Effects of dietary inulin supplementation on the composition and dynamics of cecal microbiota and growth-related parameters in broiler chickens

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ABSTRACT Inulin, a prebiotic, is an attractive alternative to antibiotic growth promoters in chickens. Dietary supplementation with inulin can improve growth performance, carcass yield, immune system activity, and serum biochemical parameters in chickens. A few studies investigated the impact of dietary inulin supplementation on chicken intestinal microbiota. In this study, we investigated how and why dietary supplementation with 1, 2, and 4% inulin can affect body weight gain, feed intake, food conversion rate, immunological parameters, serum biochemical parameters, and composition and dynamics of the cecal microbiota of Tegel broiler chickens using quantitative fluorescence in situ hybridization (qFISH). We showed that inulin inclusion has a negative effect on growth performance parameters before day 21 and a positive effect subsequently up to day 42. Quantitative FISH data revealed an age-dependent change in the cecal microbiota in the control broilers fed no inulin. Thus, relative abundances of *Firmicutes* and *Actinobacteria* decreased from 52.8 to 48.3% of total cells and from 8.7 to 1.4% at days 7 and 42, respectively. However, relative abundances of *Bacteroidetes* and *Proteobacteria* gradually increased from 9.3 to 26.9% of the total cells and from 10.7 to 21.1%, respectively, over the same periods. Inulin inclusion appeared to lower the relative abundances of *Lactobacillus johnsonii* and *Bifidobacterium* species at an early bird age, but it subsequently significantly ($P < 0.05$) increased their relative abundances. Such increases positively correlated with body weight gain of the birds, determined after day 21. Thus, dietary supplementation with inulin together with the addition of *L. johnsonii* and *Bifidobacterium* (*B. gallinarum* and *B. pullorum*) cultures at an early age may help overcome its early negative influence on growth performance. We believe that these findings can improve our knowledge on how inulin can change the intestinal microbiota of broiler chickens and help in developing an inulin feeding regime to optimize its beneficial role in chicken development.

Key words: broiler, inulin, cecal microbiota, growth performance, qFISH

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INTRODUCTION

Subtherapeutic levels of antibiotics have been used widely as antibiotic growth promoters (**AGP**) to improve health, optimize feed efficiency, and promote growth performance in the poultry industry (Brown et al., 2017). However, there is growing concern on how their widespread use can affect the spread of antibiotic resistant bacteria in the microbiota of the meat we consume (Bucław, 2016). The need to reduce antibiotic intake and eliminate antibiotic residues in poultry meat has encouraged the search for alternatives to AGP. Prebiotics, probiotics, exogenous enzymes, synbiotics, and plant extracts have all been proposed as possible alternatives (reviewed by Gadde et al., 2017). Prebiotics, non-metabolized food ingredients that pass through the gastrointestinal tract and change the gut microbiome in a manner that chicken production is improved, have attracted increasing attention (Teng and Kim, 2018).

Inulin is a powerful prebiotic and a valuable alternative to AGP (Bachanek et al., 2016). It consists of fructose subunits linked by 2,1 glycosidic linkages with a glucose terminal unit and is present in many vegetables, fruits, and cereals (Kozlowska et al., 2016). Dietary supplementation of inulin has been claimed to improve chicken growth performance, carcass yield, activity of the immune system, and important serum biochemical parameters (reviewed by Boguslawska-Tryk et al., 2012; Kozlowska et al., 2016). However, the rationale for using inulin is not clear, and the positive effects of inulin inclusion claimed above are not widely reported (Biggs et al., 2007; Ortiz et al., 2009; Li et al., 2018). Furthermore, it seems that any positive

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impact of dietary supplementation of inulin depends on its source and inclusion level, together with the basal diet composition, individual animal characteristics, and experimental conditions of hygiene (Verdonk et al., 2005).

Understanding the functions of the cecal microbiota of broilers is important because of its roles in preventing pathogen colonization, detoxifying harmful substances, recycling nitrogen, and degrading and absorbing additional nutrients, affecting host metabolism and its immunological activity (reviewed by Kogut 2018; Sood et al., 2019). Few studies have examined the effects of dietary inulin supplementation on chicken intestinal microbiota, focusing on its possible effects on the intestinal probiotic and pathogenic populations. Inulin supplementation has been reported to stimulate growth of members of *Bifidobacterium* and *Lactobacillus* in some (Rebolé et al., 2010; Shang et al., 2010; Nabizadeh, 2012a; Krismiyanto et al., 2014; Liu et al., 2018) but not in all studies (Bachanek et al., 2016; Li et al., 2018). Moreover, most of these studies used culture-dependent techniques, focused on a small number of functional microbial groups, and most importantly, were based on single cecal samples taken usually at the end of their experiments. Thus, data revealing the dynamics of the cecal microbiota in response to inulin inclusion, essential to understanding its mode of action, remain scarce.

In this study, we investigated the effects of dietary supplementation of inulin on body weight (**BW**) gain, feed intake (**FI**), food conversion rate (**FCR**), immunological parameters, and serum biochemical parameters of broiler chickens fed a basal corn-based diet. Furthermore, we examined the composition and dynamics of gene-probe targeted members in the cecal microbiota using quantitative fluorescence in situ hybridization (**qFISH**). We believe that the data presented here add additional knowledge on how inulin supplementation can modify the intestinal microbiota of broiler chickens and contribute towards optimizing a more beneficial chicken feeding program.

Table 1. Composition of the experimental diet.

Ingredient	Starter (0 to 21 D)	Grower (22 to 42 D)
Corn (%)	58.00	61.80
Soybean meal (%)	27.00	23.70
Corn gluten meal (%)	5.00	6.90
Fish meal (%)	2.80	0.00
Soybean oil (%)	3.00	3.20
Calcium hydrogen phosphate (%)	1.45	1.68
Fine stone powder (%)	1.15	0.76
Coarse stone powder (%)	0.00	0.40
Salt (%)	0.23	0.33
Methionine (%)	0.17	0.06
Lysine (%)	0.16	0.17
Premix[1] (%)	1.00	1.00
Total	100	100
Calculated nutrition composition		
ME (KC/kg)	3050	3100
CP (%)	22.0	20.0
Ca (%)	1.0	1.06
P (%)	0.70	0.74
Zn (mg/kg)	185	210
Fe (mg/kg)	337	404
Mn (mg/kg)	226	278
Mg (%)	0.17	0.19
Cu (mg/kg)	59.0	70.5
Na (%)	0.24	0.34
K (%)	0.81	0.72
AP (%)	0.45	0.40
NaCl (%)	0.35	0.35
Crude protein (%)	23.6	22.6
Crude fiber (%)	2.70	2.33
Crude fat (%)	4.82	4.49
Dry matter (%)	89.2	89.9
Tryptophane (%)	0.26	0.23
Aspartic acid (%)	1.61	1.79
Threonine (%)	0.71	0.79
Serine (%)	0.95	1.04
Glutamic acid (%)	3.65	4.26
Glycine (%)	0.76	0.79
Alanine (%)	0.82	0.97
Cysteine	0.36	0.39
Valine(%)	0.53	0.57
Methionine (%)	0.33	0.33
Isoleucine (%)	0.79	0.88
Leucine (%)	2.15	2.52
Tyrosine (%)	0.61	0.76
Phenylalanine (%)	0.98	1.08
Histidine (%)	0.45	0.44
Lysine (%)	1.14	1.17
Argnine (%)	1.02	1.10
Proline (%)	1.13	1.10

