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Article *in* Poultry Science · May 2019

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Amorphous cellulose feed supplement alters the broiler caecal microbiome

Celine De Maesschalck,[*] Venessa Eeckhaut,[*] Luc Maertens,[†] Loek De Lange,[‡] Leon Marchal,[§] Georges Daube,[#] Jeroen Dewulf,[‖] Freddy Haesebrouck,[*] Richard Ducatelle,[*] Bernard Taminau,[#] and Filip Van Immerseel[*,1]

[*]*Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium;* [†]*Animal Science Unit, Institute for Agricultural and Fisheries Research, Scheldeweg 68, B-9090 Melle, Belgium;* [‡]*Schothorst Feed Research, Meerkoetenweg 26, NL-8200 AM Lelystad, The Netherlands;* [§]*ForFarmers BV, Kwinkweerd 12, NL-7241 CW Lochem, The Netherlands;* [#]*Laboratory of Food Microbiology, Department of Food Sciences, Fundamental and Applied Research for Animal and Health (FARAH), Faculty of Veterinary Medicine, University of Liège, Quartier vallée 2, Avenue de Cureghem 10, B-4000 Liège, Belgium; and* [‖] *Veterinary Epidemiology Unit, Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 13, B-9820 Merelbeke, Belgium*

ABSTRACT The grains that form the basis of most commercial chicken diets are rich in cellulose, an unbranched β-1,4-linked D-glucopyranose polymer, used as a structural molecule in plants. Although it is a predominant polysaccharide in cereal hulls, it is considered an inert non-fermentable fiber. The aim of the current study was to analyze the effect of in-feed supplementation of cellulose on the gut microbiota composition of broilers. Administration of cellulose to chickens, on top of a wheat-based diet, changed the caecal microbiota composition, as determined using pyrosequencing of the 16S rRNA gene. At day 26, a significantly ($P < 0.01$) higher relative abundance of the *Alistipes* genus was observed in the caeca of broilers fed the cellulose-supplemented diet, compared to animals fed the control diet. An in vitro batch fermentation assay showed a significant ($P < 0.01$) growth stimulation of *Alistipes finegoldii* in the presence of cellulose. In conclusion, in-feed supplementation of cellulose alters the microbiota composition at the level of the phylum *Bacteroidetes,* specifically the *Alistipes* genus. This suggests that cellulose is not essentially inert but can alter the gut microenvironment.

Key words: cellulose, broilers, microbiota, *Alistipes*

2019 Poultry Science 0:1–7
http://dx.doi.org/10.3382/ps/pez090

INTRODUCTION

In non-carnivorous animals and in humans, fiber in the diet mostly consists of plant cell wall substances, including, and in addition to cellulose, hemicellulose, oligosaccharides, pectins, and gums. These dietary fibers resist hydrolysis by small bowel digestive enzymes, and thus are available for fermentation by the microorganisms in the distal intestine (James et al., 2003). Correlations have been found between consumption of dietary fiber and metabolic health in humans (Lattimer and Haub, 2010). In poultry diets, however, dietary fiber may have either harmful or beneficial effects on growth performance and on intestinal health, depending on the source and form of the fiber (Mateos et al., 2012).

Cellulose is an insoluble dietary fiber composed of a linear chain of β-1,4-linked D-glucopyranosyl residues (O'Sullivan, 1997, Gilbert, 2010). Cellulose microfibrils represent a major structural element in cereal cell walls and form the residue after alkaline extractions of the cell wall material (Paterson, 1995). The in vitro incubation of human feces with cellulose results in the formation of only small amounts of short chain fatty acids (**SCFA**). Therefore it was concluded that cellulose was largely resistant to bacterial degradation (Vince et al., 1990). Further in vitro fermentation studies showed that the human gut microbiota is not adapted to ferment cellulose, although cellulose is present in various human food products (Johathan et al., 2012). Consequently, cellulose has been recommended as a bulking agent in order to decrease transit time or to enhance water holding capacity of intestinal content (Hetland et al., 2004, Montagne et al., 2003). For many years, cellulose has been considered as an inert bulking and filling material also in chicken feed, to the point that it was routinely used as such in experimental feeding trials (Langhout

Received December 13, 2017.
Accepted April 23, 2019.
[1]Corresponding author: filip.vanimmerseel@ugent.be

et al., 1999). In a recent study also, cellulose was found to be an essentially inert component in broiler feed, as neither nutritive value nor effects on growth were observed compared to the silica sand fed as feed additive to control animals (Wils-Plotz et al., 2013).

