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The effect of coffee husks used as pellet bedding material on the intestinal barrier, immune-related gene expression and microbiota composition in the broiler chicken caecum

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Abstract

Introduction: Using coffee husks as waste material for bedding contributes to sustainable development. A sustainable choice of bedding has also, however, to be a safe choice for poultry. The study analysed immune-related gene expression in the intestinal mucosa and indicator bacteria in caecal content collected from broiler chickens bedded on material with coffee husk addition. **Material and Methods:** One-day-old Ross 308 chickens were divided into four groups of 10 birds each in five replicates: C, the control group kept on wheat straw bedding; CH10, a group kept on bedding of 10% coffee husks and 90% wheat straw; CH25, a group kept on bedding of 25% husks and 75% straw; and CH50, a group kept on bedding of 50% husks and 50% straw. After 42 days, the birds were slaughtered, the caecal mucosae were removed for RNA isolation and the caecal content was collected for bacterial DNA isolation. The expression of genes involved in intestinal immune response and host organism defence and the relative abundance of indicator bacteria were analysed. **Results:** Upregulation of the expression of genes related to the immune response and intestinal tightness was correlated with an increase in the percentage of coffee husks in the pellet. Coffee husk pellets at 50% bedding content caused a significant numerical increase in *Bifidobacterium* and a statistically significant increase in *Lactobacillus*. A significant reduction in *E. coli* bacteria was also demonstrated in this group. Coffee husk pellets at all content percentages resulted in a statistically significant diminution of the level of *Streptococcus* bacteria. **Conclusion:** The addition of coffee husks to poultry litter effects beneficial changes in the expression of genes related to intestinal health and the caecal bacterial profile.

Keywords: coffee waste, litter, poultry, caecum, one health.

Introduction

Safe broiler chicken production is the basis for good quality meat. Nowadays, producers and consumers are increasingly aware that the health of birds bears heavily on the quality of their meat. Many factors influence poultry growth performance, the meat's quality and the birds' resistance to diseases. Flock management should extend to housing, feeding and routine production activities, and the crucial nature of bedding and litter management in broiler production means that they cannot be overlooked. Poor litter hygiene, whether because the litter is wet or made of inappropriate material, may pose a risk to broiler health (24). Bedding material may vary depending on its availability and the region where the production occurs. The most commonly used materials are wood shavings, sawdust, bark, rice hulls, peanut and nut hulls, straw, shredded paper, peat and sand (11). There are many options for alternative bedding materials that are often waste products from other industries, coffee husks that come from coffee bean roasting being one such material. Their utilisation would improve waste management and align with the trend to find ecological solutions. Coffee husks can be used as a fertiliser, so it is concluded that the manure of chickens bedded on these husks may be a good plant fertiliser (4, 14). As described by Hidayat *et al.* (14), coffee husks contain many nutrients, including nitrogen (0.41%), phosphorus (0.29%) and potassium (1.47%). Its weakness is the content of caffeine, tannins and polyphenols. Coffee husks in litter have been proved to benefit poultry specifically, by reducing the incidence of footpad dermatitis (1).

Chicken manure is a mixture of bedding material and enteric bacteria excreted by chickens. Bedding management and the material used in its production may

affect the intestinal health of broilers (30), because chicks start to consume small fractions of litter from the first day; therefore, they colonise their digestive tract with bacteria found in the litter. The intestinal microbiota of the chicken becomes diversified and complex during rearing, influenced by many successions. The gut microbiota depends directly on the litter, which shapes the developing immune system and is critical for maintaining homeostasis (16). Researchers have shown that the type of litter influenced the host's immune response to pathogens and cytokine mRNA expression; for example, a study by Torok *et al.* (28) showed that birds kept on softwood sawdust had different communities of caecal microbes to those of birds kept on cut wheat litter. The available literature contained comparisons of fresh and used litter and showed that in chickens kept on fresh litter, the presence of *Lactobacillus* spp. in the ileum was dominant (6). Using waste from coffee production can confer sustainability to production of poultry meat, also by enriching the manure with *Lactobacillus* spp. and other bacteria to a sufficient extent to render it valuable as fertiliser. At the same time, the essence of high-standard broiler chicken production is prioritising their robust immune health, which obligates the selection of litter material which supports this.

