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The effect of β-glucan, carotenoids, oligosaccharides and anthocyanins on bacteria groups of excreta in broiler chickens

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SUMMARY

This study was conducted to examine the effect of natural compounds, such as β-glucan, carotenoids, oligosaccharides, and anthocyanins in the diet on bacteria gropus of excreta in Ross 308 broiler chickens. Chickens were fed 5 diets: control (basal) diet, a diet supplemented by β-glucan at 0.05%, and diets supplemented by carotenoids, oligosaccharides, or anthocyanins at 0.5% of each compound. On experimental day 19, excreta were collected to determine the proportion of Lactobacillus, Bifidobacterium, Campylobacter, Clostridium, and Escherichia coli. Samples were collected aseptically and snap-frozen in liquid nitrogen. Bacterial DNA was isolated from samples, then polymerase chain reaction using primer pairs designed to the 16S rDNA of bacterial groups were applied to define the proportion of the mentioned bacteria. Another universal primer pair was used to amplify a region of 16S rDNA of all the examined bacteria. Proportion of each bacterial groups was determined relatively to the intensity of universal PCR product band by gel documenting system and ImageLab software. Based on the results, carotenoids and anthocyanins increased the proportion of Bifidobacterium, which might imply the beneficial effects of the mentioned compounds on the bacteria composition of excreta.

Keywords: *natural compounds, chicken, microbiota, PCR*

INTRODUCTION

Nowadays, natural agents receive great attention in livestock production, since they contain immunostimulating compounds (Siddiqui and Moghadasian, 2020). Many probiotics, prebiotics, and synbiotics have been proved to retain health and rebalance the dysbiotic intestinal microbiota (Pandey et al., 2015). Other potential natural compounds can be also applied to alter the gut microbiota beneficially. β-carotene can affect the cecal microbiota by decreasing the levels *of Escherichia coli* and increasing the levels of *Lactobacillus* (Hui et al., 2020). Anthocyanin-rich berry pomace extracts could result in increased population of beneficial bacteria, such as *Lactobacillus* and *Enterococcus* in the cecum (Das et al., 2020). The endogen intestinal microbiota involves microorganisms living in the gastrointestinal tract, which composition and function play a major role in maintaining the health status of broiler chicken. The microbiome affects several physiological activities, such as nutrition, metabolism, and immunity (Stanley et al., 2013; Mancabelli et al., 2016). It implies hundreds of bacterial species (Clavijo and Flórez, 2018). Among them, *Lactobacillus* and *Bifidobacterium* are probiotic bacteria and they enhance the growth and activity of other beneficial bacteria (Lucchini et al., 1998; Mikkelsen et al., 2003). *Lactobacillus* produce antibiotics and organic acids, which can decrease the pH value of the intestine, through they inhibit the activity of pathogens (Huyghebaert et al., 2011; Gibson and Roberfroid, 1995). *Bifidobacterium* genus has a major role in maintaining the microbial balance, influencing immune response, and also preventing the activity of harmful bacteria (Rossi and Amaretti, 2010). Species of *Clostridium*, including *Clostridium perfringens*, are natural residents of the human and animal gut and are widely found in nature (Brandt et al., 1999). *Escherichia coli* is a pathogenic bacteria habiting the intestine, which can cause enteric infections for animals and humans as well (Dozois et al., 2003). The chicken intestine involves other pathogens, such as *Campylobacter*, which is a human enteropathogen originating from the chicken intestinal microbiota and causes acute bacterial infection (Moreno et al., 2001). The intestinal microbial composition takes an important part in immune homeostasis. Consequently, the immunological imbalance can be often attributed to the intestinal imbalance, which also affects the health status (Diaz-Carrasco et al., 2019). The gut microbial population behaves like a physical barrier and prohibits the adherence and colonization of pathogens and the production of toxic metabolites. Thus, the innate immune cells secrete cytokines, such as interleukin (IL)-1β, IL-6, and chemokines, etc. (Keestra et al., 2013). The interrelationship between the microbiota and innate immunity also results in the development of

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acquired immune response (Pan and Yu, 2014). In the case of infection of the gastrointestinal tract, host immune system reacts through a complex interconnecting system of pathways involving the innate and adaptive immunity (Diaz-Carrasco et al., 2019).