It was recently stated, however, that cellulose should not be considered as a non-fermentable carbohydrate for humans (Brotherton, 2015). Indeed, in vivo studies in humans have shown that 34% of cellulose from wheat bran is fermented (Nyman et al., 1986). Furthermore, fermentation of cellulose might depend on the form cellulose is presented to the microbes (crystalline vs. amorphous vs. integrated in larger plant cell wall fragments). To the best of our knowledge, whether and how cellulose could be fermented by the chicken microbiome in vivo has not been investigated yet, although field experience with cellulose supplementation to broiler diets suggests beneficial effects. Therefore, the purpose of the present study was to investigate the effects of an amorphous cellulose supplement on the microbiota composition in broilers. We found that the amorphous cellulose supplement stimulates the genus *Alistipes*.

MATERIAL AND METHODS

Additive

Commercial cellulose (Arbocel B800, Rettenmaier and Söhne, Rosenberg, Germany), used in the in vivo study, consisted of 99.5% amorphous cellulose with an average fiber length of 130 μm and an average fiber thickness of 20 μm. The bulking density was within the range of 155 g/L to 185 g/L and the pH between 5.5 and 7.5.

Animals and Diets

A total of 310 day-old Ross-308 male broiler chicks were housed in pens on solid floor covered with wood shavings with 31 chicks per pen. Light schedule was set to provide 18 h light and 6 h darkness. Animals were fed a mash wheat-based diet of which the composition is shown in Table 1. No non-starch polysaccharide enzymes (i.e., xylanase) were included. All chickens received a starter feed from day 1 till day 13, a grower feed from day 14 till day 26, and a finisher feed from day 27 till day 39. The chickens of the treatment group (4 pens of 31 chickens per pen) were administered feed supplemented with 0.5% cellulose during starter period and 1.0% cellulose during grower and finisher period. The animals from the control group (6 pens of 31 chickens per pen) were given the non-supplemented feed. At day 13, 26, and 39, all broilers were individually weighed, as well as fed leftovers, to calculate the feed conversion ratio (**FCR**). At day 26, 3 chickens from each pen were euthanized by intravenous injection of an overdose of sodium pentobarbital 20% (Kela, Hoogstraten, Belgium). The content of caecum and colon was collected and stored at −70°C until DNA was extracted.

Table 1. The composition and nutrient content of the wheat-based diet administered as a mash. Starter diet was given from day 1 until 13, grower diet was given from day 14 until 26, and finisher diet was given from day 27 until 39. The feed of the treatment group was supplemented with 0.5% cellulose during starter period and 1.0% cellulose during grower and finisher period.

	Starter diet	Grower diet	Finisher diet
Feedstuff (%)			
Wheat	50.48	55.00	58.94
Soybean meal (48)	21.12	15.22	11.51
Soybeans	5.00	5.00	5.00
Sunflower meal 27	5.35	6.00	6.00
Rapeseed meal	7.50	7.50	7.50
Animal fat	5.63	6.68	6.70
Soy oil	2.80	2.82	2.61
Vitamin + trace elements	1.00	1.00	1.00
$CaCO_3$	0.32	0.30	0.34
Di-Ca-phosphate	1.55	1.25	0.95
NaCl	0.21	0.25	0.25
Na-bicarbonate	0.16	0.10	0.06
L-Lys-HCl	0.31	0.35	0.39
DL-Methionine	0.26	0.24	0.23
L-Threonine	0.09	0.10	0.11
Phytase	0.02	0.02	0.02
Calculated nutrient composition (% as fed)			
Crude protein	21.50	19.60	18.27
Crude fat	9.00	10.00	10.00
Crude fiber	4.80	4.67	4.53
Non-starch polysaccharides	18.07	17.64	17.35
Metabolisable energy (MJ/kg)	11.65	12.10	12.30
Starch	29.0	31.5	33.7
Lysine, digestible	1.15	1.05	1.00
Sulfur amino acids, digestible	0.86	0.79	0.75
Threonine, digestible	0.75	0.68	0.65
Valine, digestible	0.80	0.76	0.70
Arginine, digestible	1.24	1.10	1.00
Isoleucine, digestible	0.74	0.66	0.60
Leucine, digestible	1.35	1.22	1.10
Ca	0.91	0.82	0.75
Available P	0.40	0.35	0.30
NaCl + KCl (mEq/kg)	240	207	182
Linoleic acid (18:2)	2.17	2.28	2.23

