The Journal of Animal & Plant Sciences, 27(1): 2017, Page: 98-105
ISSN: 1018-7081

COMPARATIVE EFFECTS OF DIFFERENT DIETARY CONCENTRATIONS OF β-GALACTO-OLIGOSACCHARIDES ON SERUM BIOCHEMICAL METABOLITES, SELECTED CAECEL MICROBIOTA AND IMMUNE RESPONSE IN BROILERS

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ABSTRACT

A total of, 200, day old Hubbard broiler chicks, were randomly divided into four treatment groups (n = 50) with five replicates (n = 10) in each group and allotted four treatments. Birds in the control group **(β-GOS-0)** were given a corn based diet, whereas, birds in other three experimental groups were given same diets supplemented with different concentrations of β-GOS i.e., 0.1% **(β-GOS-1)**, 0.2% **(β-GOS-2)** and 0.5% **(β-GOS-5)** for a period of 35 days. The birds were vaccinated against the Newcastle disease virus. Moreover, birds were also sensitized against 2, 4-dinitrochlorobenzene for determination of cell mediated immunity. At the end of experiment, ten birds from each group were slaughtered to collect blood for serum biochemical metabolites and humoral immunity determination and caecal digesta for selected caecal microbiota enumeration. Data were analyzed using one-way ANOVA. In case of significant F-value ($P < 0.05$), Tukey's post hoc test was applied. Results demonstrated that cell mediated immunity was higher ($P < 0.05$) in the β-GOS-1 group. Concentrations of caecal Lactobacilli were higher in the β-GOS-2 and β-GOS-5 groups. The serum biochemical metabolites, humoral immunity and counts of Clostridia and coliforms were not affected by dietary supplementation of β-GOS. In conclusion, dietary supplementation of β-GOS conferred better effects in terms of increased caecal Lactobacillus counts and immuno-modulation in broilers.

Keywords: β-galacto-oligosaccharides, serum metabolites, cell mediated immunity, humoral immunity, caecal microbiota, broilers.

INTRODUCTION

Gastrointestinal tract **(GIT)** health is a prime focus of interest because impairment in gastrointestinal functioning or gut microbial dysbiosis can adversely affect the digestion, metabolism and availability of nutrients. These factors, altogether, result in poor growth performance, poor feed efficiency and high mortality in birds (Dibner and Richards, 2005). Although, gastrointestinal microbes, ubiquitous and heterogenous, play complex and important role in nutrition, metabolism, growth and health of growing animals (Dibner and Buttin, 2002; Torok *et al.,* 2011), the nutrients in the GIT are competitively partitioned between host-animal and gut microflora for energy. Therefore, more partitioning of nutrients towards host-animal is another desirable factor that can positively influence the performance and health of growing animals. This is managed, in part, by fortification of poultry feed with sub-therapeutic levels of antibiotics (Huyghebaert *et al.,* 2011). However, after the ban, imposed by European commission (2006), on addition of antibiotics as growth promoter in feed, investigators and producers start seeking alternate of antibiotics. Number of feed additives, termed as eubiotics, including prebiotics, probiotics, phytobiotics, essential oils, enzymes and organic acids are proposed to improve nutrients digestibility and utilization and gut health, which eventually improve overall performance of the birds (Wenk, 2002; Huyghebaert *et al.,* 2011).

The prebiotic, galacto-oligosaccharides (GOS), produced by enzymatic transgalactosylation of lactose by bacterial or fungal β-galactosidase (Prenosil *et al.,* 1987), has been studied in human beings (Moro *et al.,* 2002; Boehm *et al.,* 2005; Haarman and Knol, 2005; Vulevic *et al.,* 2008), pigs (Smiricky-Tjardes *et al.,* 2003), rats (Rowland and Tanaka, 1993), mice (Vos *et al.,* 2006) and poultry (Biggs *et al.,* 2007; Jung *et al.,* 2008). These studies indicate that dietary GOS supplementation increases the populations of Bifidobacteria and Lactobacilli in feces or digesta. In addition to increasing populations of Bifidobacteria and Lactobacilli, GOS supplementation has been shown to enhance delayed-type hypersensitivity response in murine influenza vaccination model (Vos *et al.,* 2006), natural killer cells activity in human beings (Vulevic *et al.,* 2008) and mice (Gopalakrishnan *et al.,* 2012), and to decrease pro-