[1]2.5 kg of vitamin premix contains: 10.8 g retinal, 1.6 g calcidiol, 72 g tocopheryl acetate, 8 g menadione, 7.2 g thiamine, 26.4 g riboflavin, 40 g niacin, 120 g calcium pantothenate, 12 g pyridoxine, 4 g folic acid, 0.06 g cyanocobalamin, 1000 g choline chloride, 0.4 g biotin.

MATERIALS AND METHODS

Birds, Diet, and Management

A total of 400 male 1-day-old Tegel broiler chicks were obtained from a local commercial hatchery (Yunling Guangda Breeder Ltd., Kunming, China) and randomly divided into 5 experimental dietary groups on the basis of similar BW (46 ± 0.5 g). Each group had 80 broilers arranged in 4 replicate stainless steel cages with plastic mesh floors (1.5 m^2 floor area/pen) and with 20 birds per cage. Birds were fed with a "starter" diet (Table 1) from day 1 to 21 and a "grower" diet from day 22 to 42. Both starter and grower diets were formulated to meet the nutrient requirements of broilers as recommended by NRC (1994). Five dietary treatments were applied: basal diet (**BD**) (control), BD plus 400 ppm flavomycin, BD plus 1% inulin (10 g inulin/kg), BD plus 2% inulin (20 g inulin/kg), and BD plus 4% inulin (40 g inulin/kg). Flavomycin (10% active ingredient content) was purchased from a local commercial poultry antibiotic supplier (Lukang Biological Manufacture Co., Shandong, China). Inulin derived from chicory roots with a polymerization degree of 10–60 was purchased from OraftiGR (BENEO-Orafti B 3300, Tienen, Belgium). Inulin and flavomycin were supplied in powder form and were mixed with the BD to reach the

designated concentrations by replacing the same amounts of corn powder.

Birds were housed under environmentally controlled conditions. Each cage was equipped with feeding and water troughs placed outside the cage. Diets were offered twice daily (8:00 am and 6:00 pm) and water was provided ad libitum. House temperature was maintained at 34°C for the first 5 D and gradually decreased to 24°C and maintained until the end of the experiment. Light was continuously on throughout the experiment. The birds were vaccinated against the ND virus (Weike Biological Engineering Ltd., Yangzhou, China) at days 10 and 26 via the spraying method. All experimental procedures were approved, and the birds were cared for according to the guidelines of the Institutional Animal Care and Use Committee of Yunnan Agricultural University, Kunming, China.

Measurement of Performance

Residual feed and birds were weighed individually every week to determine their FI, BW gain, and FCR.

Collection of Samples

Cecal content samples for qFISH were taken on days 7, 14, 21, and 42. On each sampling day, 12 birds from each group (3 chicks per replicate) were selected randomly from each cage and humanely killed by cervical dislocation. Gastrointestinal tracts were removed from the carcasses, and the ceca were cut open with sterile scissors. Fresh cecal contents of 12 birds were removed with sterile metal spatulas onto alumina paper. Aliquots (1 g) of cecal contents from individual birds in each group were pooled immediately and suspended in 50 mL sterilized 1 × PBS (0.1 M, pH 7.0) preheated at 37°C. The suspensions were subjected to mechanical pummeling for 6 min in a Colworth Stomacher 400 (A. J. Seward & Co., Ltd., London) before filtration through a 6-layer sterilized cheesecloth. Filtrates were immediately centrifuged at 800 × *g* for 10 min, and the supernatants were collected into 10-mL centrifuge tubes. The suspensions were centrifuged (14,000 × *g*, 10 min) again, the supernatants discarded, and the pellets resuspended in 5 mL 1 × PBS. A portion of this suspension was fixed in ice-cold ethanol (50% v/v final concentration) or paraformaldehyde (4% w/v final concentration) for FISH probing of Gram-positive (Roller et al., 1994) and Gram-negative (Amann, 1995) bacteria, respectively.

Blood samples were collected on days 21 and 42 from the subwing vein of birds, transferred to centrifuge tubes, and allowed to sit for approximately 1 h. Serum was obtained by centrifugation at 2200 × *g* for 15 min at 4°C, and the supernatant stored at −20°C for biochemical and immunoglobulin analyses. Immediately after blood sampling, the birds were sacrificed by cervical dislocation. After the gastrointestinal tract was removed, breast meat, thigh meat, and abdominal fat were immediately excised from each carcass and weighed. The mean values of the organ yield of each animal were calculated as percentages of BD to determine the ratios of each individual carcass components.

Analyses of Blood Parameters and Serum Immunoglobulin

Serum triacylglycerol (**TG**), total cholesterol (**TC**), high-density lipoprotein cholesterol (**HDL-C**), and low-density lipoprotein cholesterol (**LDL-C**) were estimated colorimetrically on an automatic analyzer (TBA-120 FR, Toshiba Medical System Co., Ltd., Tochigi, Japan) according to manufacturer's instructions (Nanjing Jianchen Bioengineering Institution, Nanjing, China). Serum immunoglobulin levels IgA (Cat. no. E33–103), IgG (Cat. no. E33–104), and IgM (Cat. no. E33–102) were assessed with the double-antibody sandwich method using ELISA quantitation kits (Bethyl Laboratories Inc., Montgomery, TX). Activities of complement components C3 and C4 were measured using the chicken-specific ELISA kits SJ155987 and SJ155986 (Yangyei Biological Engineering Institute, Shanghai, China), respectively, as per the manufacturer's protocol.

FISH Probing of Cecal Microflora

Oligonucleotide probes for FISH analyses were selected from probeBase (Greuter et al., 2016) based on identities of dominant microflora found in earlier reports on chicken cecal microbiome data (Mancabelli et al., 2016; Ferrario et al., 2017) and purchased from Sangon Biotech (Shanghai, China). All probes were labeled with fluorochrome Cy3. Their names, specificities, and optimal formamide hybridization concentrations (**OFH**) are listed in Table 2. Formamide concentrations for Probe Lab9057_570 designed for *Lactobacillus johnsonii* and *Lactobacillus gasseri* have not been optimized but were estimated in this study using DECIPHER (Wright et al., 2014). An OFH of 30% was used. If necessary, the pretreatments (Ramm et al., 2012) used in catalyzed reporter deposition-FISH to improve the permeability of cells were also adopted.