Since this trial concerns a feeding experiment in which no invasive or stressful procedures were carried out on the chickens during their life, and that measurements were carried out after humane euthanasia of the chickens, it was not necessary to obtain prior ethical approval based on the Belgian and European legislation (EU directive 2010/63/EU).

Microbiota Composition

16S rRNA Gene Sequencing DNA was extracted from caecum and colon content from 3 chickens per pen, using the CTAB method (Griffiths et al., 2000, Kowalchuk et al., 2000). For each caecum and colon sample (9 chickens of each group), 16S rRNA Polymerase chain reaction (**PCR**) libraries specific for bacteria were generated using primers E9-29 and E514-430 (Brosius et al., 1981) targeting hyper variable region V1-V3. The 454 Life Sciences's sequencing oligonucleotide design included 2 different titanium adapters named A or B (Roche Diagnostics, Vilvoorde,

Belgium) and multiplex identifiers fused to the 5′ end of each primer. The amplification mix contained 5U of FastStart high fidelity polymerase (Roche Diagnostics, Vilvoorde, Belgium), 1x enzyme reaction buffer, 200 μm dNTPs (Eurogentec, Liège, Belgium), 0.2 μm of each primer, and 100 ng of extracted DNA in a volume of 100 μl. Thermocycling conditions consisted of a denaturation at 94°C for 15 min followed by 25 cycles each of 94°C for 40 s, 56°C for 40 s, 72°C for 1 min and a final elongation step of 7 min at 72°C. These amplifications were performed on an Ep Master system gradient apparatus (Eppendorf, Hamburg, Germany). The PCR products were run on a 1% agarose gel electrophoresis and the DNA fragments were plugged out and purified using the SV PCR purification kit (Promega Benelux, Leiden, The Netherlands). The quality and quantity of the products were assessed with a Picogreen dsDNA quantitation assay (Isogen, St-Pieters-Leeuw, Belgium). All libraries were run in the same titanium pyrosequencing reaction using Roche multiplex identifiers. All amplicons were sequenced using the Roche GS-Junior Genome Sequencer instrument (Roche, Vilvoorde, Belgium), the sequence number of each sample was normalized to 1,836 reads.

The 16S rRNA sequence reads were processed with the MOTHUR package (Schloss et al., 2009). The quality of all sequence reads were denoised using the Pyronoise algorithm implemented in MOTHUR and filtered with the following criteria: minimal length of 425 bp, an exact match to the barcode, and one mismatch allowed to the proximal primer. The sequences were checked for the presence of chimeric amplifications using Uchime (Edgar et al., 2011). The resulting read sets were compared to a reference dataset of aligned sequences of the corresponding region derived from the SILVA database 1.15 of full-length rDNA sequences (http://www.arb-silva.de/) implemented in MOTHUR (Pruesse et al., 2007). The final reads were clustered into operational taxonomic units (**OTU**s) using the nearest neighbor algorithm using MOTHUR with a 0.03 distance unit cut-off. At the OTU level of analysis (OTU definition level for a 0.02 distance matrix), a total of 5,967 OTUs were created. A taxonomic identity was attributed to each OTU by comparison with the SILVA database (80% homogeneity cut-off).

