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RESEARCH ARTICLE

Ileal and Cecal Microbial Population and Short-Chain Fatty Acid Profile in Broiler Chickens Fed Diets Supplemented with Lignocellulose

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ABSTRACT

The study was conducted to evaluate the performance indices, gut microbiota, short-chain fatty acid (SCFA) and lactic acid concentration and pH value in ileal and cecal digesta of 42-day-old broilers fed a diet supplemented with lignocellulose. A total of 48, 21-day-old male Ross 308 chickens were divided into 4 treatment groups with 6 replicates per treatment. Experimental diets were based on maize, wheat, triticale, soybean meal and varied in the amount of lignocellulose: 0, 0.25, 0.5 and 1.0%. There was no significant effect of lignocellulose inclusion into the diet on broiler performance indices. Ileal *Lactobacillus spp.* as well as ileal and cecal *Bifidobacterium spp.* populations increased ($P<0.05$) and the number of *Escherichia coli* and *Clostridium spp.* significantly decreased in birds fed diets supplemented with lignocellulose. The sum of SCFAs and lactic acid in both intestine segments as well as acetic and propionic acids in ileal and lactic acid level in cecal digesta of chickens fed a diet with 0.5% lignocellulose were significantly higher compared to the control birds. The results show that inclusion of lignocellulose in the chicken diet, especially at a dose of 0.5%, promotes the growth of *Lactobacillus spp.* and *Bifidobacterium spp.*, reduces the number of *E. coli* and *Clostridium spp.* as well as enhances the concentration of SCFAs and lactic acid with no substantial effect on the pH of ileal and cecal digesta. The intensity of fermentation and substantial amounts of the lactic acid and individual SCFAs, particularly acetic and propionic acids, may suggest the prebiotic effect of lignocellulose on the broiler gastrointestinal tract.

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INTRODUCTION

It is commonly known that the sub-therapeutic use of antibiotic growth promoters (AGP) in poultry production may result in the development of antibiotic-resistant pathogenic bacteria, which may be hazardous to human health. In search of effective alternatives to AGP, a special attention is given to their effect on gut microbial community which contributes to the intestine function. Until now, the interest has been focused mainly on fermentable functional feed ingredients, like fructans, or mannanoligosaccharides that exhibit beneficial effect on gut microflora, integrity of intestinal mucosa, enzymes activity and performance parameters in broiler chickens (Kim *et al*., 2011; Bogusławska-Tryk *et al*., 2012; Nabizadeh, 2012). An insoluble, non-fermentable fiber fraction, including cellulose and lignin, is conventionally considered as a diet diluent which can influence energy balance of broilers (Svihus and Hetland, 2001; Krás *et al*., 2013), whereas little attention is given to the effect of cellulose or lignin on the gastrointestinal microflora population. However, studies show that cellulose, as an effective feed ingredient, may influence the number of gut bacteria, especially beneficial *Bifidobacterium* and *Lactobacillus* as well as potential pathogens and its effect depends on the level of cellulose supplementation and bird age (Cao *et al*., 2003; Shakouri *et al*., 2006; Saki *et al*., 2010). It is generally accepted that phenolic fragments of purified lignin exhibit the antimicrobial properties (Baurhoo *et al*., 2008). Studies show, however, that the

inclusion of a low level (1.25%) of purified lignin promotes the growth of beneficial bacteria (*Lactobacillus* and *Bifidobacterium*), lowers the *E. coli* population in the ceca and improves gut integrity, and in this aspect it turns to be a potential replacement for AGP in broiler diets (Baurhoo *et al*., 2007a,b). It can be assumed that the beneficial influence of cellulose and lignin on the intestinal microbiota will affect the fermentation profile and composition of SCFAs in the intestinal digesta, which play an important role as an energy source for the peripheral tissues and colonocytes and affect the glucose and protein metabolism in the liver (Bogusławska-Tryk *et al*., 2012). To our knowledge, there is a lack of information about the effect of cellulose or lignin on the SCFA profile in the gastrointestinal tract of broilers. Moreover, there were no studies evaluating the effect of lignocellulose on bacterial population and fermentation intensity in the gut. Therefore, the purpose of the present study was to determine the effect of supplementation of a broiler diet with lignocellulose on the microflora, SCFAs and lactic acid content in ileal and cecal digesta as well as growth performance in broiler chickens.