inflammatory cytokines production in humans (Vulevic *et al.,* 2008). *In-vitro* immuno-modulatory properties of β-GOS were also tested on human colonic NCM 460 cells. It was found that inclusion of β-GOS in the cell culture medium of NCM 460 cells significantly reduced TNF-α-induced IL-8 and MIP-3α secretions and, therefore, it had the ability to modulate inflammatory response of immune cells (Dubert-Ferrandon *et al.* 2008).

Keeping in view the significance of GOS from literature, the present study was designed to evaluate the effects of different dietary concentrations of second generation β-galacto-oligosaccharides (Bimuno^{TM}, Clasado Limited, United Kingdom) on serum biochemical metabolites, selected caecel microbial populations and immune response in broilers.

MATERIALS AND METHODS

Experimental Design: A total of 200, day-old broiler chicks (Hubbard), were purchased from hatchery and housed in an environmentally controlled shed (Phool-nagar, Pattoki) carpeted with wood shaving litter. The birds were equally divided into four treatment groups (n = 50/group) with five replicas (n = 10) in each treatment group. During the brooding period, first week, temperature was kept at 35 ± 1°C and relative humidity was maintained at 65 ± 5%. Temperature was decreased 3°C on weekly basis until it reached 26°C which was then maintained for the rest of experimental period, whereas, relative humidity was kept constant at 65 ± 5% throughout the experiment. The birds were fed a corn-based diet (Table 1), formulated free of antimicrobials and coccidiostats to meet or exceed the recommendations of the "National Research Council" (NRC, 1994), or the same diet supplemented with different concentrations of β-galacto-oligosaccharides **(β-GOS)**.

Four experimental diets were prepared by mixing 0%, 0.1%, 0.2% and 0.5% β-GOS and fed to the experimental birds. The birds in the control **(β-GOS-0)** group were provided the diet (0% β-GOS) and birds in other groups were provided same diets supplemented with 0.1% β-GOS **(β-GOS-1)**, 0.2% β-GOS **(β-GOS-2)** and 0.5% β-GOS **(β-GOS-5)**, *ad libitum* for 35 days.

The birds were vaccinated against Newcastle disease virus **(NDV)** and infectious bursal disease virus **(IBDV)**. Live attenuated NDV vaccine (Ceva-Phylaxia, Budapest, Hungary) was given intraocularly on day 4 and repeated on day 20 in drinking water. Similarly, live intermediate strain IBDV vaccine (Lohman Animal Health GmbH, Cuxhaven, Germany) was administered intraocularly on day 8 and booster of live attenuated IBDV vaccine was given on day 24 in drinking water.

Sampling Protocols: At the end of experimental period, 10 birds from each group (2 birds / replica) were slaughtered by exsanguination. Blood samples were collected in plane plastic test tubes and kept at 4°C for 4 hours. Blood samples were centrifuged at 4,000 rpm at 4°C for 20 minutes in a temperature controlled centrifuge machine. Top clear layer of serum was harvested in aliquots using micropipette and stored at -40°C for serum biochemical metabolites and NDV antibodies titer analyses.

Abdominal cavity of each bird was exposed to collect caecal digesta. Caecal digesta was collected in sterile plastic air tight test tubes and immediately transferred to laboratory for enumeration of cecal microbiota.

Serum Biochemical Metabolites: Frozen serum samples were thawed at room temperature for determination of serum biochemical metabolites including glucose, total proteins, albumin, cholesterol, triglycerides, uric acid, liver enzymes including alanine aminotransferase and aspartate aminotransferase and thyroid hormones including tri-iodothyronine and thyroxine. Serum globulins were determined by subtracting albumin from total proteins. Serum metabolites and liver enzymes were determined using commercial kits (Randox, UK) and reactions were read by spectrophotometer, whereas, thyroid hormones were determined using commercial competitive ELISA kits (BioCheck, CA).