FISH analyses with probes Str, Lab9057_570, Lab2185_87, ALF968, Ent, Bif228, and MB1174 were carried out according to the methods described by Amann (1995), whereas those for probes Erec482, Bfa602, and Bdis656 were performed as described by Franks et al. (1998). To determine total microbial cell numbers, 4' 6-diamidino-2-phenylindole (**DAPI**) staining was carried out after FISH according to Kong et al. (2010). The FISH-probed cecal digesta samples were examined with an epifluorescence microscope (Leica DM6000B) equipped with a Leica DFC500 camera. Hybridization signals were detected, visualized,

Table 2. Oligonucleotide probes used in fluorescence in situ hybridization (specificity checked on 13 January 2019).

Probe name[1]	Sequence (5′-3′)	Specificity	FA[2]%
Erec482	GCTTCTTAGTCARGTACCG	Most of the *Lachnospiraceae* (1674/2092)	0
Str	CACTCTCCCCTTCTGCAC	Most *Streptococcus* species (1055/1256)	30
Lab9057_570	TGAACCGCCTGCACTCGCTTTAC	*Lactobacillus johnsonii* and *Lactobacillus gasseri*	30
Lab2185_87	TGGTGATCCATCGTCAATCAGGTG	*Lactobacillus reuteri*	40
Bfra602	GAGCCGCAAACTTTCACAA	Most *Bacteroides* species (1242/1488)	30
Bdis656	CCGCCTGCCTCAAACATA	*Parabacteroides distasonis*	0
ALF968	GGTAAGGTTCTGCGCGTT	*α-Proteobacteria*	20
Ent	CCCCCWCTTTGGTCTTGC	*Enterobacteriaceae* (4438/4600)	30
Bif228	GATAGGACGCGACCCCAT	Most *Bifidobacterium* species (1340/1380)	40
MB1174	TACCGTCGTCCACTCCTTCCTC	Most species in *Methanobacterium*, *Methanobrevibacter*, *Methanosphaera*	45

[1]All the probes are described in Probebase (Greuter et al.; 2016). Values in the parentheses represent the numbers of isolates their 16S rRNA sequences perfect match the sequence of a probe (numerator) out of the total numbers of isolates (denominator) in a phylogenetic group.
[2]Formamide concentration used in fluorescence in situ hybridization.

and randomly captured using Cy3 and DAPI filters for samples mounted on Citifluor (Citifluor Ltd., London, UK). The percentages of the cecal digesta cells hybridizing with each probe were estimated as percentages of total cell numbers of the same microscopic field stained by DAPI. Cell enumerations based on FISH images were performed according to the method described by Kong et al. (2010). At least 60 sets of images taken with the ×100 objective lens from 3 different slide wells (20 images from each) were counted for each sample.

Statistical Analysis

Physiochemical data for the broiler chickens were subjected to 1-way ANOVA to examine any effects from treatment with inulin and flavomycin. When significant differences were found, Duncan's multiple range test was used to rank the treatment groups. Linear regression was used to reveal the abundances of any probe-defined group or species that were positively linked to the tested individual growth-related parameters. Differences between the means of individual physiochemical data and the relative abundances of each probe-defined microbial group or species associated with different dietary treatments were compared using least significant differences. A P-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL).

RESULTS

Growth Performance of Chickens

The effects of the different dietary supplementations with inulin or flavomycin on growth performance of broilers are summarized in Table 3. Inclusion of inulin at 1, 2, and 4% levels or 400 ppm flavomycin in the control diet had no effect on either the BW gain or FI of birds by the end of the experiment (day 42). However, birds fed diets with either 2 or 4% inulin or 400 ppm flavomycin showed increases ($P < 0.05$) in BW gain from day 22 to 42. Those receiving these dietary supplements had lower ($P < 0.05$) FI from day 8 to 14 than those fed the control diet. None of the dietary treatments had significant ($P > 0.05$) effects on FCR or mortality of birds at any period during the experiments.

Carcass

The effects of dietary inclusion of inulin or flavomycin on abdominal fat deposition and thigh and breast muscle weights of birds are summarized in Table 4. After day 14, birds receiving 1, 2, and 4% inulin in their diets had lower ($P < 0.05$) relative thigh weight than those receiving the control diet. At the end of the experiment (day 42), only those fed 1% inulin had lower ($P < 0.05$) relative thigh weights. After day 14, birds receiving 1, 2, or 4% inulin feed supplement had lower ($P < 0.05$) relative breast muscle weights than those receiving the control diet, while those fed diets containing 2 or 4% inulin or 400 ppm flavomycin had higher ($P < 0.05$) relative abdominal fat weight than those receiving the control diet after day 42.

Serum Cholesterol

The effects of diet supplementation with inulin or flavomycin on selected serum biochemical parameters of birds are summarized in Table 5. While birds receiving 2 or 4% inulin in their diet had higher ($P < 0.05$) HDL-C concentrations after day 42 than those receiving 1% inulin, those receiving 4% inulin or 400 ppm flavomycin had lower ($P < 0.05$) TG than birds receiving the control diet at day 21. In birds fed diets with 1, 2, and 4% inulin or 400 ppm flavomycin, no significant ($P > 0.05$) effects on LDL-C and TC levels were observed at days 21 and 42.

Serum Immunoglobulins and Complement Components

Effects of dietary supplementation with inulin and flavomycin on blood immunoglobulins and complement

Table 3. Effects of dietary supplementation with 0 (control), 1, 2, and 4% inulin and 400 ppm flavomycin on the growth performance (1 to 42 D) of broiler chickens fed a corn-based diet.