MATERIALS AND METHODS

Birds and diets: All animal care procedures were approved by the Local Ethical Committee on Animal Testing at the University of Technology and Life Sciences in Bydgoszcz. A total of 48 twenty one-day-old male Ross 308 broiler chicks were obtained from a commercial farm. After delivering to the biological tests laboratory with automatically controlled humidity, temperature and air exchange, chickens were weighed and allocated to one of four treatments (n=12). Two birds were housed in one metabolism cage (6 replicates per treatment). The chickens were provided with continuous light and reared according to the technological recommendations for the breed. During the 3-week experiment (from 21 to 42 d of age) all birds had free access to water and were fed *ad libitum* an antibiotic and coccidiostats-free standard finisher mixture based on maize (24.1%), wheat (23.1%), triticale (23.6%), and a soybean meal (23.8%) supplemented with soybean oil, essential amino acids, vitamin and mineral premix and Ronozyme phytase (Nutrition Specification for Ross 308, 2007). Experimental diets varied in the amount of lignocellulose (Arbocel® RC, J. Rettenmaier & Söhne GmbH + CO) incorporated into the basal mixture: 0.0 (control group), 0.25, 0.5 and 1% as an expense of wheat. According to the manufacturer's data, the lignocellulose preparation contained about 70% acid detergent fiber (ADF) and 24% acid detergent lignin (ADL), the length of fiber fraction is 200-300µm and the water absorption is about 500-700%. The analyzed chemical composition and fiber fractions of experimental diets are presented in Table 1. The density of ME/kg of diets was calculated from the analyzed chemical composition according to the DLG standard (Anonymous, 1999).

Experimental procedures: The body weight of individual chickens, feed intake and feed conversion (FCR) within each replicate group were measured at the 42nd day of age. FCR was determined as the ratio between the feed intake and weight gain. European Efficiency Index (EEI) was calculated for each replicate as follows:

EEI = (livability × live weight, kg / length of rearing period, days × FCR) × 100

At the 42nd day of age after BW measurements, all chickens were stunned and killed by decapitation and gastrointestinal tracts were quickly removed. The samples of the digesta from the ileum (from Meckel's diverticulum to the ileocecal junction) and the ceca were immediately collected from each bird into sterile tubes, pooled (1 digesta sample from the ileum and 1 sample from ceca / metabolic cage) and put on ice until they were delivered to the laboratory for enumeration of bacterial population, for SCFA and lactic acid analyses. The pH of fresh ileal and cecal digesta were measured using pH-meter CP-411 (Elmetron, Poland).

Analytical methods: The dietary content of dry matter, crude protein, fat, fiber, ash and dry matter content in ileal and cecal digesta were determined in duplicate according to AOAC (1990). The concentration of neutral detergent fiber (NDF), ADF and ADL was analyzed according to the Van Soest method (1963) using the fiber analyzer (ANKOM 220, US).

The quantitative determination of microbiota of ileal and cecal digesta was performed by the standard Koch's plate method. The samples were suspended in buffered 1% peptone water (1:9 w/v), then serial decimal dilutions were prepared. The following bacteria species were identified with the original medium (MERCK, Germany): the total number of aerobic and anaerobic bacteria on Plate Count agar; *Lactobacillus spp.* on MRS agar; *Escherichia coli* on Chromocult TBX agar; *Bifidobacterium spp.* on RCA agar and *Clostridium spp.* on DRCM agar. All the plates, in triplicate, were incubated at 37°C. Total aerobic bacteria, *Lactobacillus spp.* and *E. coli* were incubated under aerobic conditions for 48, 72 and 24h, respectively; total anaerobic bacteria for 48h and *Bifidobacterium spp.* and *Clostridium spp.* for 72h, in the Ruskinn Concept 400 anaerobic chamber (Biotrace International, UK).

SCFA (formic, acetic, propionic and butyric) and lactic acid concentration in digesta samples was determined in duplicate using HPLC system (Thermo Scientific, USA) equipped with refractive index (RI 150) and ultraviolet (UV, wavelength 210 nm) detectors. Analyses were performed using a BioRAD AMINEX HPX-87H (300 x 7.8 mm) column (Hercules, USA). Sulfuric acid (0.005M) was used as a mobile phase with a flow rate of 6 µl/min. The total run time was approximately 40 min, at a column temperature 60°C. All the solutions were filtered through 0.22 µm syringe filters and a sample of 10 µl volume was injected into the HPLC system to provide standard curves. The concentration of individual SCFAs and lactic acid was estimated using external standard solution.

Statistical analysis: Data are expressed as means and SEM. The results were determined by one-way ANOVA and the differences between treatments were analyzed

post hoc by Tukey's test using STATISTICA 8 software (StatSoft, Inc®).