Caecal Microbiota: Caecal digesta collected from two birds in each replica was pooled to get a single sample. After pooling, five samples per treatment were obtained for enumeration of different microbial populations including coliforms, Clostridia and Lactobacilli using conventional plate culturing method.

Bacterial specific growth media were prepared by dissolving agar, under continuous stirrer at 40°C on temperature control magnetic hot plate, in distilled water. All media were autoclaved and then 20 mL medium was poured in sterile glass petriplate. Medium was allowed to solidify at room temperature. After solidification of medium, all petriplates were incubated at 37°C, in temperature control incubator, for overnight to confirm sterility of media.

One gram digesta from each sample was taken, homogenized and serially diluted (10-folds) in sterile saline solution. Hundred microliters diluted samples were inoculated and spread on bacterial specific growth media in petriplates. Coliforms were grown on MacConkey's agar at 37°C for 24 hours under aerobic conditions. Clostridia were grown on Reinforced Clostridial agar at 37°C for 24 hours under 5% CO_2 conditions. Lactobacilli, being strict anaerobe, were grown on de Man, Rogossa and Sharpe (MRS) agar at 37°C for 48 hours under strict anaerobic conditions induced in anaerobic jars by anaerobic sachets (Oxoid, UK). All samples were run in triplicate and average values were used in statistical analysis, after $\log_{10}$ transformation.

Immunity

Humoral Immunity: Antibody titers against NDV were used to determine humoral immunity. Antibody titers were determined by employing haemagglutination inhibition **(HI)** test (Brugh and Beard, 1980). The HI test was conducted using haemagglutination of chicken red blood cells **(CRBC)**. Chicken red blood cells were obtained from a healthy chicken. For this purpose, blood was collected in heparinized vaccutainer using sterile needle. Blood was centrifuged at 4,000 rpm for 20 minutes. Upper layer of plasma and middle buffy coat were removed and CRBC were washed thrice with normal saline by centrifugation. Washed CRBC were diluted with saline solution to prepare 1% CRBC suspension. The 1% CRBC suspension was used to determine 4 haemagglutination **(HA)** units of antigen as well as antibody titers against NDV. Four HA units of antigen were determined by dispensing 25 µL of NDV antigen, in 96 well round bottom microplate, 2-fold serially diluting with equal volume of saline solution and agglutination of CRBC. Highest dilution of NDV antigen exhibiting agglutination of CRBC was used to calculate 4 HA units.

The HI test was performed by 2-fold serial dilution of 25 µL serum with equal volume of saline solution up to 11^{th} well of 96 well microplate. Thereafter, same volume of 4 HA antigen was added to each well including well 12 of same row. Microplate was incubated at 37°C for 30 minutes. After incubation, 25 µL CRBC (1%) was added to each well, microplate was shaken and again incubated 37°C for 30 minutes. Clear and sharp button formation of CRBC at the bottom of well indicates positive result i.e., HI. Well 12 served as a negative control.

Cell Mediated Immunity: Cell mediated immunity **(CMI)** was determined by employing dermis delayed type hypersensitivity **(DHT)** reaction (Tiwary and Goel, 1985). For this purpose, 1% solution of 2, 4-dinitrochlorobenzene **(DNCB)** was prepared in acetone. Two birds from each replicate were selected and sensitized with 250 µL of 1% DNCB solution. Breast skin of each bird was gently defeathered and 2 cm^2 area was marked. Solution of DNCB was dribbled slowly on marked area and dried with continuous air blow. Thigh skin of each sensitized bird was defeathered gently and challenged with 250 µL of 0.1% DNCB solution after 14 days of sensitization. Skin DHT was determined by measuring thigh skin thickness on day 0, 1, 2 and 3 post-challenge dose.