As a secondary analysis all unique sequences for each OTU were compared to the SILVA dataset 1.15 using BLASTN algorithm (Altschul et al., 1990). For each OTU, a consensus detailed taxonomic identification was given based upon the identity (less than 1% of mismatch with the aligned sequence) and the metadata associated with the best hit (validated bacterial species or not). The raw sequences were deposited in Genbank (accession number pending).

Quantitative PCR The number of *Alistipes* bacteria in the caeca of 3 chickens per pen was determined via qPCR using forward (5′-TTAGAGATGGGCATGCGTTGT-3′) and reverse (5′-TGAATCCTCCGTATT-3′) primers (Vigsnaes et al., 2012). The amplification and detection was performed using the CFX384 Biorad detection system (Biorad, Nazareth-Eke, Belgium). Each reaction was done in triplicate in a 12 μl total reaction mixture using 2x SensiMix SYBR No-ROX mix (Bioline, Kampenhout, Belgium), 3.0 μm final primer concentration, and 2 μl of (50 ng/μl) DNA. The amplification program consisted of 1 cycle at 95°C for 10 min followed by 40 cycles of 15 s at 95°C, 30 s at 52°C, and 20 s at 72°C. The fluorescent signal was detected at the last step of each cycle.

In vitro Fermentation

Bacterial Strain and Growth Conditions *Alistipes finegoldii* DSM 17242^T was purchased from the Leibniz institute DSMZ-German collection of microorganisms and cell cultures. This strain was grown on Columbia blood agar or in Tryptone Soya broth (**TSB**) in an anaerobic chamber (Ruskinn technology, Bridgend, United Kingdom) with 84% N_2, 8% H_2, and 8% CO_2 at 37°C for 2 D.

The in vitro fermentation study was conducted using TSB without glucose (17 g/L pancreatic digest of casein, 3 g/L enzymatic digest of soya bean, 5 g/L NaCl, 2.5 g/L K_2HPO_4) supplemented with 1 mg/mL cysteine-HCl, and a mixture of SCFAs (final concentrations: acetate (31 mM); propionate (9 mM); isobutyrate, isovalerate, and valerate (1 mM each). A 5% stock solution of the cellulose that was used in the in vivo trial, was prepared in TSB without glucose, autoclaved and diluted to a final concentration of 0.5% (v/v). Non-supplemented TSB without glucose was used as a control (blank). The final pH of the medium was adjusted to 7.0 ± 0.1. The media were pre-incubated in an anaerobic cabinet. *Alistipes finegoldii,* pre-cultured in TSB, at 37°C under anaerobic conditions for 48 ± 1 h without shaking, was diluted 100 times in the supplemented and non-supplemented TSB without glucose. After 5 D of anaerobic incubation at 37°C, 10-fold dilutions of the cultures were plated on Colombia blood agar and incubated to determine the titer.

Statistical Analysis

GraphPad Prism software version 5 was used for the statistical analysis of the performance data except for body weight (**BW**). Differences in FCR, feed intake, and average daily gain (**ADG**) between the treatment and the control group were analyzed with an independent samples t-test. The BW and qPCR were analyzed by means of a linear mixed effect model with pen included as random effect, using S-Plus. The differences were considered statistically significant at P value ≤ 0.05 and considered as a tendency at P value ≤ 0.1. The in vitro fermentation, growth, and succinate

Table 2. Effect of in-feed supplementation of cellulose on feed conversion ratio (FCR), body weight (BW) (g), feed intake (FI) (g/d/bird), and average daily gain (ADG) (g/d/bird). Values are presented as the mean ± standard error and were determined at 3 different time points (D13, D26, and D39).