RESULTS

During the whole experimental period, all broilers were healthy and the performance at the 42nd day of age was satisfactory. There was no significant effect of lignocellulose inclusion into the diets on chicken body weight gain, feed conversion and EEI value (Table 2). The results of microbial counts determined in ileal and cecal digesta are shown in Table 3. There were no differences (P>0.05) in total aerobes and anaerobes population in ileal and cecal digesta but counts of *Lactobacillus spp.*, *Bifidobacterium spp.*, *E. coli* and *Clostridium spp.* were significantly affected by the presence of lignocellulose in the diets. There was an increase (P<0.05) in ileal *Lactobacillus spp.* as well as ileal and cecal *Bifidobacterium spp.* populations in broilers fed the diets containing 0.25, 0.5 and 1.0 % of lignocellulose compared to the control group. The number of *Escherichia coli* and *Clostridium spp.* was significantly lower in ileal and cecal digesta of chicks fed diets supplemented with 0.25 and 0.5% of lignocellulose than in the control birds.

Dry matter content, digesta pH, SCFA and lactic acid concentration in the intestine are shown in Table 4. Dry matter content and pH value were not affected (P>0.05) by lignocellulose inclusion, however there was a tendency for the lower pH value in chickens fed a diet with fiber addition, especially at the level of 0.5%. Supplementation of the diet with 0.5% lignocellulose significantly increased (approx. 63%) the sum of SCFAs and lactic acid in ileal digesta compared to the control group as well as in cecal digesta compared to the control and 1.0% lignocellulose diet (approx. 31% and 26%, respectively). Similarly to the sum of SCFAs and lactic acid, the level of all the examined individual SCFAs, except for butyric acid in the ceca, was the highest in ileal and cecal digesta of birds fed the diet with 0.5% lignocellulose. Significant differences in ileal digesta were detected in the acetic and propionic acids pool (0.5% lignocellulose vs. control and 0.25% supplemented diets), as well as in cecal digesta lactic acid content (0.5% lignocellulose vs. control and 1% lignocellulose). It should be mentioned that, generally, the addition of lignocellulose to the chicken diet, regardless of the dose, caused an increase in the formic, acetic, propionic and lactic acid content in ileal and cecal digesta as well as the butyric acid concentration in the ileal content.

Table 1: Analyzed chemical composition of the experimental diets (g/kg, as-fed basis)

Item	Lignocellulose in the diet (%)			
	0.0	0.25	0.5	1.0
Dry matter	908.9	906.9	907.9	905.7
ME (MJ/kg)	12.8	12.9	12.8	12.8
Crude protein (N x 6.25)	195.7	197.1	192.5	196.9
Ether extract	41.4	40.5	41.1	40.6
Crude fiber	28.7	29.2	30.0	32.3
N-free extractives[a]	587.1	576.7	582.6	575.8
Fiber fractions:				
NDF	81.5	81.1	86.6	90.5
ADF	26.9	29.0	32.8	36.6
ADL	4.9	4.9	5.3	6.7

[a]by difference: N-free extractives = dry matter – (crude protein + crude fat + crude fiber + ash).

Table 2: Performance indices (mean±SEM) of broiler chickens at 42 day of age

Indices	Lignocellulose in the diet (%)			
	0.0	0.25	0.5	1.0
Initial BW, g	739±4.31	729±3.38	735±1.25	728±3.53
Final BW, g	2422±87.33	2423±48.36	2436±50.87	2429±55.55
Daily BW gain, g	80.15±4.07	80.65±2.21	81.00±2.41	81.02±2.67
FCR, kg/kg	1.865±0.02	1.876±0.03	1.854±0.02	1.875±0.03
EEI[a]	620±26.22	616±8.54	626±17.86	618±22.13

[a]EEI – European Efficiency Index

DISCUSSION

To our knowledge, in the available literature, there is a lack of data concerning the effect of lignocellulose in broiler diets on growth, gut microbial population and bacterial fermentation activity. Previous studies focused on the effect of cellulose or lignin on bird production and physiological indices, and the results obtained are ambiguous. In the present study, inclusion of 0.25-1.0% lignocellulose did not affect the performance parameters. These results confirm the findings of most studies conducted in recent years with pure cellulose (Svihus and Hetland, 2001; Cao *et al*., 2003; Jiménez-Moreno *et al*., 2009), lignin (Baurhoo *et al*., 2007a,b) or lignocellulose (Farran *et al*., 2013) as a chicken diet supplement. However, Sarikhan *et al*. (2010) report that the addition of moderate amounts (0.25-0.75%) of an insoluble fiber concentrate, containing 86.5% ADF fraction, to a broiler diet can positively influence birds' growth and feed efficiency, mainly in the grower period.

