens

Ileum morphology	Treatments						*p*-value
	Control (LPS)	**Control (Saline)**	**β-glucan**	**Carotenoids**	**Oligosaccharides**	**Anthocyanins**	
Villus height (µm)	774,31±20,3^{a}	712,02±13,1^{a}	998,93±11,9^{b}	908,94±11,8^{b}	977,08±24,1^{b}	921,84±17,8^{b}	$p < 0,0001$
Crypt depth (µm)	140,38 ±5,9^{b,c}	107,31±2,9^{a}	120,43 ±3,6^{a,b}	160,27±7,5^{c}	179,90±6,2^{d}	134,47±3,9^{b}	$p < 0,0001$
Villus height: crypt depth ratio	5,83 ±0,2^{a,b}	6,81±0,2^{b,c}	8,57±0,2^{d}	6,19±0,3^{a,b,c}	5,70±0,2^{a}	7,12±0,2^{c}	$p < 0,0001$
Total mucosa thickness (µm)	1156,89±24,2^{a,c}	1137,47±19,2^{a}	1350,07±28,7^{b}	1251,06 ±14,2^{b,c}	1346,49±28,6^{b}	1286,38±20,5^{b}	$p < 0,0001$

a, b, c, d = Different letters in a row indicate significant differences.

3.1.3 Effects of bioactive compounds on gut microbiota composition of broiler chicken

Relative proportions of PCR fragments of *Lactobacillus*, *Bifidobacterium*, *Salmonella*, *Campylobacter*, and *Escherichia coli* bacterial groups contrasted to the total number of bacteria in the control group and the treatments are shown in *Figure 5*.

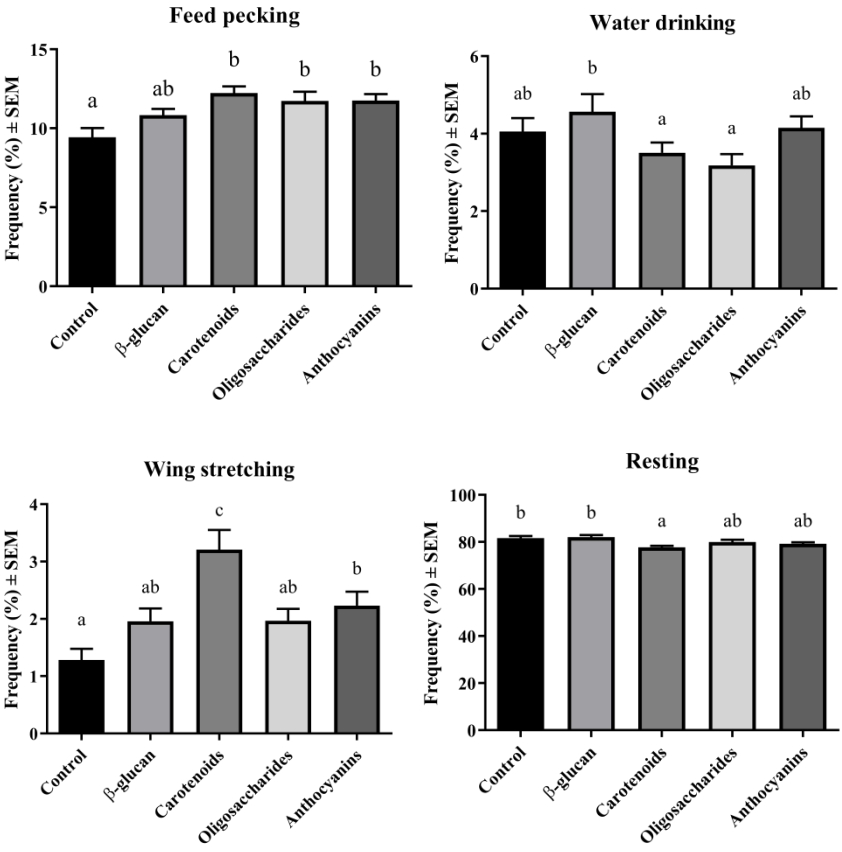


a, b: $p < 0.05$

Figure 5. **The relative proportion of PCR products of bacterial groups determined from the excreta of broiler chickens**

Among treatments, carotenoids and anthocyanins could increase the relative proportion of *Bifidobacterium* compared to the control group. *Bifidobacterium* is considered to be beneficial bacteria that enhance the growth and activity of other health-promoting bacteria (LUCCHINI et al., 1998; MIKKELSEN et al., 2003). Therefore, carotenoids and anthocyanins may enhance the growth of the *Bifidobacterium* genus in the gastrointestinal tract of broiler chickens. The relative proportion of the *Clostridium* genus was increased in the intestine of anthocyanin-fed birds contrasted to the control and β-glucan (positive control) treatment. *Clostridium* species, involving *C. perfringens*, are widely found in the environment and are a natural inhabitants of human and animal intestinal microbiota (BRANDT et al., 1999).