Item	Control	Flavomycin	1% inulin	2% inulin	4% inulin	SEM	*P*-value
Mean weight (g)							
1 D	46.40	46.04	46.31	46.92	46.70	0.20	0.7390
8 D	96.62	97.15	93.08	94.02	94.64	0.72	0.3600
15 D	222.53	222.40	219.64	208.03	222.52	2.16	0.1270
22 D	412.57^{a}	388.01^{b,c}	374.63^{c}	370.63^{c}	402.71^{a,b}	4.80	0.0020
42 D	1125.37^{c,d}	1250.51^{a}	1109.45^{d}	1140.15^{c}	1161.93^{b}	13.47	0.0000
Body weight gain (g/bird)							
1 to 7 D	50.17	51.11	46.76	47.03	47.94	0.80	0.3472
8 to 14 D	125.86	123.31	126.00	121.36	125.10	1.30	0.8171
15 to 21 D	189.78	165.56	173.34	176.12	176.92	3.06	0.1443
22 to 42 D	718.90^{d}	853.57^{a}	723.62^{c,d}	765.98^{b}	754.47^{b,c}	13.53	0.0000
1 to 42 D	1078.74	1205.07	1063.09	1093.30	1113.65	18.70	0.1235
Feed intake (g/bird)							
1 to 7 D	57.98	60.94	58.48	58.06	60.50	0.42	0.2202
8 to 14 D	189.93^{a}	178.02^{b,c}	184.38^{a,b}	170.48^{c}	176.52^{b,c}	2.24	0.0261
15 to 21 D	319.17	294.93	291.15	279.16	294.77	4.67	0.0664
22 to 42 D	1586.06	1713.74	1589.90	1665.66	1653.67	30.00	0.6912
1 to 42 D	2275.28	2353.11	2238.85	2284.03	2296.98	32.53	0.9006
Feed conversion (feed:gain)							
1 to 7 D	1.16	1.20	1.25	1.24	1.26	0.02	0.3126
8 to 14 D	1.43	1.44	1.46	1.41	1.41	0.03	0.9812
15 to 21 D	1.68	1.79	1.68	1.59	1.67	0.03	0.4024
22 to 42 D	2.24	2.01	2.20	2.17	2.19	0.04	0.3817
1 to 42 D	2.11	1.95	2.11	2.09	2.06	0.03	0.5997
Mortality (%)							
1 to 7 D	1.59	0.00	2.38	2.38	2.38	0.72	0.8922
8 to 14 D	1.59	0.00	0.00	0.00	0.00	0.43	0.7103
15 to 21 D	1.59	0.00	0.00	0.00	0.00	0.43	0.7105
22 to 42 D	1.52	0.00	0.00	0.00	0.00	0.41	0.7102
1 to 42 D	4.69	0.00	2.38	2.38	2.38	0.74	0.8169

Values are the means of 4 pens of at least 10 birds per pen. Values with different superscripts in the same row are significantly different ($P < 0.05$).

Table 4. Effects of dietary supplementation with 0 (control), 1, 2, or 4% inulin or 400 ppm flavomycin on the relative weight of thigh, breast, and abdominal fat of broiler chickens fed a corn-based diet.

Carcass parameters	Age (D)	Control	Flavomycin	1% inulin	2% inulin	4% inulin	SEM	*P*-value
Thigh	7	8.06	8.17	7.05	7.69	7.85	0.16	0.1750
(% body weight)	14	10.01^{a}	10.39^{a}	8.85^{b}	8.79^{b}	8.83^{b}	0.18	0.0010
	21	10.94	11.66	11.81	10.55	11.23	0.22	0.3480
	42	12.76^{a}	12.84^{a}	11.58^{b}	13.24^{a}	13.47^{a}	0.16	0.0000
Breast muscle	7	5.14	5.05	4.68	5.11	5.11	0.16	0.7030
(% body weight)	14	8.06^{a}	7.50^{a,b}	6.68^{c}	7.17^{c}	6.96^{b,c}	0.13	0.0030
	21	9.32	8.51	9.24	8.14	8.92	0.16	0.0860
	42	9.74	10.18	10.04	9.99	9.85	0.14	0.6630
Abdominal fat	7	n/d*	n/d	n/d	n/d	n/d	n/d	n/d
(% body weight)	14	0.56	0.36	0.45	0.45	0.48	0.02	0.2020
	21	0.84	0.88	0.95	0.90	0.86	0.06	0.7420
	42	0.61^{b}	1.00^{a}	0.57^{b}	1.02^{a}	1.01^{a}	0.05	0.0000

Values are the means of 4 pens of 3 birds per pen. Values with different superscripts in the same row are significantly different ($P < 0.05$). *n/d: not determined.

components of birds are summarized in Table 6. Although inclusion of 1, 2, and 4% inulin in diets increased blood C3 and IgM levels at day 21, no such effects were observed at day 42. In contrast, inclusion of 400 ppm flavomycin increased ($P < 0.05$) blood IgM levels only in the birds at day 21, whereas inclusion of 1, 2, and 4% inulin or 400 ppm flavomycin had no significant ($P < 0.05$) impact on concentrations of blood C4, IgA, and IgG after days 21 and 42.

Composition and Dynamics of FISH Probe-Targeted Members in the Ceca of Chickens Fed Diets With or Without Inulin

Compositions and dynamics of FISH probe-targeted members in the cecal microbiota of broilers fed a BD supplemented with or without inulin or flavomycin were investigated at days 7, 14, 21, and 42 using qFISH. Trends of relative percentage abundances (**RPA**) of

Table 5. Effects of dietary supplementation with 0 (control), 1, 2, or 4% inulin or 400 ppm flavomycin on the serum biochemical parameters of broiler chickens fed a corn-based diet.

Serum indices	Age (D)	Control	Flavomycin	1% inulin	2% inulin	4% inulin	SEM	*P*-value
LDL-C (mmol/L)	21	0.95	0.62	0.69	0.74	0.65	0.05	0.1712
	42	0.77	0.76	0.85	0.88	0.95	0.04	0.6345
HDL-C (mmol/L)	21	2.25	2.06	1.89	2.31	2.13	0.06	0.1363
	42	1.87^{a,b}	1.91^{a,b}	1.59^{b}	2.01^{a}	2.23^{a}	0.07	0.0334
TC (mmol/L)	21	3.00	2.50	2.40	2.92	2.58	0.09	0.0956
	42	2.62	2.70	2.40	2.88	3.08	0.08	0.0921
TG (mmol/L)	21	0.52^{a}	0.34^{b}	0.38^{a,b}	0.51^{a}	0.34^{b}	0.03	0.0367
	42	0.45	0.61	0.49	0.55	0.54	0.02	0.1781

LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; TC: total cholesterol; TG: triacylglyceride. Values are the means of 4 pens of 3 birds per pen. Values with different superscripts in the same row are significantly different ($P < 0.05$).

Table 6. Effects of dietary supplementation with 0 (control), 1, 2, or 4% inulin or 400 ppm flavomycin on immunoglobulins and complement components of broiler chickens fed a corn-based diet.

