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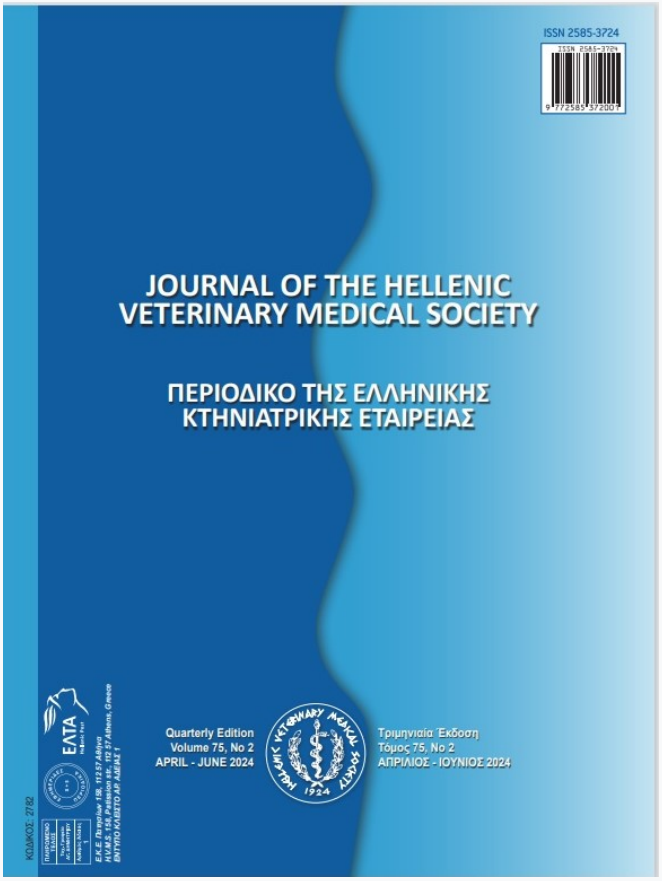

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Research article
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Evaluation of using probiotics, prebiotics and symbiotic as growth promoters in broilers and their effects on some growth performance-related genes

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ABSTRACT: This study was conducted in an attempt to evaluate the impact of dietary addition of probiotics (*Enterococcus faecium*, *Lactobacillus acidophilus*, *Bacillus subtilis* and *Saccharomyces cerevisiae*) and prebiotics (β-glucanand mannan oligosaccharides) on broiler diets with respect to growth related genes (mucin2, chicken growth hormone (cGH), and insulin-like growth factor-1 (IGF-1) gene expression. It was also our aim to evaluate the growth performance and economic efficiency of the diet. A total of 350 one-day-old male broiler chicks (ROSS) were randomly divided into 14 groups, each containing 25 birds that were fed different doses of probiotics, prebiotics, and symbiotics, except for the control group. The results showed that there was a significant improvement in body weight gain (BWG) in the probiotic, prebiotic, and symbiotic treatments compared to the control group. The best result was T8, 2007.5± 23.88, which contained probiotics (108 cfu/ml) + 250 ppm prebiotics/ton. The same treatment (T8) also showed a clear improvement in feed intake (FI), as the birds consumed the least amount of feed (3064 ± 26.53) compared to other groups, with the best feed conversion rate 1.52 of 0.01. The liver of birds fed T8 had higher IGF1 and cGH expression compared to other treatments 7.60±1.33 and 8.66±1.38 respectively. Enhanced expression of muc2 was found in treatments fed with probiotics, prebiotics, and symbiotics; the best result was T8 8.70±1.29. The economic evaluation showed that birds fed the symbiotic at a 250 ppm level of prebiotics were the best treatments. It could be concluded that supplementation with probiotics, prebiotics, and symbiotic had beneficial effects on total BWG, FI, FCR and IGF1, cGH and muc2 expression in broiler chickens. They also enhanced the expression of some growth-related genes, so they can be used as an alternative to antibiotics.