Since the composition of intestinal microbiota is strongly correlated to the immune status promotes the production of food animals and due to the relatively few studies, this research aimed to investigate the effects of some bioactive compounds on selected bacteria groups of chicken excreta. In this study, the applied bioactive agents were extracted from by-products of fruit and vegetable industry, for those which processing are expensive and unresolved. Accordingly, carotenoids, oligosaccharides extracted from red sweet pepper and anthocyanins originated from sour cherry were used to examine their effect on the proportion of *Lactobacillus*, *Bifidobacterium*, *Clostridium*, *Campylobacter*, and *Escherichia coli* of chicken excreta. Therefore, samples were collected, then bacterial DNA isolation, polymerase chain reaction (PCR) and agarose gel-electrophoresis were carried out to define the proportion of the mentioned bacteria groups.

MATERIALS AND METHODS

Animal ethics

The experiments were confirmed by the University of Debrecen Committee of Animal Welfare, Hungary (Permit number: DEMAB/12-7/2015).

Preparation of extracts

β-glucan was commercially available and originated from *Saccharomyces cerevisiae*. Carotenoids were extracted from Hungarian red sweet pepper powder. Extraction was carried out with HPLC as previously described (Nagy et al., 2017). Major carotenoid compounds were determined by Diode Array Detector detection on 460 nm and 350 nm. Based on the HPLC profile, carotenoid compounds with the greatest areas were the following: capsanthin, cis-capsanthin, β-carotene, zeaxanthin (Csernus et al., 2020).

Oligosaccharides with high arabino-galactose content were extracted from Hungarian red sweet pepper retained from industrial food waste. HP 5890 Gas chromatograph with SP 2380 capillary column and Flame Ionization Detector were applied for detection of the monomer units of oligosaccharides as glucose, arabinose, xylose, galactose, mannose were identified (Csernus et al., 2020).

Anthocyanins were extracted from Hungarian sour cherry using a VWR-Hitachi ChromasterUltraRs UHPLC with a Phenomenex Kinetex® column as published earlier (Nemes et al., 2018). The main anthocyanin compounds were cyanidin-3-O-glucosyl-rutinoside, cyanidin-3-O-rutinoside, and cyanidin-3-O-monoglucoside which were determined according to Homoki et al. (2016).

Birds and experimental diet

A total of 900, 1-day-old mixed-sex broiler chickens (Ross 308) were used in a 19-day feeding trial. Chickens were allocated to 5 treatments in 3 floor pens (replicates) of 60 broilers per pen covered with wood shavings in a thermostatically controlled house at a stocking density of 650 cm^2/bird. Diets were based on corn, wheat, and soybean meal. The 5 dietary treatments were: basal diet (control group) (*Table 1*), a basal diet supplemented with 0.05% β-glucan, a basal diet supplemented with 0.5% carotenoids, a basal diet supplemented with 0.5% oligosaccharides, and a basal diet supplemented with 0.5% anthocyanins. The basal diets (pre-starter, starter) were fed in mashed form. Feed and clean drinking water were available ad libitum throughout the entire feeding trial. The chickens were exposed to light according to Olanrewaju et al. (2006).

Table 1: **Composition and nutrient level of the basal diets**

Basal Ingredients	Value	
	Pre-starter (Day 1–9)	Starter (Day 10–19)
Corn, %	33	34
Wheat, %	27	29
Soybean meal, solvent extracted (46.0% CP), %	29	24
Soybean meal, extruded (46.0% CP), %	4	6
Sunflower meal, extracted, %		1
Feed yeast, %	1	
DDGS, %		1
Plant fats, %	2	1
Premix, %	4	4
Total, %	100	100
Nutrient Level		
Dry matter, %	89.06	89.03
AME_n poultry, MJ kg^{-1}	12.23	12.47
Crude protein, %	21.58	20.28
Crude fat, %	4.61	4.83
Crude fiber, %	3.37	3.51
Lysine, %	1.37	1.27
Methionine, %	0.57	0.54
Methionine + Cysteine, %	0.94	0.9
Calcium, %	0.85	0.73
Phosphorus, %	0.63	0.55