Parameter	Age (D)	Control	Flavomycin	1% inulin	2% inulin	4% inulin	SEM	*P*-value
C3 (μg/L)	21	1273.67^{b}	1333.82^{b}	1572.49^{a}	1556.57^{a}	1589.88^{a}	35.766	0.0021
	42	1678.35	1730.74	1714.85	1751.68	2043.86	50.553	0.1372
C4 (μg/L)	21	544.25	626.09	640.90	650.67	681.05	20.380	0.2906
	42	769.38	794.61	795.99	799.45	812.48	15.171	0.9428
IgA (ng/mL)	21	357.85	347.50	363.38	377.22	394.37	13.808	0.8367
	42	428.81	498.87	434.30	448.15	485.59	20.012	0.5741
IgG (ng/mL)	21	238.19	241.86	234.45	225.66	223.50	10.389	0.9750
	42	242.87	254.44	277.96	285.57	267.70	10.113	0.7023
IgM (μg/mL)	21	6.55^{b}	8.29^{a}	8.74^{a}	8.36^{a}	9.03^{a}	0.287	0.0455
	42	10.10	10.81	12.13	10.91	10.99	0.410	0.6637

Values are the means of 4 pens of 3 birds per pen. Values with different superscripts in the same row are significantly different ($P < 0.05$).

individual probe-defined microbial groups or species are shown in Table 7. In total, these FISH probes hybridized with 66.7 (± 7.0)–102.7 (± 10.4)% of total number of microbial cells in each of the individual cecal samples examined after days 7, 14, 21, and 42 (Table 7). In all samples examined, members of the *Firmicutes*, comprising *Lachnospiraceae*, hybridizing with FISH probe Erec482, *Streptococcus* and FISH probe Str, *Lactobacillus* and FISH probes Lab9057_570 and Lab2185_87, and *Bacteroidetes* (comprising *Bacteroides* and *Parabacteroides* hybridizing with FISH probes Bfra602 and Bdis656, respectively) constituted the majority (32.5 to 55.6% and 9.3 to 34.1%, respectively) of the total number of cells in the cecal microbiota. On the contrary, the FISH data suggested that members of the *α-Proteobacteria* and *Enterobacteriaceae* hybridizing with probes ALF968 and Ent, respectively, and the *Actinobacteria* (comprising *Bifidobacterium* hybridizing Bif228) contributed 8.6 to 22.7% and 0.8 to 11.9% of total cells, respectively.

Supplementation with 1, 2, and 4% inulin decreased the RPA (Table 7) of *Lachnospiraceae* (Figure 1A) and *Methanobacteriaceae* (Figure 1B) and increased the RPA of *L. johnsonii* group (Figure 1C), *Bifidobacterium* (Figure 1D), and *Parabacteroides distasonis* (Figure 1E). In contrast, supplementation with inulin at the same 3 concentrations increased the RPA (Table 7) of *Lactobacillus reuteri* (Figure 1F), *Bacteroides* (Figure 1G), and *α-Proteobacteria* (Figure 1H) in samples taken before day 17, but their RPA subsequently decreased. Diet supplementation with inulin had no increasing effects ($P > 0.05$) on the RPA of either *Streptococcus* (Figure 1I) or members of the *Enterobacteriaceae* (Figure 1J).

Composition and Dynamics of FISH Probe-Targeted Members in the Cecal Microbiota of Chickens Supplemented With or Without Flavomycin

Inclusion of 400 ppm flavomycin in the diets decreased significantly ($P < 0.05$) the RPA (Table 7) of *Lachnospiraceae* and *Bifidobacterium*, while increasing ($P < 0.05$) the RPA (Table 7) of *L. johnsonii* group and *P. distasonis*. However, it had no greater ($P < 0.05$) effect on the RPA (Table 7) of *Streptococcus*, *L. reuteri*, *Bacteroides*, *α-Proteobacteria*, *Enterobacteriaceae*, and *Methanobacteriaceae*.

Do Changes in the Composition of the Cecal Microbiome Explain BW Gain?

Linear regression analyses were used to identify the RPA of individual probe-defined populations that correlated positively with the changes in BW. The RPA of the *L. johnsonii* group hybridizing with probe Lab9057_570 in ceca of birds fed diets containing 2 or 4% inulin positively or tended towards positively (*P*-value of 0.044 and 0.053, respectively) correlated

Table 7. Effects of dietary supplementation of 0 (control), 1, 2, or 4% inulin, or 400 ppm flavomycin on related percentage abundances of oligonucleotide probe-defined microbial group or species in the cecal microbiota of broiler chickens fed a corn-based basal diet.

Probe-defined microbial group or species	Age (D)	Control	Flavomycin	1% inulin	2% inulin	4% inulin	SEM	*P*-value
Bacteria (%)								
Lachnospiraceae	7	0.280^{b}	0.202^{c}	0.307^{ab}	0.301^{c}	0.335^{a}	0.017	0.0000
(probe Erec482)	14	0.262	0.171	0.244	0.136	0.253	0.023	0.2550
	21	0.216^{a}	0.124^{a,b}	0.138^{a,b}	0.102^{b}	0.199^{a}	0.020	0.0360
	42	0.204^{a}	0.111^{b}	0.114^{b}	0.039^{c}	0.033^{c}	0.017	0.0000
Streptococcus spp.	7	0.048	0.030	0.032	0.035	0.028	0.003	0.3240
(probe Str)	14	0.059	0.055	0.047	0.043	0.040	0.003	0.0700
	21	0.072	0.067	0.058	0.052	0.059	0.004	0.5640
	42	0.128	0.087	0.089	0.064	0.107	0.008	0.0740
Lactobacillus johnsonii group	7	0.166	0.134	0.100	0.067	0.147	0.014	0.1820
(probe Lab9057_570)	14	0.160	0.133	0.120	0.096	0.156	0.008	0.0590
	21	0.136	0.168	0.187	0.146	0.168	0.014	0.5580
	42	0.100^{b}	0.213^{a}	0.200^{a}	0.200^{a}	0.189^{a}	0.013	0.0110
Lactobacillus reuteri	7	0.034	0.047	0.065	0.056	0.046	0.009	0.5210
(probe Lab2185_87)	14	0.045^{b}	0.047^{b}	0.102^{a}	0.072^{a,b}	0.080^{a,b}	0.007	0.0160
	21	0.066^{b}	0.101^{a}	0.043^{b,c}	0.053^{b,c}	0.023^{c}	0.008	0.0010
	42	0.051^{a,b}	0.079^{a}	0.027^{b,c}	0.023^{b,c}	0.012^{c}	0.008	0.0090
Bacteroides spp.	7	0.059^{c}	0.066^{b,c}	0.082^{b}	0.065^{b,c}	0.119^{a}	0.006	0.0000
(probe Bfra602)	14	0.087^{c}	0.090^{c}	0.134^{b}	0.095^{c}	0.152^{a}	0.007	0.0000
	21	0.106^{c}	0.117^{b,c}	0.259^{a}	0.153^{b}	0.222^{a}	0.017	0.0000
	42	0.176	0.151	0.161	0.108	0.158	0.016	0.6240
Parabacteroides distasonis	7	0.034^{b}	0.042^{b}	0.051^{b}	0.069^{a}	0.070^{a}	0.004	0.0020
(probe Bdis656)	14	0.071	0.090	0.062	0.066	0.070	0.004	0.1600
	21	0.106	0.116	0.082	0.089	0.098	0.008	0.9030
	42	0.093^{b}	0.126^{a}	0.135^{a}	0.132^{a}	0.122^{a}	0.005	0.0140
Bifidobacterium spp.	7	0.087	0.044	0.025	0.041	0.067	0.010	0.3630
(probe Bif228)	14	0.056^{b}	0.029^{c}	0.045^{b,c}	0.055^{b}	0.056^{a,b}	0.007	0.0020
	21	0.037^{c}	0.012^{d}	0.049^{b,c}	0.066^{b}	0.091^{a}	0.006	0.0000
	42	0.014^{b}	0.008^{b}	0.085^{a}	0.097^{a}	0.119^{a}	0.013	0.0000
α-Proteobacteria	7	0.098	0.083	0.095	0.093	0.092	0.003	0.6620
(probe ALF968)	14	0.129^{b,c}	0.109^{c}	0.157^{a,b,c}	0.195^{a}	0.170^{a,b}	0.010	0.0250
	21	0.153	0.1861	0.097	0.135	0.148	0.012	0.1780
	42	0.208^{a}	0.224^{a}	0.065^{b}	0.091^{b}	0.116^{b}	0.020	0.0100
Enterobacteriaceae	7	0.010^{a}	0.003^{b}	0.009^{a}	0.007^{a,b}	0.005^{b}	0.001	0.0140
(probe Ent)	14	0.004	0.002	0.006	0.005	0.007	0.001	0.1930
	21	0.004	0.003	0.005	0.004	0.005	0.000	0.5080
	42	0.003	0.003	0.002	0.002	0.001	0.000	0.0790
Archaea (%)								
Methanobacteriaceae	7	0.026	0.016	0.011	0.024	0.024	0.003	0.3710
(probe MB1174)	14	0.032^{a,b}	0.019^{c}	0.025^{a}	0.030^{a}	0.026^{b,c}	0.002	0.0020
	21	0.036^{a}	0.027^{a,b}	0.021^{b,c}	0.026^{a,b,c}	0.014^{c}	0.002	0.0170
	42	0.044^{a}	0.033^{a,b}	0.016^{b,c}	0.026^{a,b,c}	0.012^{c}	0.004	0.0250