It is widely known that both the density and diversity of bacterial population in the different segments of chicken's gastrointestinal tract significantly change with age. Moreover, this labile microflora can be influenced by many factors, like health status, maintenance conditions and diet composition (Lu *et al*., 2003; Shakouri *et al*., 2006; Rehman *et al*., 2007; Saki *et al*., 2010). The results of the current study demonstrate that the addition of lignocellulose can have a stimulatory effect on the growth of beneficial bacteria (*Lactobacillus spp.* and *Bifidobacterium spp.*) and limit the population of potential pathogens (*E. coli* and *Clostridium spp.*) in ileal and cecal digesta of 42-day-old broilers. The mechanism of lignocellulose action on bacterial population in the gastrointestinal tract is not fully understood. It can be assumed that it is the result of different action mechanisms of cellulose and lignin on the intestinal environment. It is documented that insoluble dietary fiber degradation (NDF fraction) by bacterial enzymes in chicks is about 35% (Jamroz *et al*., 2001) but the effect on microbial population results mainly from its physicochemical properties. The abrasive action of insoluble dietary fiber on the intestine mucosal surface favors the elimination of pathogenic bacteria capable of adhering to the mucus layer, such as *Clostridium perfringens* (Mateos *et al*., 2012). This effect, in turn, can support the growth of beneficial microbiota. The earlier studies demonstrate that the inclusion of cellulose to a broiler diet can modify gut microflora by stimulating the growth of *Lactobacilli* and *Bifidobacteria* (Cao *et al*., 2003; Saki *et al*., 2010). These microorganisms, through the release of bacteriocins, inhibit the further proliferation of pathogens in the gut (Kawai *et al*., 2004; O'Shea *et al*.,

Table 3: Microbial counts (log_{10} CFU/g digesta) in ileal and cecal digesta of chickens fed diets supplemented with lignocellulose

Item	Lignocellulose in the diet (%)			
	0.0	0.25	0.5	1.0
Aerobes				
Ileum	9.37±0.11	9.42±0.08	9.59±0.01	9.57±0.03
Ceca	9.39±0.12	9.44±0.08	9.51±0.08	9.43±0.04
Anaerobes				
Ileum	9.20±0.12	9.36±0.09	9.33±0.12	9.29±0.09
Ceca	9.57±0.05	9.56±0.09	9.62±0.06	9.51±0.06
Lactobacilli				
Ileum	7.94±0.03^{a}	8.54±0.06^{b}	8.62±0.09^{b}	8.37±0.08^{b}
Ceca	8.16±0.03	8.42±0.09	8.40±0.14	8.40±0.09
Bifidobacteria				
Ileum	7.95±0.00^{a}	8.57±0.07^{b}	8.59±0.09^{b}	8.48±0.11^{b}
Ceca	8.21±0.05^{a}	8.75±0.07^{b}	8.77±0.05^{b}	8.69±0.03^{b}
Escherichia coli				
Ileum	6.82±0.04^{a}	6.25±0.13^{b}	6.23±0.14^{b}	6.51±0.16^{ab}
Ceca	6.75±0.06^{a}	6.23±0.13^{b}	6.23±0.14^{b}	6.67±0.07^{a}
Clostridia				
Ileum	5.78±0.04^{a}	5.38±0.08^{b}	5.32±0.09^{b}	5.59±0.10^{ab}
Ceca	5.79±0.03^{a}	5.42±0.08^{b}	5.36±0.08^{b}	5.59±0.09^{ab}

Mean±SEM in a row with different letters differ significantly (P<0.05).

Table 4: Dry matter content (%), pH value, short-chain fatty acids and lactic acid concentration (µmol/g digesta) in ileal and cecal digesta of chickens fed diets supplemented with lignocellulose

Measurements	Lignocellulose in the diet (%)			
	0.0	0.25	0.5	1.0
Dry matter				
Ileum	18.09±0.31	18.64±0.24	18.27±0.55	18.46±0.28
Ceca	18.23±1.19	18.08±0.69	17.08±0.12	17.63±0.56
pH				
Ileum	6.58±0.25	6.20±0.13	6.01±0.27	6.11±0.21
Ceca	6.08±0.15	6.04±0.19	5.74±0.14	6.00±0.17
Formic acid				
Ileum	6.74±1.96	9.34±2.61	11.95±1.96	8.04±1.30
Ceca	5.65±0.65	6.30±0.87	6.30±0.65	5.87±0.43
Acetic acid				
Ileum	9.16±2.33^{ac}	7.16±2.66^{a}	19.98±1.83^{b}	18.32±2.83^{bc}
Ceca	29.31±3.16	30.14±4.33	32.81±4.16	25.98±3.00
Propionic acid				
Ileum	3.78±1.48^{a}	9.72±3.64^{ab}	17.41±2.83^{b}	9.18±3.10^{ab}
Ceca	22.00±2.16	24.97±0.81	26.05±2.02	23.76±0.81
Butyric acid				
Ileum	4.54±1.36	5.22±1.02	5.33±1.02	4.54±0.68
Ceca	6.70±0.57	6.58±0.45	6.36±0.57	5.79±0.23
Lactic acid				
Ileum	28.75±4.11	29.64±5.33	31.64±2.33	29.53±1.22
Ceca	60.28±2.00^{a}	82.48±7.22^{ab}	90.48±8.21^{b}	67.16±2.78^{a}
Sum of SCFA and lactic acid				
Ileum	52.97±7.17^{a}	61.08±8.67^{ab}	86.31±4.32^{b}	69.61±3.61^{ab}
Ceca	123.94±4.75^{a}	150.47±10.07^{ab}	162.00±8.78^{b}	128.56±4.69^{a}