Intervals in days	Treatment	FCR	BW (g)	FI (g/d/bird)	ADG (g/d/bird)
0 to 13	− **cellulose**	1.46 ± 0.02	354 ± 4.34	34.7 ± 0.40	23.8 ± 0.48
	+ **cellulose**	1.37 ± 0.03	381 ± 4.80	35.4 ± 0.38	25.8 ± 0.36
	P-value	0.04	0.05	0.23	0.04
14 to 26	− **cellulose**	1.57 ± 0.01	1210 ± 14.27	103.9 ± 1.14	66.2 ± 0.97
	+ **cellulose**	1.55 ± 0.03	1258 ± 15.86	107.2 ± 1.18	69.3 ± 1.52
	P-value	0.48	0.18	0.07	0.11
27 to 39	− **cellulose**	1.82 ± 0.01	2545 ± 30.50	183.9 ± 1.39	101.1 ± 1.15
	+ **cellulose**	1.86 ± 0.02	2539 ± 35.64	183.5 ± 2.82	98.8 ± 2.06
	P-value	0.11	0.93	0.76	0.35
0 to 26	− **cellulose**	1.54 ± 0.01		69.3 ± 0.71	45.0 ± 0.68
	+ **cellulose**	1.50 ± 0.02		71.3 ± 0.44	47.6 ± 0.70
	P-value	0.11		0.07	0.05
0 to 39	− **cellulose**	1.66 ± 0.01		107.5 ± 0.85	64.8 ± 0.44
	+ **cellulose**	1.65 ± 0.02		108.7 ± 1.21	65.8 ± 0.98
	P-value	0.91		0.75	0.59

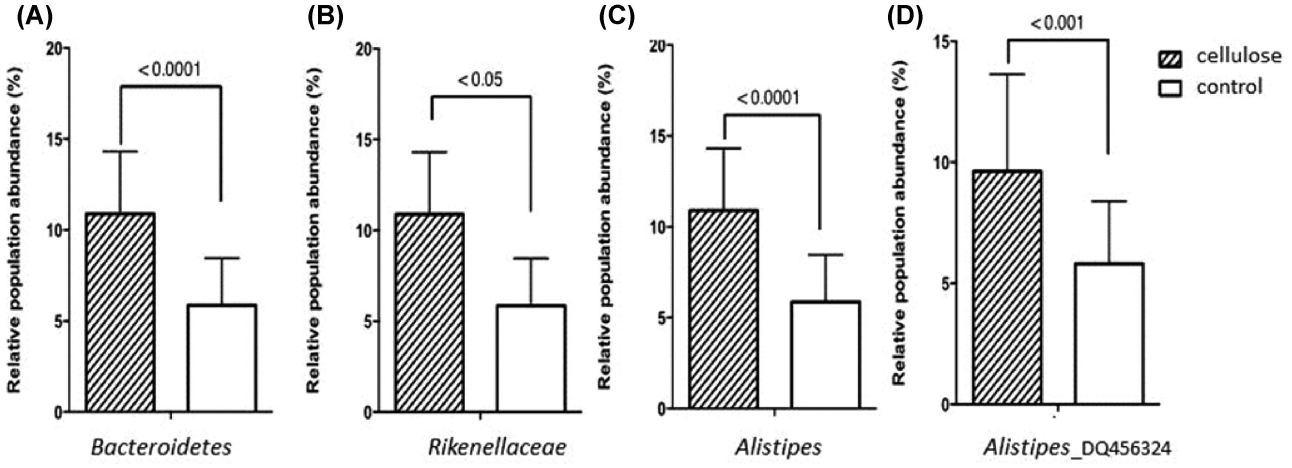

Figure 1. Bar charts showing relative population abundance percentage of (A) the phylum *Bacteroidetes*, (B) family *Rikenellaceae*, (C) genus *Alistipes,* and (D) unknown species DQ456324 within the genus *Alistipes* in the caecum, at day 26, in broilers fed a wheat-based diet either or not supplemented with cellulose.

concentration were analyzed with an independent samples t-test.

RESULTS

Performance

Broiler performance data are shown in Table 2. During the starter period, supplementation of cellulose resulted in a significant increase of the ADG ($P = 0.047$) and a significant decrease of the FCR ($P = 0.029$). Feed supplementation of cellulose tended to increase the BW at day 13 ($P = 0.053$). No significant differences were determined for ADG, FCR, and BW at day 26 and 39 between the treatment and the control groups. The feed intake did not differ significantly between the treatment and the control group during the whole trial. Mortality was low and not significantly different between groups.