Statistical Analysis: Data were analyzed using Statistical Package for Social Science (SPSS for Windows Version 13.0, SPSS Inc., Chicago, IL, USA). Data were subjected to Shapiro-Wilk test for confirmation of normal distribution. After confirmation, all data, except CMI data, were analyzed by one-way analysis of variance **(ANOVA)**. The CMI data were analyzed by repeated measuring ANOVA (Steel *et al.*, 1997). In case of significant F-values, data were subjected to Tukey's post-hoc test. Significance level was set at $P < 0.05$ and data were presented as means ± SE.

RESULTS

Serum Biochemistry: Concentrations of all serum biochemical metabolites, liver enzymes and thyroid hormones were remained unchanged among all the experimental groups except cholesterol which was significantly higher in the 0.5% β-GOS supplemented birds compared with the control birds (Table 2). The concentrations of cholesterol, however, were within the physiological range defined for broiler chickens.

Caecal Microbial Populations: Populations of coliforms and Clostridia in cecal digesta of all the experimental birds remained unchanged. However, populations of Lactobacilli in cecal digesta of the 0.2% and 0.5% β-GOS supplemented groups was higher ($P < 0.05$) compared with the control group (Figure 1).

Immune Response: Supplementation of β-GOS at different inclusion concentrations did not affect the antibodies titres against the NDV vaccine compared to the non-supplemented group (Figure 2). Cell mediated immune response remained same in all the experimental groups for first 48 hours post-challenging period. However, cell mediated immune response was greater ($P < 0.05$) in next 24 hours (i.e., 72 hours post-challenging) in 0.1% β-GOS supplemented group compared with the control group (Figure 3).

Table 1. Feed ingredients, proximate composition and calculated AME of the starter (1-21 days of age) and grower diets (22-35 days of age) for broilers.

	Starter	Grower
Feed Ingredients (%)		
Corn	40.15	57.57
Rice broken	15.0	----
rice polish	----	4.00
Wheat bran	1.34	----
Soya meal	11.54	9.60

Sunflower meal	12.00	13.00
Canola meal	9.00	5.00
Rapeseed meal	5.00	7.60
Guar meal	1.00	----
Molasses	2.00	----
Dicalcium phosphate	1.73	1.96
Premix*	1.00	1.00
Sodium chloride	0.21	0.21
Sodium bicarbonate	0.03	0.065
Proximate Composition (%)		
Crude protein	19.6	18.5
Crude fat	2.16	2.35
Crude fiber	1.26	1.80
Total ash	5.77	5.40
Calculated AME (Kcal/kg)	2,750	2,850

*Vitamin mineral premix (each kg contained): Ca, 195 g; K, 70 g; Na, 18 g; Mg, 6 g; Zn, 4,000 mg; Fe, 8,000 mg; Cu, 800 mg; Mn, 6,200 mg; Se, 15 mg; Co, 35 mg; I, 40 mg; vitamin A, 200,000 IU; vitamin D3, 80,000 IU; vitamin E, 1072 IU; vitamin K3, 34 mg; ascorbic acid, 1,300 mg; thiamine, 180 mg; riboflavin, 350 mg; niacin, 3,500 mg; vitamin B6, 320 mg; folic acid, 50 mg; vitamin B12, 800 µg; biotin, 13,000 µg.

Table 2. Comparative effects of different dietary concentrations of β-galacto-oligosaccharides on serum biochemical metabolites of broilers.