This study aimed to analyse the effect of different proportions of coffee husks in the bedding material on the intestinal health of broiler chickens by investigating the expression of genes related to intestinal tightness, the birds' defence against pathogens and their intestinal immune response, and by quantifying the relative abundance of indicator bacteria.

Material and Methods

Animals and experimental design. Approval of the experiment by the Institutional Animal Care and Use Committee was obtained (No. 2/2022). In the study, Ross 308 broiler chickens were kept for 42 days on the premises of a small-scale farm in a poultry house. The birds were divided into four equal groups of 50, and each group was split into five replicates of 10 chickens. Each 10-chicken replicate was kept in a separate pen of 1.5 m^2 area, made of stainless steel. The chickens were kept at 32°C from day 1, and then the temperature decreased to 20°C. Relative humidity was at the level of 60–65%. The light cycle was continuous for the first three days (24 h of light), and then was modified to 18 h of light and 6 h of darkness per day. In the last three days before slaughter, the lighting duration increased to 23 h. There were bell-shaped drinkers and feeders in each pen, which were placed on the walls. Chickens had access to fresh water and complete feed, which was given appropriately for the three feeding phases. From the 1st to the 14th day, starter feed was given; from the 15th to the 35th day grower feed was supplied; and from the 36th to the 42nd day finisher feed was offered (2).

The day before the start of the experiment, the bedding was prepared. Wheat-straw pellet bedding was prepared for the control group (C). The pellet bedding was prepared in the experimental groups based on wheat straw and coffee husks with different proportions: the CH10 group was provided bedding with a 10% proportion of husks, the CH25 group had a 25% proportion and the CH50 group 50%. The coffee husks were obtained from a local coffee roasting plant in Bydgoszcz in the Kuyavian-Pomeranian Voivodeship (Poland). On the 42nd day of rearing, an 8-h ante-mortem fast was imposed. Ten birds from each group (two birds from each replicate) were randomly selected and slaughtered by decapitation (between the first cervical vertebra and the occipital condyle). Before slaughter, the chickens were stunned following the standard procedures and rules for killing birds up to 5 kg body weight.

The caecum (n = 8 per group) was cut lengthwise, washed in phosphate-buffered saline and preserved in a stabilising buffer (fixRNA; EURx, Gdańsk, Poland). The content of the caecum (n = 8 per group) was collected in a 5 mL Eppendorf tube and then frozen on dry ice. The sample was transported to the laboratory for further analysis.

RNA and bacterial DNA isolation. Using a TissueRuptor homogeniser (Qiagen, Hilden, Germany), each tissue was homogenised in 1 mL of Extracol (EURx). Chloroform (200 µL) was added to the homogenate and the mixture was shaken and centrifuged at 12,000 rpm for 15 min, after which the aqueous phase with the isolated RNA was collected. The RNA was purified using a commercial Universal RNA Purification Kit (EURx), following the manufacturer's instructions. Ribonucleic acid was eluted in nuclease-free water. Qualitative and quantitative evaluation of RNA was performed using 2% agarose gel electrophoresis and a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA). The RNA was stored at −20°C as per the isolation kit manufacturer's recommendations. Bacterial DNA was extracted from caecal content (approximately 150 mg per sample) using a GeneMATRIX Stool DNA Purification kit (EURx) according to the manufacturer's instructions. The extracted DNA was subjected to qualitative assessment by agarose gel electrophoresis and quantitative assessment with the Nanodrop2000 spectrophotometer.

Relative gene expression in caecal mucosa. Gene expression analysis was performed following the method described by Dunisławska *et al.* (8). The isolated RNA was reverse transcribed into cDNA following the manufacturer's protocol (Maxima First Strand cDNA Synthesis Kit for RT-qPCR; Thermo Scientific, Vilnius, Lithuania). The qPCR reaction was performed using the following reaction mixture: Maxima SYBR Green qPCR Master Mix (Thermo Scientific), 140 ng of cDNA, 1 µM of forward primer and 1 µM of reverse primer. The primer sequences were taken from the literature data and previously published scientific reports (8) and are listed in Table 1. A LightCycler 480 II

instrument (Roche Diagnostics, Basel, Switzerland) was used to run the thermal reaction programme. It consisted of an initial denaturation (20 min, 95°C) followed by 40 cycles of amplification (15 s, 95°C), annealing (20 s, 58°C - melting point for each primer pair (according to the optimisation of starters); expected for IL12 - 65 °C) and elongation (20 s, 72°C). Each qPCR reaction was performed in duplicate. Relative gene expression analysis was conducted separately for each experimental group by the ΔΔCt method using *ACTB* and *G6PDH* as reference genes. The geometric means of cycle threshold values (Ct) of reference genes were used in the analysis. Statistical analyses were performed by comparing the Ct value of each experimental group with that of the control group by Student's *t*-test ($P \leq 0.05$).