Values are the means of 4 pens of 3 birds per pen. Values with different superscripts in the same row are significantly different ($P < 0.05$).

with chicken BW gain of the birds. A similar trend was observed for *Bifidobacterium* spp. in response to the same diets (*P*-value of 0.038 and 0.070, respectively). Furthermore, the RPA of *P. distasonis* with diets supplemented with 1, 2, or 4% inulin also positively or tended towards positively (*P*-value of 0.023, 0.031, and 0.057, respectively) correlated with BW gain of the birds.

DISCUSSION

Inulin is a prebiotic that has the potential to replace AGP in chicken diets to maintain productivity and animal health in poultry industry (Bucław, 2016). However, its mode of action is complex, and its effectiveness in poultry nutrition seems to depend on several factors (Bucław, 2016). In this study, incorporating inulin into a basic control diet at 3 different concentrations did not significantly improve the overall growth performance of Tegel broilers after 42 D. However, it led to a binary effect on their growth performance. Thus, a negative impact was observed from approximately day 1 to 21. During that early period, birds fed these diets had lower ($P < 0.05$) percentages of thigh and breast muscle mass after day 14, and a lower FI at higher levels of 2 and 4% inulin from day 8 to 14 than birds receiving the BD had. However, birds fed diets containing 2 and 4% inulin had higher ($P < 0.05$) BW gains than did the control birds from day 21 to 42 (Table 3).

We measured serum biochemical parameters, immunoglobulin level, and complement components to investigate any effects of dietary inulin supplementation on lipid metabolism and activity of their immune system. Data suggest that serum TG level decreased by