CP: crude protein

DDGS: Distillers Dried Grains with Solubles

AMEn poultry: apparent metabolizable energy corrected for nitrogen

Sample collection, bacterial DNA isolation and PCR

On experimental day 19, excreta were collected aseptically and snap-frozen in liquid nitrogen and stored at -80 °C for later determination of the proportion of bacterial groups. Determination of the proportions of the bacterial composition of excreta was started with bacterial DNA isolation performed by E.Z.N.A.® Stool DNA Kit (Omega Bio-tek, Inc.,

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Norcross, USA) following the manufacturer's protocol. The purity and concentration of each sample were measured by NanoDrop ND-1000 spectrophotometer. Primers were designed for the 16S rRNA region of *Lactobacillus*, *Bifidobacterium*, *Campylobacter*, *Clostridium*, and *Escherichia coli* (*Table 2*). A universal primer pair was designed using the invariant region in the 16S rDNA of bacteria to determine the total microbiota population (Amit-Romach et al., 2004). Target sequences of the bacterial groups were amplified by polymerase chain reaction using PCRmax Alpha Cycler (PCRmax, Staffordshire, UK). Each reaction included 10 ng DNA template, 0.5U DreamTaq polymerase, 100 nM of each primer, 10x DreamTaq Green Buffer, 10 nM dNTP, 25 nM $MgCl_2$ and distilled water in 20µl final volume. PCR conditions consisted of polymerase activation at 95 °C for 1 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 1 min, and a final elongation step at 72 °C for 5 min.

Table 2: **Primer sequences of the examined bacterial groups**

Bacterial group	Primer sequence (5'→3')	Reference	Product length (bp)
Universal bacteria	F: CGTGCCAGCCGCGGTAATACG R: GGGTTGCGCTCGTTGCGGGACTTAACCCAACAT	Amit-Romach et al. (2004)	611
Lactobacillus	F: CATCCAGTGCAAACCTAAGAG R: GATCCGCTTGCCTTCGCA	Wang et al. (1996)	286
Bifidobacterium	F: GGGTGGTAATGCCGGATG R: CCACCGTTACACCGGGAA	Langendijk et al. (1995)	510
Campylobacter	F: ATCTAATGGCTTAACCATTAAAC R: GGACGGTAACTAGTTTAGTATT	Denis et al. (2001)	857
Clostridium	F: AAAGGAAGATTAATACCGCATAA R: ATCTTGCGACCGTACTCCCC	Amit-Romach et al. (2004)	722
Escherichia coli	F: GACCTCGGTTTAGTTCACAGA R: CACACGCTGACGCTGACCA	Candrian et al. (1991), Wang et al. (1996)	585

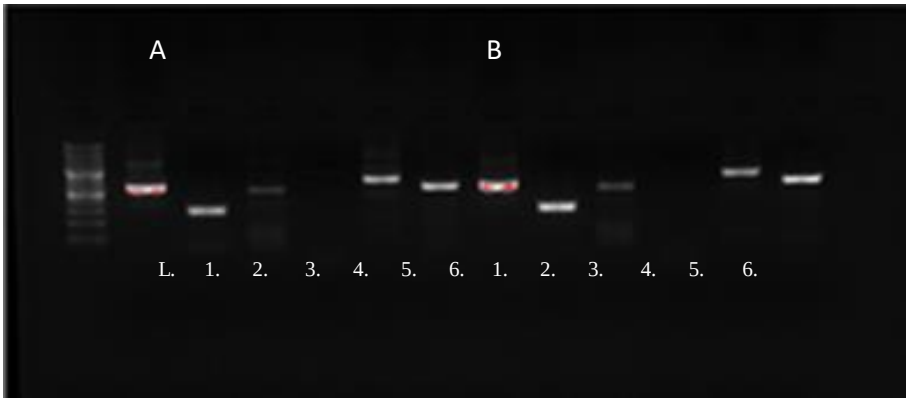
Determination of bacterial proportion

PCR products were visualized by 2% agarose gel electrophoresis stained with ethidium bromide and are shown in *Figure 1*. Agarose gel bands were analyzed by Bio-Rad Gel Doc XR+ gel document system (Bio-Rad Laboratories, California, USA) using the „relative quantity" option of ImageLab software. The evaluation of the PCR products was normalized to the intensity of the band of the total bacteria. To define the proportion of each bacterium, all of the PCR products were expressed relative to the intensity of the universal PCR band that was set at 100% (Amit-Romach et al., 2004).