Relative abundance of indicator bacteria. A qPCR reaction in the LightCycler 480 II was used to analyse the relative abundance of bacteria in accordance with the method described by Dunisławska *et al.* (9). The reaction mixture included Maxima SYBR Green qPCR Master Mix intercalating dye (Thermo Fisher Scientific, Waltham, MA, USA), a primer specific to the 16S rRNA region of indicator bacteria (1 µM each, synthesised by Sigma-Aldrich, Schnelldorf, Germany; presented in Table 2), and DNA (20 ng). Two technical replicates of the qPCR reaction were run. The qPCR reaction thermal profile was initial denaturation for 5 min at 95°C, followed by 40 amplification cycles of denaturation for 15 s at 95°C, annealing for 15 s at 58°C and elongation for 45 s at 72°C. Fluorescence was measured at the end of each extension step. After amplification, a melting curve was generated by increasing the temperature to 98°C and measuring the fluorescence of melting amplicons. The calculation was based on the separate reaction of five dilutions of the bacterial DNA template (1×, 0.5×, 0.25× 0.125×, and 0.0625×). Using the formula $(1+E_{universal})^{Ct\ universal}/(1+E_{target})^{Ct\ target}$, the relative quantification of the bacteria in the caecal content was calculated. The expression $E_{universal/target}$ is the efficiency for universal/target bacterial primers and $Ct_{universal/target}$ is the quantification cycle for universal/target bacterial primers (5). Statistical analysis was performed using Student's t-test (P-value ≤ 0.05) comparing the experimental and control groups.

Table 1. Primer sequences used in the RT-qPCR reaction

Gene	Forward primer	Reverse primer
ACTB	CACAGATCATGTTTGAGACCTT	CATCACAATACCAGTGGTACG
G6PDH	CGGGAACCAAATGCACTTCGT	GGCTGCCGTAGAGGTATGGGA
CLDN1	TCTTCATCATTGCAGGTCTGTC	AACGGGTGTGAAAGGGTCAT
TJAP1	AGGAAGCGATGAATCCCTGTT	TCACTCAGATGCCAGATCCAA
MUC6	TTCAACATTCAGTTCCGCCG	TTGATGACACCGACACTCCT
AvBD1	AAACCATTGTCAGCCCTGTG	TTCCTAGAGCCTGGGAGGAT
CATHL2	AGGAGAATGGGGTCATCAGG	GGATCTTTCTCAGGAAGCGG
IL1B	GGAGGTTTTTGAGCCCGTC	TCGAAGATGTCGAAGGACTG
IL4	GCTCTCAGTGCCGCTGATG	GGAAACCTCTCCCTGGATGTC
IL6	AGGACGAGATGTGCAAGAAGTTC	TTGGGCAGGTTGAGGTTGTT
IL10	CATGCTGCTGGGCCTGAA	CGTCTCCTTGATCTGCTTGATG
IL12B	TTGCCGAAGAGCACCAGCCG	CGGTGTGCTCCAGGTCTTGGG
IFNB	ACCAGATCCAGCATTACATCCA	CGCGTGCCTTGGTTTACG
IFNG	ACACTGACAAGTCAAAGCCGC	AGTCGTTCATCGGGAGCTTG

Table 2. Primer sequences for the identification of bacteria

Bacteria	Forward primer	Reverse primer	Reference
Universal bacteria	ACTCCTACGGGAGGCAGCAGT	GTATTACCGCGGCTGCTGGCAC	(27)
Clostridium difficile	TTGAGCGATTTACTTCGGTAAAGA	TGTACTGGCTCACCTTTGATATTCA	(23)
Faecalibacterium prausnitzii	ACCATGAGAGCCGGGGGG	GGTTACCTTGTTACGACTT	(18)
Bifidobacterium spp.	GCGTGCTTAACACATGCAAGTC	CACCCGTTTCCAGGAGCTATT	(23)
Lactobacillus spp.	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG	(13)
Escherichia coli	CATGCCGCGTGTATGAAGAA	CGGGTAACGTCAATGAGCAAA	(23)
Streptococcus spp.	AGATGGACCTGCGTTGT	GCTGCCTCCCGTAGGAGTCT	(29)