Parameters	Treatment Groups			
	β-GOS-0	**β-GOS-1**	**β-GOS-2**	**β-GOS-5**
Glucose (mg/dL)	273.87 ± 10.75[a]	246.55 ± 7.03[a]	277.54 ± 10.40[a]	267.61 ± 8.84[a]
Total Proteins (g/dL)	3.16 ± 0.26[a]	3.69 ± 0.27[a]	3.41 ± 0.35[a]	3.79 ± 0.20[a]
Albumin (g/dL)	1.83 ± 0.05[a]	1.92 ± 0.04[a]	1.95 ± 0.06[a]	1.92 ± 0.04[a]
Globulins (g/dL)	1.37 ± 0.11[a]	1.77 ± 0.10[a]	1.14 ± 0.13[a]	1.83 ± 0.17[a]
Cholesterol (mmol/L)	2.11 ± 0.24[b]	2.49 ± 0.30[ab]	2.88 ± 0.19[ab]	3.42 ± 0.28[a]
Triglycerides (mmol/L)	0.91 ± 0.07[a]	1.11 ± 0.14[a]	1.11 ± 0.09[a]	1.26 ± 0.08[a]
Uric acid (µmol/L)	460.48 ± 35.10[a]	454.44 ± 46.08 [a]	489.80 ± 35.18[a]	483.22 ± 37.52[a]
Aspartate Aminotransferase (U/L)	138.98 ± 22.00[a]	122.48 ± 16.26[a]	129.87 ± 13.79[a]	140.34 ± 12.75[a]
Alanine Aminotransferase (U/L)	14.47 ± 3.10[a]	13.10 ± 2.42[a]	10.23 ± 1.35[a]	11.24 ± 2.02[a]
Tri-iodothyronine (ng/dL)	184.33 ± 22.41[a]	176.67 ± 8.82[a]	176.33 ± 11.89[a]	160.33 ± 11.29 [a]
Thyroxine (µg/dL)	1.63 ± 0.26[a]	2.03 ± 0.63[a]	1.77 ± 0.42[a]	1.34 ± 0.35[a]

[a-b]Different superscripts in a column differed significantly (P < 0.05)

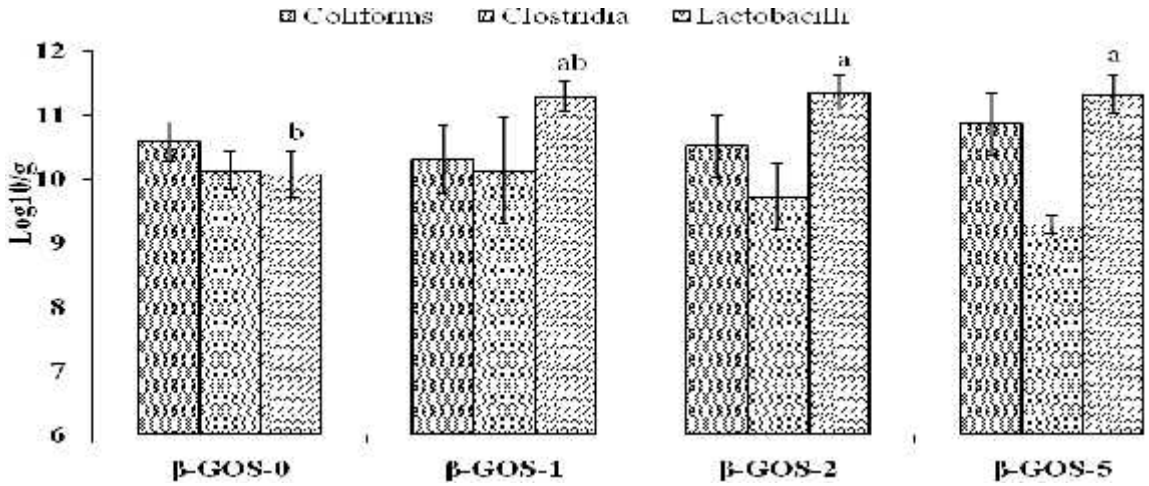

Figure 1. Comparative effects of different dietary concentrations of - -galacto-oligosaccharides on Coliforms, Clostridia and Lactobacilli populations (Log_{10}±SE) in broilers. [a-b]Different superscripts on lines differed significantly (P < 0.05).