3.1.4 Effects of bioactive compounds on chicken behavior patterns



a, b: $p < 0.05$

Figure 6. **Results of the behavioral observation of broiler chickens**

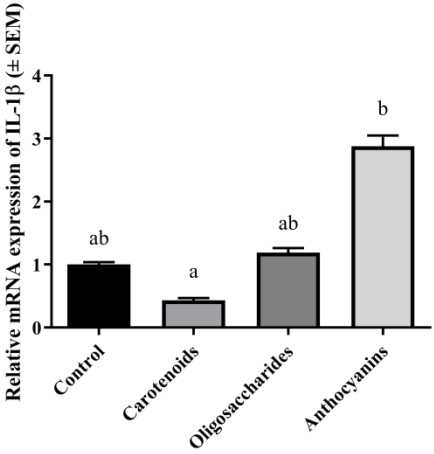
The frequency of feed pecking (*Figure 6.*) was increased by carotenoid ($p < 0.0001$), oligosaccharide ($p = 0.0012$) and anthocyanin ($p = 0.0011$) treatments. The frequency of water drinking occurred the most frequently in β-glucan supplementation (4.56%). Wing stretching indicating comfort feeling was observed in the highest proportion in carotenoid

supplementation. The treatment was followed by anthocyanin supplementation (p = 0.0387), where 2.2% of the observed birds stretched their wings. Most resting birds were in the control group (81.65%) and β-glucan treatment (82.06%), while the lowest resting birds (p = 0.0018) were in the carotenoid treatment group (77, 71%). Approximately 3.1-3.8% of the broilers were walking during the observations. The frequency of walking was affected by none of the bioactive compounds.

3.2 Results of the fish experiment

3.2.1 Effects of bioactive compounds on immunological parameters of common carp

Effects of the applied carotenoids, oligosaccharides, and anthocyanins at 1% in feed were determined on gene expression levels of carp cytokines. In this study, no significant differences were observed in gene expression levels of splenic and intestinal *IL-1β*, *IL-8*, *TNF-α*, and *IRF-1* when carotenoids were applied as feed additives compared to the control treatment. However, the gene expression level of intestinal IL-1β could decrease in carotenoid-fed carp compared to anthocyanin-fed ones (*Figure 7*.).



a, b: $p < 0.05$

Figure 7. **Effects of bioactive compounds on IL-1β gene expression level in the mid intestine of common carp**

LI et al. (2019) reported decreased gene expression levels of pro-inflammatory cytokines, such as *IL-1β* and *TNF-α*, when astaxanthin (carotenoid compound) was applied at 50, 100, or 200 mg/kg of body weight in snakehead (*Channa argus*) under LPS-induced inflammation. Consequently, the authors discussed astaxanthin reduced the inflammatory responses, since inhibition of the inflammatory cytokines was observed.

In our study, oligosaccharides could not alter the relative mRNA levels of examined cytokines. YOUSEFI et al. (2018) investigated the effect of galactooligosaccharides at 0.5, 1, and 2% in feed on innate immune parameters in zebrafish (*Danio rerio*). Similar to our results, the authors found no significant differences in *IL-1β* mRNA level when galactooligosaccharides were applied at 1% and 2% in feed, and the gene expression level of *TNF-α* was not altered when galactooligosaccharides were used at 2%, either. In contrast, the authors defined significantly decreased *IL-1β* gene expression levels when treatment involved galactooligosaccharides at 0.5%. Also, significantly increased *TNF-α* gene expression levels were identified in treatments that involved galactooligosaccharides at 0.5 and 1%. The authors explained that prebiotics may impact the immune parameters and immune-related gene expression and suggest a possible immunomodulatory effect of galactooligosaccharides at a molecular level (YOUSEFI et al., 2018). In contrast, the other immune-related gene expression levels, such as *IL-1β* and *TNF-α* were increased in the spleen when blackberry syrup was used at 7.5 g/kg and discussed as blackberry syrup could produce more innate components and improved the immune parameters (YILMAZ, 2019).

3.2.2 Effects of bioactive compounds on intestinal morphology of common carp

Increased villus height and width provide a larger surface for efficient nutrient absorption (HEIDARIEH et al., 2018). Several compounds, such as oligosaccharides can enhance mucus secretion by enterocytes, which can improve digest's viscosity (TORRECILLAS et al., 2011). Improvement in digest viscosity can stimulate the intestinal mucus layer development (YUJI-SADO et al., 2015).

Anthocyanin-fed carp had lower villus height in the mid intestine compared to the control group, carotenoid and oligosaccharide supplementations (*Table 3*.).Villus width was also the highest in the control group, while other treatments decreased the mentioned morphological parameter.

ZHOU et al. (2010) reported the same and fold height was not affected by fructooligosaccharides, galactooligosaccharides, mannan oligosaccharides, and galacto-gluco-mannans at 10 g/kg in feed through 8 weeks in the proximal, middle or distal intestine of red drum (*Sciaenops ocellatus*).

In contrast, YUJI-SADO et al. (2015) measured higher intestinal fold height on experimental day 30, when mannan oligosaccharides were applied at 0.2 and 0.4% in feed in the proximal intestine of nile tilapia (*Oreochromis niloticus*).