Microbiota Composition as Determined by 16S rRNA Sequencing and qPCR

There were no significant differences in microbiota composition in the colon between the treatment and the control group. In the caecum, supplementation of cellulose resulted in a significant ($P < 0.0001$, Figure 1) increase of bacteria belonging to the phylum *Bacteroidetes*. This increase was the result of a significant ($P < 0.05$) increase of the family *Rikenellaceae* and within this family solely in the genus *Alistipes* ($P < 0.0001$, Figure 1). One unknown species (DQ456324) within this genus was significantly ($P < 0.001$) more abundant when cellulose was supplemented. A qPCR using primers that specifically amplify *Alistipes* bacteria confirmed the increase of the genus in the caecal microbiota of broilers fed the cellulose-supplemented feed (Figure 2).

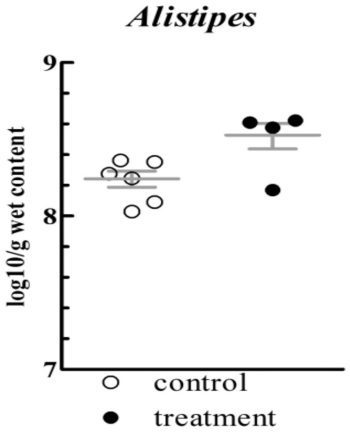

Figure 2. Number of *Alistipes* bacteria expressed as $\log_{10}$ copy number of the 16S rRNA gene per gram of wet caecal content in 26-day old chickens fed a wheat-based diet either or not supplemented with cellulose. Each dot represents the mean value of 3 chickens per pen. The grey line shows the overall mean and standard error of the mean. ○, control group; •, treatment group.

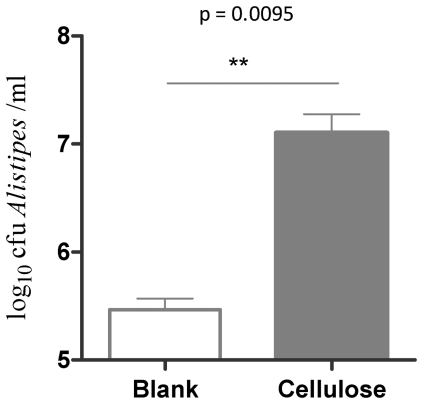

Figure 3. Log_{10} cfu *Alistipes finegoldii* per mL Tryptone Soya broth without cellulose (blank) or with cellulose after 5 D of incubation. The in vitro experiment was done twice in triplicate. Statistical analysis was done with GraphPad Prims 5, using an independent t-test.

The unknown species of the 16S sequencing data showed highest 16S rRNA gene sequence similarity with *Alistipes putredinis* (96.48%) and *Alistipes finegoldii* (95.44%) using the EzTaxon database.

In vitro Fermentation

The in vitro culture of *Alistipes finegoldii* showed a significant ($P = 0.01$, Figure 3) increase in growth in the presence of cellulose as sole carbon source after 5 D of anaerobic incubation.

DISCUSSION

Many studies have shown that dietary changes, such as the addition of dietary fiber, may result in a shift in the intestinal microbiota composition (Knarreborg et al., 2002; Shakouri et al., 2006). In rats harboring a human microbiota, the addition of a mix of short chain fructo-oligosaccharides and inulin induced a bifidogenic effect in the caecum (Kleessen et al., 2001). In another study in rats, supplementation of arabinoxylan induced an increase in SCFA producing species such as *Roseburia intestinalis* and *Eubacterium rectale* (Van den Abbeele et al., 2011). Courtin *et al.* observed a bifidogenic effect upon administration of arabinoxylo-oligosaccharide and soluble arabinoxylan to the diet of broilers (Courtin et al., 2008). In another broiler study, the in-feed supplementation of xylo-oligosaccharides increased caecal levels of members of the butyrate producing *Clostridium* cluster XIVa (De Maesschalck et al., 2015).