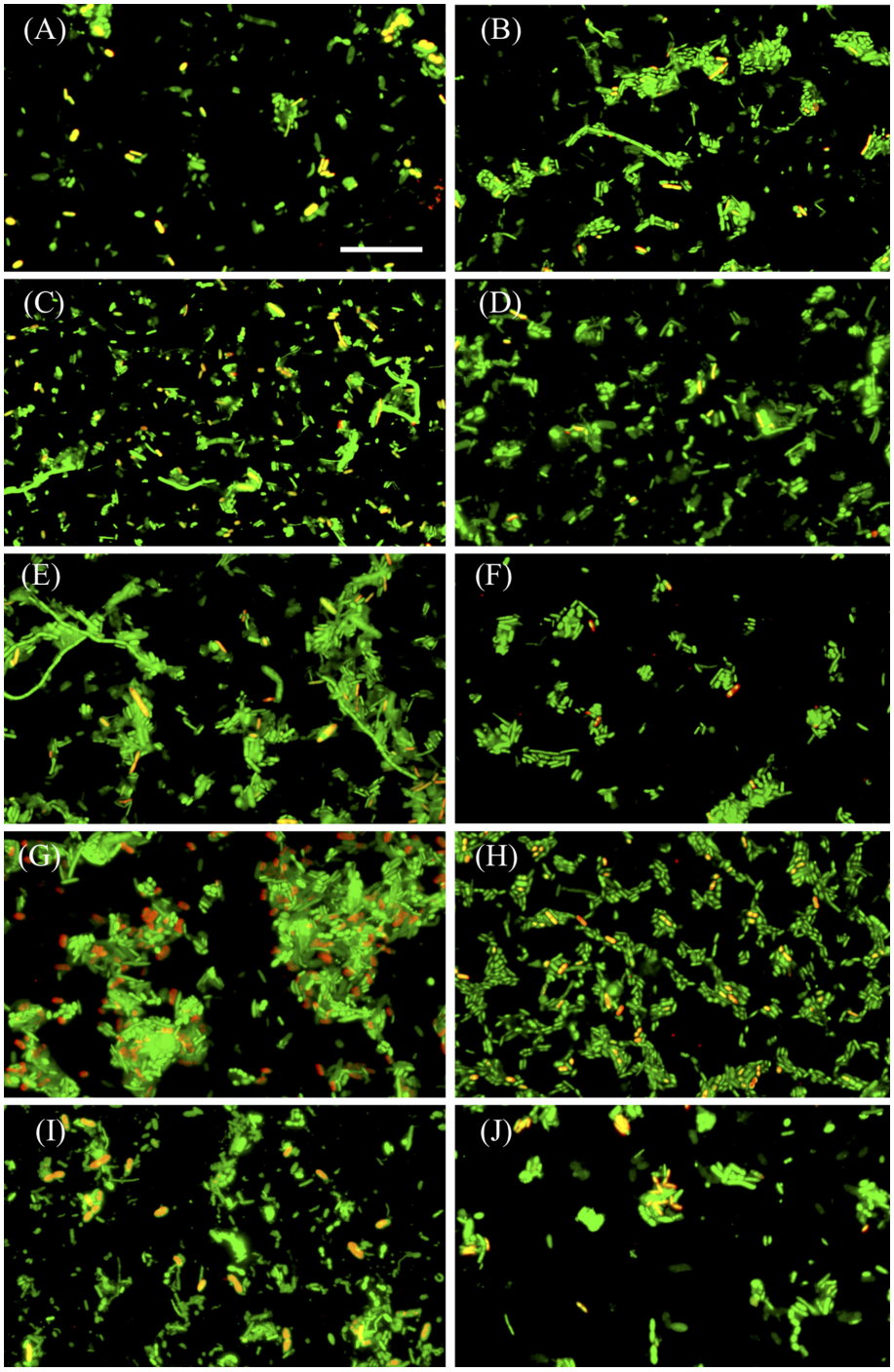

Figure 1. Images of cecal samples from the broiler chickens fed a corn-based diet after color combination. Images from probes are labeled in red, and those from DAPI staining are in green. The yellow (combination of red and green), including those partly colored cells in panels F to H, hybridized with probes Erec482 targeting *Lachnospiraceae* (A), MB1174 targeting *Methanobacteriaceae* (B), Lab9057_570 targeting *Lactobacillus johnsonii* group (C), Bif228 targeting *Bifidobacterium* spp. (D), Bdis656 targeting *Parabacteroides distasonis* (E), Lab2185_87 targeting *Lactobacillus reuteri* (F), Bfra602 targeting *Bacteroides* spp. (G), ALF968 targeting α-*Proteobacteria* (H), Str targeting *Streptococcus* spp. (I), and Ent targeting *Enterobacteriaceae* (J), respectively. Bar, 10 μm.

41.2 and 31.3% with 1 and 4% inulin, respectively, on day 21. Park and Park (2011) reported a significant decrease in TG (by 11.75 to 13.45%) with inulin exposure. Serum concentrations of immunoglobulins IgA, IgG, and IgM produced by B cells reflect humoral immunity of the broilers (Sharma, 1997). Here dietary supplementation with inulin at all tested levels and 400 ppm flavomycin increased significantly serum concentrations of IgM. Thus, IgM levels in the birds fed diets with 1, 2, and 4% inulin and flavomycin were 133.4, 127.6, 137.9, and 126.6% higher at day 21 than those of the control broiler group. This outcome is in line generally with the findings of other studies (Park and Park, 2011; Nabizadeh, 2012b), all of which showed that

dietary inulin supplementation improved their humoral immunity. Furthermore, the C3 levels in broiler groups fed diets with 1, 2, and 4% inulin were 123.5, 122.2, and 124.8% higher than those in the control group, affirming the positive effect inulin has on immune systems of these broilers.

Quantitative FISH analysis of cecal contents has allowed a comprehensive compositional profile of the cecal microbiota in birds fed these different diets. The cocktail of FISH probes used here hybridized with 66.7 (±7.0)–102.7 (±10.4)% of total microbial cells in each of the individual cecal samples (Table 7). Considering the 7 to 10% detection deviation for qFISH measurements, the majority of cecal prokaryotic cells were thus identified and quantified using these probes (Table 2). As expected from other similar 16S rRNA gene-based studies (Mancabelli et al., 2016; Ferrario et al., 2017; Ocejo et al., 2019), the cecal microbiota of chickens comprised largely members of the *Firmicutes* and *Bacteroidetes*, with the remainder being mainly populations of *Proteobacteria* and *Actinobacteria.*

Age-dependent changes in intestinal microbiota of broiler chickens have been well documented (Shaufi et al., 2015; Awad et al., 2016; Ocejo et al., 2019). Thus, using 16S rRNA Illumina high-throughput sequencing, Ocejo et al. (2019) found that age was the strongest influencing factor influencing the cecal microbiota composition. Our qFISH data support this. Thus, in ceca of the control birds, a gradual decrease in the RPA of *Lachnospiraceae* (Figure 1A) and *Enterobacteriaceae* (Figure 1J) was detected, with similar increases in the RPA of *Bacteroides* (Figure 1G) and *P. distasonis* (Figure 1E) across the whole growth cycle.

An increase in the RPA of *Bifidobacterium* strains in chicken ceca in response to inulin exposure has been reported previously (Rebolé et al., 2010; Shang et al., 2010; Nabizadeh, 2012a; Krismiyanto et al., 2014; Liu et al., 2018). Such an increase is considered desirable since *Bifidobacterium* spp. are thought to act as immunostimulators, as well as competing with pathogenic bacteria for cell adhesion sites and producing essential volatile fatty acids for energy production (reviewed by Binda et al., 2018). Data from our study support this. Thus, ceca of birds fed diets supplemented with inulin had higher RPA ($P < 0.001$) of *Bifidobacterium* spp. cells after days 21 and 42 than those fed the control diet (Figure 1I). Furthermore, such changes in RPA of *Bifidobacterium* positively correlated with improvements in BW gain of these birds over the same period. The bifidogenic effect of inulin is thought to arise from changes in the expression levels of β-fructofuranosidase genes, encoding invertase-, β-fructosidase-, and inulinase-type enzymes (Pokusaeva et al. 2011). Furthermore, Bifidobacteria possess a unique fermentative pathway called the "bifid shunt" that allows them to produce more ATP from glucose or fructose than that from the conventional homo- and hetero-fermentative pathways used by lactic acid bacteria (De Vries and Stouthamer, 1967). The result is increased activities of oligofructose and inulin degradation mechanisms and kinetics that serve to favor bifidobacterial competitiveness against other putative inulin degraders (De Vuyst et al., 2013).

However, the phylogeny of members of the genus *Bifidobacterium* is complex, currently containing at least 54 species (Mattarelli and Biavati, 2018). No FISH probes targeting individual *Bifidobacterium* populations are available. Our Illumina high-throughput sequencing (Y Xia, unpublished data) of 16S rRNA gene analysis using chickens after 42 D show that *Bifidobacterium saeculare* was the only *Bifidobacterium* species identified, and its RPA increased from 0.5% of the total 16S rRNA reads in the control group to 2.1, 4.0, and 17.5% in ceca of birds supplemented with 1, 2, and 4% inulin, respectively. *Bifidobacterium saeculare* was isolated originally from rabbit feces, and its presence in human and poultry intestinal tracts has seldom been reported. *Bifidobacterium saeculare* shares 99.3 to 99.9% similarity in its 16S rRNA sequence with *Bifidobacterium gallinarum* and *Bifidobacterium pullorum* (Matsuki et al., 2003), both of which have been isolated from chicken ceca (Trovatelli et al., 1974; Watabe et al., 1983), suggesting a possible misidentification here of *B. saeculare.*