In conclusion, nutrient absorption can be the most effective in the control group, while smaller villus height, villus width, and intestinal muscular layer are not beneficial for appropriate digestion of the supplemented groups.

Table 3.

Effects of bioactive compounds on carp intestinal morphology

Intestinal morphology	**Treatment**				***p*-value**
	Cont.	**Carot.**	**Oligos.**	**Anth.**	
Villus height (µm)	360,74±2,52 ^{b}	372,88±4,32 ^{b}	379,93±3,66 ^{b}	338,20±3,13 ^{a}	$p < 0,0001$
Villus width (µm)	44,62±0,88 ^{b}	38,62±0,54 ^{a}	40,74±0,78 ^{a}	40,80±0,63 ^{a}	$p < 0,0001$
Muscular thickness (µm)	47,96±0,42 ^{b}	45,52±0,69 ^{ab}	48,18±0,51 ^{b}	44,91±1,01 ^{a}	$p = 0,0048$

a, b = Different letters in a row indicate significant differences. Cont. = control group; Carot. = carotenoid treatment; Oligos. = oligosaccharide treatment; Anth. = antochyanin treatment.

4. New Scientific Results

1. The applied carotenoids, oligosaccharides, and anthocyanins could partially affect the growth performance of broiler chickens. None of the compounds impacted the body weight and average daily gain of chickens. β-glucan (83 g/day/bird), oligosaccharides (82 g/day/bird), and anthocyanins (88 g/day/bird) increased average daily feed intake during the whole experimental period (1-42 days).

2. Carotenoids, oligosaccharides, and anthocyanins used in this research may be potential immunomodulators of inflammatory response in broiler chickens. Each compound could decrease the mRNA levels of pro-inflammatory *IL-1β*. Carotenoids resulted in 80%, oligosaccharides about 70%, and anthocyanins almost 80% lower *IL-1β* gene expression. Carotenoids also reduced the pro-inflammatory *IL-6* gene expression levels by more than 80%.

3. The applied β-glucan and oligosaccharides can improve the humoral immune response of broiler chickens since these compounds increased plasma IgG levels to 252,1 pg/ml and 253,5 pg/ml contrasted to the control (196,4 pg/ml).

4. β-glucan supplementation influenced positively the heights of villus in ileum segments by 22%, carotenoids by about 14%, oligosaccharides by 20%, and anthocyanins by 16%, which can point to a beneficial effect on absorption functions. Higher villus height to crypt depth ratios were shown in the β-glucan (8,57) and anthocyanin (7,12) treatment compared to control (5,83); diets supplemented with β-glucan, oligosaccharide and anthocyanin thickened the mucosa by 14, 14, and 11%, respectively. These findings indicate an increased absorption area in the ileum of the treated birds.

5. Carotenoids and anthocyanins may influence the gut microbiota composition beneficially since these compounds increased the relative proportion of *Bifidobacterium* by almost 40%.

6. Bioactive compounds influenced the behavioral patterns of broiler chickens. Carotenoids, oligosaccharides, and anthocyanins enhanced feed pecking, and carotenoids and anthocyanins further increased the frequency of wing stretching indicating comfort feeling.

7. The potential immunomodulating effect of carotenoid, oligosaccharide, and anthocyanin treatment was not observed in carp and did not affect intestinal morphology, either.

5. Applicability of the results

Based on the results of the thesis, the beneficial effects of each bioactive substance on production can be stated:

1. *β-glucan* can be useful for increasing the ileal absorption surface. It can be a potential immunomodulator of humoral immunity as well.

2. The applied *carotenoids* are being suggested to decrease inflammation through an acute phase response and to affect nutrient absorption positively. By increasing the *Bifidobacterium* genus in the gastrointestinal tract of broiler chickens, it can maintain the microbial balance.

3. The applied *oligosaccharides with high arabino-galactose content* (prebiotics) are suggested to use to alleviate inflammatory parameters, therefore improve cellular immunity, enhance humoral immune response, and affect morphometric factors, such as villus height and mucosa thickness beneficially.

4. The anti-inflammatory effect of the applied *anthocyanins* is also proved by decreasing inflammation and effective absorption functions are also indicated. Anthocyanins may increase the relative proportion of useful microorganisms (*Bifidobacterium*) of chicken intestinal microbiota.

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7. List of publications

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List of publications related to the dissertation

Foreign language scientific articles in Hungarian journals (2)

1. **Csernus, B.**, Biró, S., Babinszky, L., Stündl, L., Gálné Remenyik, J., Pesti-Asbóth, G., Oláh, J., Czeglédi, L.: The effect of β-glucan, carotenoids, oligosaccharides and anthocyanins on bacteria groups of excreta in broiler chickens.
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3. **Csernus, B.**, Biró, S., Babinszky, L., Komlósi, I., Jávor, A., Stündl, L., Gálné Remenyik, J., Bai, P., Oláh, J., Pesti-Asbóth, G., Czeglédi, L.: Effect of Carotenoids, Oligosaccharides and Anthocyanins on Growth Performance, Immunological Parameters and Intestinal Morphology in Broiler Chickens Challenged with Escherichia coli Lipopolysaccharide.
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