Statistical analysis

The main effects of the bioactive compounds on excreta microbiota were analyzed using one-way analysis of variance Tukey-test by GraphPad Prism 7.0.1 software. Differences among treatments were considered significant at $p<0.05$.

Figure 1: **PCR products of the examined bacterial groups**



PCR products from 2 different samples (A and B) in a 2% agarose gel for all of the examined bacterial groups (bands from 2 to 7) and the universal PCR products (band 1). L = DNA ladder (100 bp). 1 = Universal bacteria (611 bp), 2 = *Lactobacillus* (286 bp), 3 = *Bifidobacterium* (510 bp), 4 = *Campylobacter*, 5 = *Clostridium* (722 bp), 6 = *Escherichia coli* (585 bp).

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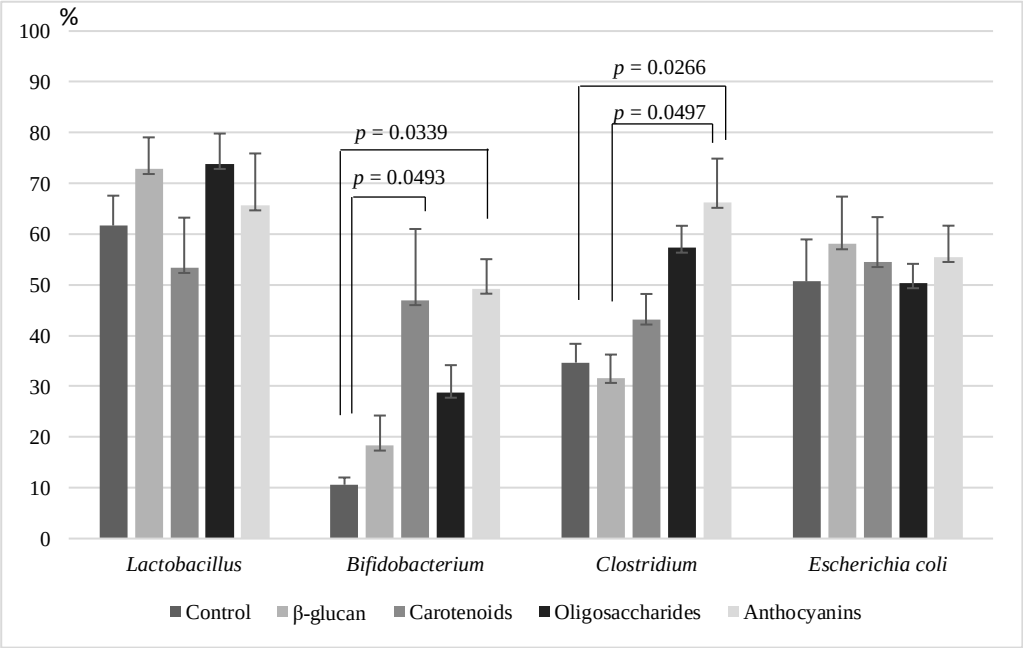
RESULTS AND DISCUSSION

The proportions of the PCR products of bacterial groups compared to the total number of bacteria are shown in *Figure 2*. At 19 days of age, the proportion of *Lactobacillus* in the control group reached 61.7%, in β-glucan treatment reached 72.8%, 53.3% was in carotenoid treatment, 73.8% was in the oligosaccharide treatment, and 65.6% was in the anthocyanin treatment. The proportion of *Bifidobacterium* in the control group was 10.6% compared to the total bacteria, in the β-glucan supplementation was 18.3%, in the carotenoid supplementation was 47%, in the oligosaccharide supplementation was 28.8%, and in the anthocyanin treatment was 49.3%. *Campylobacter* was not detected in our samples. The proportion of *Clostridium* in the control group was 34.6%, 31.6% for β-glucan supplementation, 43.2% for carotenoid supplementation, 57.3% for the oligosaccharide supplementation, 66.2% for the anthocyanin supplementation. The proportion of *Escherichia coli* in the control group was 50.6%, 58% in β-glucan-treated birds, 54.5% in carotenoid-treated chickens; 50.3% in oligosaccharide-treatment, 55.5% in anthocyanin-treated chickens compared to the total bacteria.