Inulin inclusion in the diet also promoted the RPA of members of the *L. johnsonii* group. As with *Bifidobacterium*, these bacteria produce lactate as their main fermentation end product, which can be assimilated in the cecum serving as an energy source for the chickens. *Lactobacillus* spp. are important contributors to the nutritional needs of chickens and have been held responsible for increasing both their FI and BW gain (Angelakis and Raoult, 2010; Yan et al., 2017). However, the precise identity of which *Lactobacillus* population is directly responsible for such improvements remains unclear. The phylogeny of members of the genus *Lactobacillus* is also complex, as the genus is thought to consist currently of more than 170 species and 17 subspecies (Goldstein et al., 2015). In this study, we showed that members of the *L. johnsonii* group were dominant members of the cecal microbiome microflora, and increases in their RPA correlated positively with BW gain of the birds, from day 21 to 42. Among its members are *L. johnsonii* and *L. gasseri.* Illumina sequencing (Y Xia, unpublished data) of microbiome 16S rRNA amplicons has identified *L. johnsonii* but not *L. gasseri* in the same cecal samples. *Lactobacillus johnsonii* has been reported to promote chicken growth performance (Wang et al., 2017a), to minimize the negative health impacts of the pathogens *Clostridium perfringens* (Wang et al., 2017b) and *Campylobacter jejuni* (Mañes- Lázaro et al., 2017), thus preventing subclinical necrotic enteritis by ameliorating lipid metabolism and intestinal microflora (Qing et al., 2017).

Unlike these data for *L. johnsonii*, the qFISH data failed to show any link between the RPA of *L. reuteri* and chicken growth performance. Dietary supplementation with *L. reuteri* improved feed conversion (Yu et al., 2007), protected birds against *Salmonella*-induced pullorum disease (Zhang et al., 2012), and increased BD

gain (Nakphaichit et al., 2011). Thus, its failure to correspond to improvements in the growth performance of birds here suggests that not all *Lactobacillus* species/strains had positive effects on chicken growth performance, and may only reflect individual *Lactobacillus* strains, their host animals (Mancabelli et al., 2016), and the diet used, which together emphasize the complex role of the cecal microbiome in chicken nutrition.

Parabacteroides distasonis is a core member of the human gut microbiome, and its presence is linked to a decrease in body mass index (Falony et al., 2016). It is also claimed to alleviate obesity and related disorders in both ob/ob mice and mice fed a high fat diet resulting from the production of succinate and secondary bile acids (Wang et al., 2019). Its importance or otherwise in chicken ceca is not known. Unexpected was that the FISH probe Bdis656 designed to target *P. distasonis* also hybridized positively with 3.4 to 13.5% of total cells in cecal samples in birds fed different diets (Table 7). Although the FISH probing data were often equivocal, Illumina 16S rRNA amplicon sequencing (Y Xia, unpublished data) revealed that several *Parabacteroides* spp., including *P. distasonis*, were detected in these same cecal samples, thus confirming *P. distasonis* as an important member of the bird cecal microbiota. Moreover, inulin inclusion at the 3 different levels in the fed diets enriched *P. distasonis*, and increases in its RPA were positively associated with chicken BW gain. A transcriptome analysis (Y Xia, unpublished data) of the same cecal samples has also revealed that *Parabacteroides* spp. were among the main sources of carbohydrate-degrading enzymes, including glycoside hydrolases, glycosyltransferases, and carbohydrate-binding modules, giving further support to an important role of *Parabacteroides* in the carbohydrate metabolism of chicken ceca.

As discussed here and in other studies (Biggs et al., 2007; Ortiz et al., 2009; Li et al., 2018), not all the reported effects of inulin supplementation on BW and other production parameters have been positive, but vary depending on the source of inulin used, its diet inclusion level, the composition of the basal diet, individual animal characteristics, and the state of experimental hygiene conditions (Kozlowska et al., 2016). We present data here suggesting that changing inulin feed regimes may improve its prebiotic impact. Although inulin was added to broilers diets throughout their growth period, its promoting effects on *L. johnsonii* group (Figure 1C) and *Bifidobacterium* (Figure 1D) and the positively linking of the RPA of these probiotic bacteria to chicken BW gain were only observed after day 21. Furthermore, in samples taken before day 7, the RPA of these bacteria were significantly lower than those in the control birds. Thus, inulin supply negatively impacted the RPA of these bacteria and the corresponding growth performance parameters of the birds over their early growth period (before day 21). It is possible that the chickens require time for inulin adaptation, before the cecal community, containing high RPA of *L. johnsonii* group and *Bifidobacterium* can develop to the stage where they have their positive nutritional impact on chicken growth. It could be that dietary supplementation inulin together with populations of *L. johnsonii*, *B. gallinarum*, and/or *B. pullorum* from day 1 to 7 may improve markedly chicken growth. Wahyuni et al. (2018) have reported that combining addition of *Lactobacillus* sp. and inulin had greater effects on improving protein mass and cumulative BW of chickens than those fed diets supplemented with each alone. Furthermore, Darilmaz et al. (2019) found that synbiotic treatment with inulin and *Lactobacillus* spp. together had greater effects on oxalate-degrading activity of *Lactobacillus fermentum* IP5 than those supplemented with either inulin or *Lactobacillus* spp. alone.

An inulin dose-dependent change in the RPA of *Bifidobacterium* species was observed (Table 7). Inulin inclusion at 1% level led a gradual increase in the RPA of *Bifidobacterium* species from 2.5 at day 7 to 8.5% at day 42. And the increase during the same period was from 4.1 to 9.7% with 2% inulin and 6.7 to 11.9% with 4% inulin. However, such a clear dose-dependent effect of inulin on the RPA of other probe-defined groups or species could not be seen (Table 7). This discrepancy here could be mainly due to differences in composition and physiology of different probe-defined microbial groups or species.

Flavomycin is a highly valued AGP (Pfaller, 2006). It was employed in this study as an antibiotic control. It too affected the same FISH probe-defined bacterial groups as inulin, but there were striking differences in their effects. As with inulin, flavomycin inclusion in the diet decreased the RPA of members of the *Lachnospiraceae* group while increasing the RPA of the *L. johnsonii* group after 42 D. However, flavomycin inclusion had no effect on the RPA of members of the *α-Proteobacteria,* although these decreased with inulin supplementation. Furthermore, no effects were observed on the RPA of *Bifidobacterium* strains, whereas, with inulin dietary inclusion, their RPA increased. Explanations for such marked differences are not immediately forthcoming and because of the high complexity of the population dynamics of the cecum, finding answers will not be straightforward.

In summary, dietary supplementation with 1, 2, and 4% inulin improved to varying extents lipid metabolism and immune activities of the broiler chickens under the conditions used in this study. Quantitative FISH data revealed an age-dependent change to the cecal microbiota, and inulin inclusion at all levels changed markedly its composition. The relative abundances of *L. johnsonii* and *Bifidobacterium* populations correlated with BW gain of the birds, thus suggesting these bacteria especially play important roles in cecal inulin metabolism.

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