Results

Relative analysis of gene expression in caecal mucosa. A panel of 12 genes related to the intestinal immune response and defence functions was analysed for gene expression in the caecal mucosa of broiler chickens (Fig. 1). Expression of 11 genes was upregulated in all analysed groups. Nine of them – *CLDN1*, *MUC6*, *AvBD1*, *CATHL2*, *IL1B*, *IL4*, *IL6*, *IL10* and *IFNG* – showed a statistically significant increase in expression, which was also correlated with an increase in the proportion of coffee husks in the bedding material. The *TJAP1* gene was downregulated in all studied groups, statistically significantly so in the CH25 group.

Relative abundance of indicator bacteria in caecal content. A panel of five indicator bacteria was analysed for relative abundance in the caecal content of broiler chickens (Fig. 2). A significant increase in *Bifidobacterium* bacteria was found in the groups reared on litter with coffee husks over the level of the bacteria in the control group, the highest *Bifidobacterium* content having been found in the group with the highest replacement of wheat straw with coffee husks (50%). A statistically significant increase was shown in the case of *Lactobacillus* bacteria, where the highest level of bacterial relative abundance was also found in the group with the largest proportion of coffee husks. The use of coffee husks in the bedding material caused a reduction in the relative abundance of *E. coli*. The highest statistically significant decrease was observed in the CH50 group. The greatest abundance of *Streptococcus* bacteria was noted in the control group, and in the experimental groups, *Streptococcus* was statistically significantly less in content. There were no significant differences in the number of *Faecalibacterium*, while the highest numerical level was found in the control and CH50 groups. The presence of *Clostridium* bacteria (Ct > 35) was not detected.

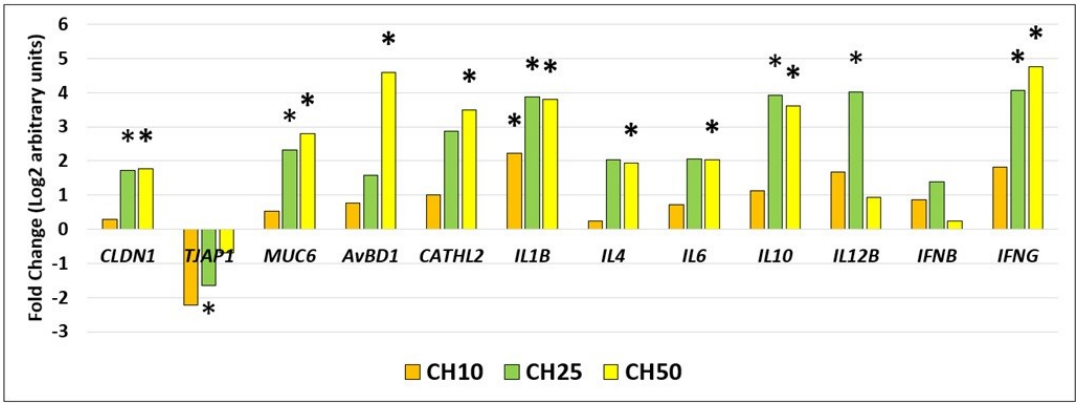

Fig. 1. Relative analysis of expression of a panel of genes related to intestinal immune response and organism defence against pathogens in the caecal mucosa of broiler chickens bedded on litter with different proportions of coffee husks and wheat straw. CH10 – broilers with litter of 10% coffee husks and 90% wheat straw; CH25 – broilers with litter of 25% coffee husks and 75% wheat straw; CH50 – broilers with litter of 50% coffee husks and 50% wheat straw; * – $P \leq 0.05$ (n = 8/group)