Results showed that carotenoids ($p = 0.0493$) and anthocyanins ($p = 0.0339$) could increase the proportion of *Bifidobacterium* compared to the control birds, and the proportion of *Clostridium* was higher in anthocyanin-fed chickens compared to the control ($p = 0.0497$) and β-glucan ($p = 0.0266$) groups.

Figure 2: **The proportions of bacterial groups in chicken excreta at 19 days of age**



The evaluation of PCR products was normalized to the intensity of the PCR product of universal primers by ImageLab software. Columns represent mean values + standard errors of the mean. Differences among treatment groups were considered significant at $p < 0.05$.

In this study, proportions of bacterial groups of the chicken excreta were examined. Results of the present study showed that the proportion of *Bifidobacterium* compared to the total bacteria was increased in carotenoid ($p = 0.0493$) and anthocyanin ($p = 0.0339$) treatment compared to the control group. *Bifidobacterium* is a common probiotic bacterium and enhances the activity of other useful bacteria, so carotenoids and anthocyanins may have positive effects on the intestinal microbial population. Carotenoid sources, such as β-carotene can support the immune system due to the beneficial effect of IgA, which also alters the microbial community (Reikvam et al., 2012; Rogier et al., 2014). The IgA response is in connenction with microbiota composition. Due to the deficiency of IgA, *Gammaproteobacteria* was increased, while *Lactobacillus* and *Bifidobacterium* were decreased in the intestinal microbiota of mice (Hui et al., 2020). Similarly, Das et al. (2020) reported an increased cecal and cloacal population of beneficial bacteria, such as *Lactobacillus* and *Enterococcus* by berry pomace extracts (contain a high level of flavonoids and anthocyanins) at 1 and 2% in the feed of broiler chickens. The mentioned bacteria have high β-glucosidase activity and metabolize anthocyanins into phenolic metabolites like p-couramic acid and benzoic acid (Petersen et al., 2019). Polyphenols from berry pomaces can also act as prebiotics and promote the growth of the beneficial bacteria, which ferment polyphenols into lactate that can be absorbed in the cecum providing energy (Xia et al., 2019). Finally, carbohydrates from berry pomaces can also enhance the

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growth of beneficial bacteria, which catabolize glycan and secrete acetate, lactate, formate, and butyrate (Ozcan et al., 2017). β-glucan and oligosaccharide supplementations did not affect the proportion of the bacterial groups. The proportion of *Clostridium* was increased in anthocyanin-treated chickens compared to the control ($p = 0.0497$) or β-glucan ($p = 0.0266$) treatment. *Clostridium* is one of the most dominant genus in broiler chickens (Luo et al., 2013). It is found in all livestock feces, but chicken fecal samples contain higher relative abundance of the genus (Kim et al., 2021). *Clostridium* involves pathogenic bacteria, such as *C. perfringens* and *C. difficile*, but the whole genus was examined in this study. Thus, presence of pathogens in a healthy gastrointestinal tract at detectable level does not show important changes in microbiota structure or diversity (Skraban et al., 2013; Stanley et al., 2014).

In our study, oligosaccharides did not impact the proportion of *Clostridium*. None of the applied carotenoids, oligosaccharides, and anthocyanins affected the proportions of *Lactobacillus*, *Campylobacter*, and *Escherichia coli*. Similarly, Rezaei et al. (2015) reported no alterations in the chicken cecal microbial population, when oligosaccharides extracted from palm kernel expeller did not change the number of logarithmic copies of *Bifidobacterium*, *Lactobacillus*, *Campylobacter*, and *Escherichia coli* on experimental day 21 and 35. Li et al. (2016) also discussed no alterations in the colonies of *Escherichia coli* in the cecal content of 21 and 42 day-old-chickens, when yeast cell wall powder was applied at 1 g kg^{-1} in feed. In contrast, Muthusamy et al. (2011) found that dietary mannan-oligosaccharides decrease the *Escherichia coli* number in the small intestine (duodenum, jejunum, ileum) in broiler chickens under *Salmonella* challenge or with poor health status.

CONCLUSIONS

In conclusion, carotenoids, and anthocyanins at the applied concentration could affect the microbial composition of chicken excreta beneficially, as the proportion of *Bifidobacterium* was increased in the feed supplemented groups. The proportion of *Clostridium*, which is one of the most dominant genus in chicken was also higher in athocyanin-treated chickens compared to control or β-glucan.

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