Cereal coproducts such as brans and hulls are concentrated sources of cellulose forming a network of crystalline cellulose microfibrils embedded in an amorphous matrix composed of hemicellullose, pectin, and lignin, and stabilized by intra- and intermolecular hydrogen bonds (Celino et al., 2014). Within microfibrils the degree of crystallinity may vary, generating regions that are more amorphous (Knudsen, 2014). Highly crystalline cellulose is particularly recalcitrant to enzymatic degradation, whereas amorphous forms are more accessible (Flint et al., 2012). As such, bacteria able to degrade amorphous cellulose could be isolated from most human individuals, although bacteria able to degrade crystalline cellulose substrates were not always recoverable (Wedekind et al., 1988; Robert and Bernalier-Donadille, 2003).

In the current trial, amorphous cellulose was supplemented to feed, of which the intrinsic cellulose concentration was calculated as less than 0.5% based on the acid detergent fiber value, which is the sum of cellulose and lignin. As a result, the effect on the microbiota composition may largely be due to the 0.5% or 1% supplemented cellulose. To break down cellulose, the gut microbiota needs specific enzymes that can degrade cellulose (Smits and Annison, 1996). In humans, members of the phylum *Bacteroidetes* have been shown to harbor several genes encoding cellulose degrading enzymes (De Flippo et al., 2010; Hamaker and Tuncil, 2014). In the present chicken trial, administration of cellulose resulted in a higher abundance of the phylum *Bacteroidetes* and more specifically in a significant increase of the genus *Alistipes*. This genus harbors anaerobic, non-spore forming, and non-motile Gram-negative bacteria isolated from the human feces (Rautio et al., 2003; Song et al., 2006; Nagai et al., 2010). *Alistipes* spp. are considered to be part of the human intestinal core microbiome (Claesson et al., 2012) and to represent more than 1% of the bacterial sequences in the chicken gut (Wei et al., 2013). Although a positive association between broiler performance and abundance of *Alistipes finegoldii* in their caeca has been shown (Torok et al., 2011), the role of *Alistipes* spp. in energy harvest from lower intestinal tract fermentation processes was hitherto unclear. In the present study, we showed the ability of *Alistipes* spp. to grow on cellulose. The major fermentation end-product of *Alistipes* bacteria is succinate (Rautio et al., 2003; Reichardt et al., 2014) that can provide energy in 2 distinct ways. First,

succinate can be taken up directly by chicken intestinal cells through a sodium-dependent transport system (Kimmich et al., 1991) and then further introduced in the tricarboxylic acid or Krebs cycle. Secondly, it can be used by numerous other *Bacteroidetes* bacteria which may convert succinate into propionate after decarboxylation, which appears to be the most prominent route for propionate formation (Reichardt et al., 2014). Propionate in turn can be used as an energy source by the epithelial cells and is known to have health-promoting effects, including an anti-inflammatory activity, which may influence performance (Hosseini et al., 2011; Vinolo et al., 2011). Besides the effect on the microbial fermentation, in-feed supplementation of cellulose has been hypothesized to induce a more intensive protein metabolism because the protein content in the pancreas as well as the activity of the proteolytic enzymes of the pancreas gland were higher in chickens that were administered cellulose into their feed, resulting in a higher BW (Boguslawska-Tryk, 2005). Conclusive data on the effect of in-feed cellulose administration to broilers on animal performance should be investigated in more detail in larger in vivo studies and cannot be concluded from our data.

In conclusion, in-feed supplementation of cellulose resulted in an increased abundance of the *Alistipes* genus in the caeca. It is hypothesized that this genus may influence performance by producing succinate as metabolic end-product.

ACKNOWLEDGMENTS

The authors are grateful to the Productschap Diervoeder of The Netherlands government for financial support of this work. The authors acknowledge the PhD students from the department of pathology, bacteriology, and avian diseases who assisted in the sampling in the in vivo trial.

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