Mean±SEM in a row with different letters differ significantly (P<0.05).

2012). Moreover, polyphenolic compounds of lignin can cause bacterial cell membrane disintegration and in this way inhibit the growth of microorganisms in the digestive tract (Baurhoo *et al*., 2008). Nelson *et al*. (1994) reported that Alcell lignin, both *in vivo* and *in vitro* reduced growth of aerobic bacteria and intestinal translocation of pathogenic bacteria in mice. Previous studies demonstrate that the positive effect of cellulose and lignin on ileal and cecal microflora is dose-dependent. Cao *et al*. (2003) reported that the high level of alpha-cellulose (10%) in the chicken diet significantly enhanced cecal *Bifidobacteria* and *Lactobacilli* amounts compared to 0 and 3.5% cellulose groups but did not affect the number of *Clostridia* and *Enterobacteriaceae*. The studies performed by Baurhoo *et al*. (2007a,b) show that the addition of a low level (1.25%), but not high level (2.5%) of purified lignin, significantly increased the cecal population of lactic acid bacteria compared with the antibiotic-free diet. Moreover, both levels of lignin reduced the cecal *E. coli* populations in birds challenged with pathogenic strains of *E. coli*, and this effect was dose related (Baurhoo *et al*., 2007a). In the present study, relatively low levels of fiber were used and the most beneficial effect on ileal and cecal microflora was observed in the birds fed a diet with 0.5% of lignocellulose.

The previous studies indicate large variations in SCFA concentration (11.9-191.4 µmol/g digesta) and pH values (5.0-7.1) in intestinal content which depend mainly on chicken age, diet composition, dietary fiber type and its fermentation degree (Marounek *et al*., 1999; van der Wielen *et al*., 2000; Rehman *et al*., 2007; Jiménez-Moreno *et al*., 2009). However, there is a lack of information regarding the SCFA profile in birds fed diets supplemented with lignin or cellulose. In our study, the beneficial microbial population in the gut results in a lower pH, the highest concentration of lactic, acetic and propionic acids as well as sum of SCFA and lactic acid in ileal and cecal digesta in chickens fed the diet with 0.5% of lignocellulose. It is probably due to more intense fermentation of a dietary fiber as a result of the increased population of lactic acid bacteria in the gut. The increase of concentration of acetic and propionic acid in the gut digesta is a desirable effect. It is documented that propionate, acetate and formate *in vitro* have a toxic effect on some pathogenic bacteria (Cherrington *et al*., 1990; van der Wielen *et al*., 2000). According to van der Wielen *et al*. (2000), there is a significant negative correlation between acetate concentration and counts of *Enterobacteriaceae* in broiler ceca.

Conclusion: The results of the study show that the inclusion of low amounts of lignocellulose to a chicken diet promotes the growth of *Lactobacillus spp.* and *Bifidobacterium spp.*, reduces the number of *E. coli* and *Clostridium spp.* as well as enhances the concentration of SCFAs and lactic acid with no substantial effect on the pH of ileal and cecal digesta. The composition of intestinal microflora, intensity of fermentation and substantial amounts of the individual SCFAs, particularly acetic and propionic acids as well as lactic acid, may suggest the prebiotic effect of lignocellulose on the broiler gastrointestinal tract. Since the effect of lignocellulose on intestinal microbial population and SCFA profile in chickens was not studied previously, further investigations are necessary to confirm the results of the present experiment.

Author's contribution: MBT and RS conceived the idea and designed experiment, analyzed and interpreted the data. AP and KB conducted the experiment, analyzed and interpreted the data. K.Ś analyzed the digesta samples. All the authors critically revised the manuscript and approved the final version.

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