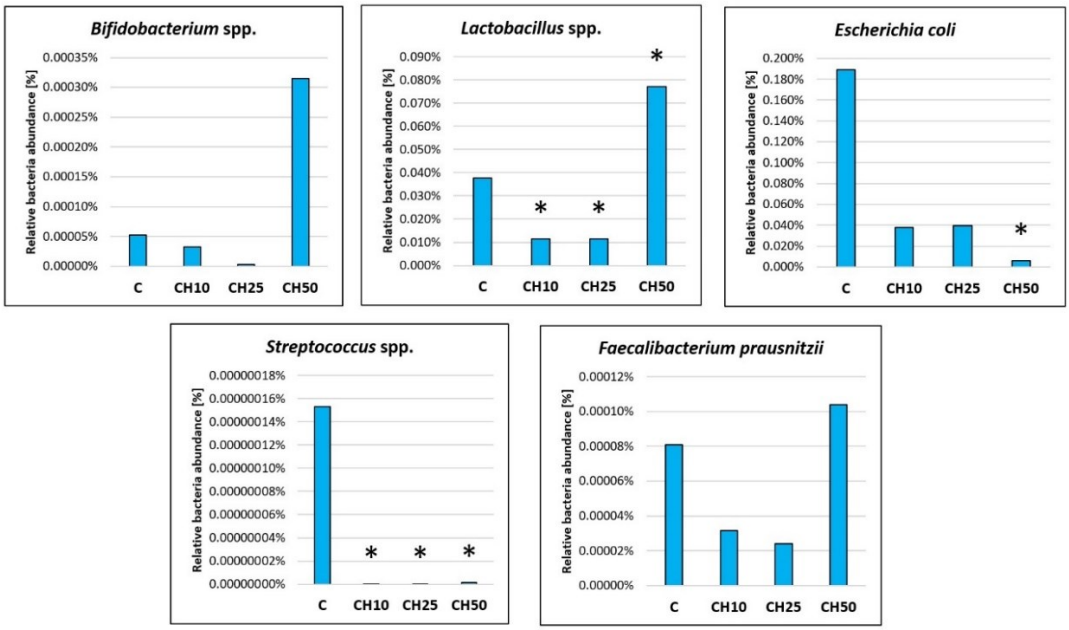

Fig. 2. Relative abundance of indicator bacteria in caecal content of broiler chickens. C – broilers in the control group with litter of 100% wheat straw; CH10 – broilers with litter of 10% coffee husks and 90% wheat straw; CH25 – broilers with litter of 25% coffee husks and 75% wheat straw; CH50 – broilers with litter of 50% coffee husks and 50% wheat straw; * – $P \leq 0.05$ (n = 8/group)

Discussion

As mentioned in the introduction, previous studies have not shown a negative impact of the inclusion in litter of coffee husks on the growth performance of broiler chickens. In the research by Biesek *et al.* (2), the body weight of chickens in all groups was similar (P-value > 0.05), ranging from 2,404.78 to 2,543.98 g, and the feed conversion ratio ranged from 1.69 to 1.77 kg/kg (P-value > 0.05). However, a significant reduction in footpad dermatitis incidence was noticed when 25 and 50% of coffee husks were used as a bedding material, which is important for the health of broiler chickens.

Coffee husks, residues from the roasting process of coffee beans, are an essential source of biologically active compounds. In recent years, interest in their potential impact on the health and efficiency of animal production, including poultry farming, has increased significantly. Coffee husks contain several compounds, including caffeine, polyphenols, chlorogenic acids and other substances with potential immunomodulatory effects. The main aim of the research was to verify whether waste material such as coffee husks could be reused in animal production while demonstrating beneficial properties in the maintenance of poultry. Up to the time of this research, the changing of gene expression and the intestinal microbiome in poultry had not been tested.

The properties of coffee husks may influence the expression of genes involved in gut barrier function, including tight junction proteins like claudins and occludins. The *CLDN1* gene, which encodes the claudin-1 protein, plays a crucial role in the formation of tight junctions between cells, particularly in epithelial tissues. Tight junctions are essential for maintaining cellular barrier integrity and selective permeability, such as those found in the gastrointestinal tract (15). Our study shows that the level of *CLDN1* expression increased with an increase in the proportion of the litter which was coffee husks and statistically significant differences were observable. This indicates the potential of coffee husks in pellets to increase intestinal tightness. Mucins play essential roles in protection and cell surface signalling. Mucin 6 (MUC6) and related mucins contribute to the protective functions of mucus in poultry (7). A significant increase in *MUC6* gene expression was observed related to the percentage of coffee husks in the pellet, and this may indicate the stimulating properties of supplementation for mucus production and the activity of protective mechanisms. By enhancing intestinal barrier integrity, coffee husks can help maintain a balanced gut microbial community and prevent the translocation of harmful bacteria into the bloodstream (26).