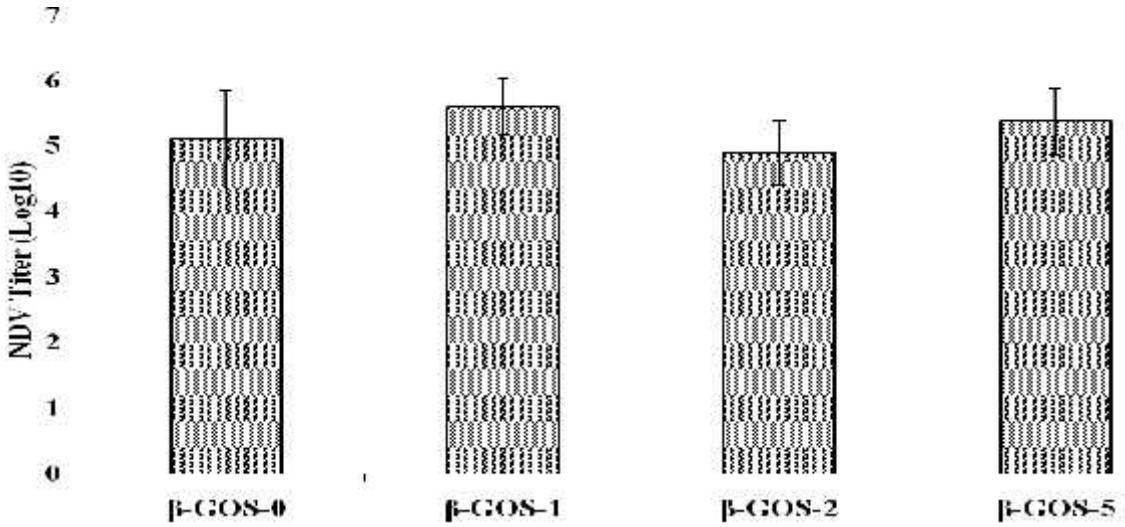

Figure 2. Comparative effects of different dietary concentrations of -galacto-oligosaccharides on NDV titer (Log $_{10}$±SE) in broilers

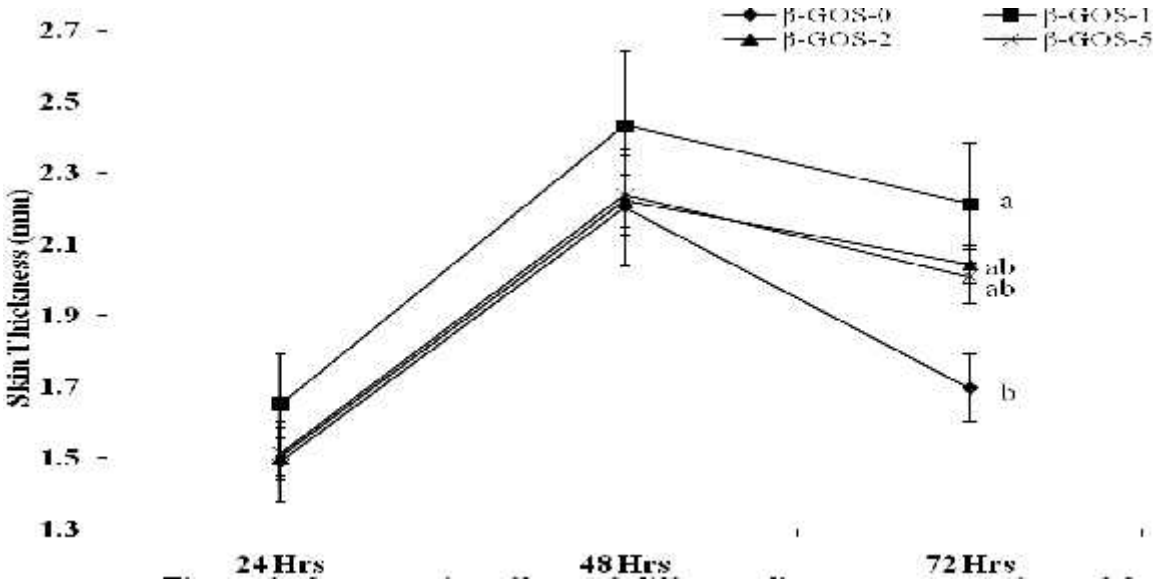

Figure 3. Comparative effects of different dietary concentrations of -galacto-oligosaccharides on cell mediated immunity (mm±SE) in broilers at different time intervals. [a-b]Different superscripts on lines differed significantly ($P < 0.05$).