As in the case of genes related to intestinal tightness, a proportion-dependent effect of pellets with the addition of coffee husks was also demonstrated in genes related to the immune response. The *AvBD1* gene, which encodes for the avian β-defensin 1 peptide, plays a crucial role in the innate immune response of poultry (3). Genes of cytokines as analysed interleukins, which are critical mediators of the immune response, may be an essential indicator of the impact of coffee hulls on poultry health. All analysed cytokines showed a similar upregulation trend, proving that coffee husks in the bedding material have immunomodulatory potential.

The effects of coffee husks on the intestinal microbiota may be dose-dependent (as in the case of changes in gene expression), with higher concentrations potentially exerting stronger effects on microbial composition and activity (25). Coffee husks contain polysaccharides and dietary fibres, which may serve as substrates for beneficial gut bacteria. These compounds can act as prebiotics, promoting the growth of beneficial microbes such as bifidobacteria and lactobacilli in the poultry gut (20). *Lactobacillus* and *Bifidobacterium* bacteria are crucial to the health of poultry. This is primarily due to their modulatory functions in the immune system (increasing the production of IgA antibodies, excreted mainly into the gastrointestinal tract) and active competition with pathogens by attaching to the intestinal membrane where pathogens may also seek to attach (19). The addition of coffee husks in the highest proportion caused an increase in the abundance of *Lactobacillus* and *Bifidobacterium* bacteria in the caecum of broiler chickens. Besides provision of an advantageous substrate and immunomodulation, coffee husks may also render the gut environment favourable for beneficial bacteria through anti-inflammatory properties. The polyphenol components of coffee husks have been reported to possess anti-inflammatory properties. By reducing inflammation in the gut, coffee husks may foster the growth of health-promoting bacteria while inhibiting that of harmful pathogens (12). Caffeine, a major component of coffee, has an antibacterial effect on *Escherichia coli* (10) that was confirmed by Okabe *et al.* (22). These results suggest that coffee may be helpful as a natural inhibitor of particular bacteria.

In our study, pellets with coffee husks reduced the abundance of bacteria in the caecal content of broiler chickens. This was especially noticeable when 50% of the wheat straw was replaced with these pellets. *Streptococcus* species are considered part of the normal microbiota in poultry but can cause diseases (21). These diseases can lead to significant economic losses in the poultry industry because they reduce production, increase mortality and incur treatment costs. Prevention and control of streptococcal infections in poultry usually include good biosecurity practices, good hygiene, vaccination, and prompt treatment with antibiotics. Genetic selection for disease resistance and management practices that minimise stress may also help reduce the risk of streptococcal infections in poultry populations (12). Complementarily to these measures, providing litter with coffee husk content appears also to be a control measure for streptococcal infections. As the results indicate, coffee husks may have the potential for

preventive use to eliminate or reduce *Streptococcus* bacteria and a potential pathogen. The introduction of pellets with the addition of all three proportions of coffee husks significantly reduced the relative abundance of *Streptococcus* bacteria.

Research on *Faecalibacterium prausnitzii* in intestinal contents suggests that bedding management patterns influence the intestinal microbiota in chickens, which may significantly impact nutritional status and intestinal health. In the study by Wang *et al.* (30), it was noted that reused litter increases the predominance of *Faecalibacterium prausnitzii* bacteria, which are responsible for the production of butyrate in the intestines. In our research, we have shown that coffee husks in the bedding material at the highest content increase the content of *Faecalibacterium prausnitzii* bacteria. The analysis of the relative abundance of bacteria based on indicator bacteria clearly indicates that intermediate doses of coffee husks in the bedding material do not significantly impact the intestinal microbiological profile. These changes are observable when the highest dose is used.

Conclusion

Coffee husks may have the potential to be a material for poultry bedding because of their immunomodulatory effects. However, further research is needed to understand better the mechanisms of action and the potential benefits and risks associated with their use. Our research has shown that 50% coffee husk content in pellets used in broiler chickens has the highest potential for immune stimulation, intestinal tightness and beneficial profiling of the intestinal microbiota.

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Animal Rights Statement: The study was carried out in compliance with Directive 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes and the Act of 15 January 2015 on the protection of animals used for scientific or educational purposes (Journal of Laws of the Republic of Poland 2015, item 266). The approval of the Institutional Animal Care and Use Committee was obtained (No. 2/2022).

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