DISCUSSION

Perbiotics are selectively fermentable compounds that are being fermented by the gut friendly bacteria termed as probiotics and, therefore, prebiotics have the ability to improve the gut health and ultimately overall performance of the host animals (Hume, 2011; Huyghebaert *et al.,* 2011). Despite the usefulness of prebiotics, many indigenous factors like type of animals and exogenous factors like type of prebiotics and concentration, feed and production unit hygiene affect the efficacy of prebiotics (Ten Bruggencate *et al.,* 2003; Verdonk *et al.,* 2005; Biggs *et al.,* 2007; Yang *et al.,* 2009). The present study reveals the effects of different supplemented concentrations of β-galacto-oligosaccharides **(β-GOS)** on serum biochemical metabolites, selected caecel microbiota and immune response in broilers.

Gut microbiota have significant effects on the nutrients digestibility, bioavailability and, therefore, on overall host response and development (Rehman *et al.,* 2008). Gut microbiota is influenced by number of variables including diet composition and type, dietary supplementations including prebiotics, probiotics, essential oils and organic acids (Klaenhammer, 2000; Peñalver *et al.,* 2005; Rehman *et al.,* 2008; Goodarzi Boroojeni *et al.,* 2014). Results of our study revealed that caecal concentrations of Lactobacilli are higher in the

0.2% and 0.5% β-GOS supplemented groups compared with the control group. Many *in vivo* and *in vitro* studies demonstrated the lactobacillogenic effects of GOS in different animal species as well as human beings (Smiricky-Tjardes *et al.,* 2003; Tzortzis *et al.,* 2005; Ben *et al.,* 2008; Jung *et al.,* 2008; Macfarlane *et al.,* 2008; Cardelle-Cobas *et al.,* 2012). Our findings are in line with above mentioned trials and, therefore, demonstrate that GOS supplementation has lactobacillogenic effects. Beneficial attributes of Lactobacilli have been well documented including maintenance of gastrointestinal mucosal barrier (Yan and Polk, 2010), bacteriocins production (Mazmanian *et al.,* 2008), immune-modulation (Huang *et al.,* 2004; Koenen *et al.,* 2004) and growth suppression or competitive exclusion of intestinal pathogens (Higgins *et al.,* 2007). These effects alone or in combination can improve the growth performance and feed conversion efficiency of host. Moreover, β-GOS has been reported to inhibit the attachment of different pathogens including *Escherichia coli* and *Salmonella enterica* serovar Typhimurium under *in vitro* "HT-29 epithelial cell line" (Tzortzis *et al.,* 2005) and *in vivo* "murine oral challenge model" (Searle *et al.,* 2009) experiments. This property of β-GOS has been attributed to α-anomeric configured oligosaccharides.

Dietary supplementation of 0.1% β-GOS increased cell mediated immunity compared with the control group, whereas, high β-GOS supplementation (0.2% and 0.5%) does not affected cell mediated immunity in the current study. Several studies have shown that GOS supplementation enhances delayed-type hypersensitivity response in murine influenza vaccination model (Vos *et al.,* 2006), natural killer cells activity and anti-inflammatory cytokines in human beings (Vulevic *et al.,* 2008) and mice (Gopalakrishnan *et al.,* 2012), and decreases pro-inflammatory cytokines production in humans (Vulevic *et al.,* 2008). *In vitro* study has shown that β-GOS partially inhibits TNF-α mediated NF-κB translocation and, therefore, reduces production of pro-inflammatory cytokines (Dubert-Ferrandon *et al.,* 2008).

In summary, β-GOS has a potential to improve the populations of Lactobacilli as well as immunity in broilers. However, studies are warranted to confirm the effects of β-GOS supplementation on molecular microbial ecology as well as indigenous immuno-modulators.

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