***Keywords*:** Probiotics; Prebiotics; symbiotic; Gene expression; Feed additives; Poultry nutrition.

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INTRODUCTION

Poultry and its products are one of the fastest growing industries in the food sector. This industry has grown exponentially over the past twenty years, and has become one of the most important industries economically. Poultry is also one of the most widespread food industries in the world. Chicken is the most commonly farmed species, with more than 90 billion tons of chicken meat produced annually (FAO 2017). During the last several decades, antibiotics have been widely used in the poultry industry to promote growth. The extensive use of antibiotics has led to an increase in the antibiotic resistance of food poisoning bacteria Antibiotic resistance (AR), which is defined as the ability of an organism to resist the killing effects of an antibiotic to which it is normally susceptible, has become an issue of global interest (Abdel-Raheem and Abd-Allah, 2011). Previous studies have reported that antibiotic residues in chickens can enter the food chain and induce resistance in the consumer's natural gut flora. An increase in drug-resistant bacteria can lead to gastrointestinal and nervous system diseases, and even death (Neogi et al., 2020). Bacteria acquire resistance through mobile genetic elements, including phages, plasmids, or transposons. This facilitates the transfer of resistance genes between bacteria and also accelerates the acquisition of antibiotic resistance (Davies and Davies, 2010). Currently, broiler lines are genetically selected for maximum productivity. The quality and composition of the meat are also affected by the treatment of the birds during rearing and the addition of biologically active substances such as probiotics, prebiotics and symbionts. This may greatly affect the quality of the meat as it regulates the immune response, metabolism, and digestion (Slizewska et al., 2019). There are must-have criteria for selecting probiotics, including non-pathogenic activity and toxins, tolerance to gastric juice, ability to adhere to intestinal epithelial cells, and antibiotic resistance. In addition, probiotics must maintain their viability and stability during feed processing and storage to ensure their viability (James and Wang, 2019).

Functionally, mucin plays important roles in mediating signal transmission between epithelial cells, forming mucous layers on various organs, the most important of which are the stomach and intestines, and providing a protective barrier against pathogenic bacteria. In addition, mucin forms an interface with commensal and pathogenic microbes, contributing to defense against pathogens (Linden et al., 2008). GH gene in broiler chickens regulates metabolism, growth, and reproduction, and affects various individual systems, such as the digestive, reproductive, endocrine, and immune systems, in a significant way. Growth hormone also stimulates the production of IGF-1 and increases the concentration of glucose and free fatty acids (Bahadoran et al., 2019). Previous studies have investigated the impact of probiotics on poultry, but studies on the use of probiotics with prebiotics (beta-glucan and MOS) are rare. Thus, the present study was planned to evaluate the effect of symbiotic (probiotics with prebiotics) on broiler performance (BWG, FI, FCR) and IGF1, cGH and muc2 expression, so they can be used as an alternative to antibiotics. From the aforementioned, it is clear that probiotics and prebiotics are important, both healthy and economical, and have had a significant impact on the health of poultry and, thus, human beings.

MATERIAL AND METHODS

Ethical approval

The experimental design and procedures were in compliance with the ethical standards of your relevant national and institutional committee on animal experimentation approved (BUAPD- 20203) by the scientific Ethics Committee, Animal Production Department, Faculty of Agriculture, Benha University, Egypt.

This study was conducted on the farm of Faculty of Agriculture, Benha University, Egypt. A total of 350 one-day-old male broiler chicks (ROSS) were obtained from Dakahlia Poultry Company, Egypt, and were randomly assigned to14 groups, each with 25 birds (Table 1). The strains of probiotics E. faecium, L. acidophilus, B. subtilis, and S. cerevisiae were supplied by the Food Safety Laboratory, Regional Center for Food and Feed (RCFF), and Agriculture Research Center (ARC) in Egypt. Probiotics were prepared and isolated according to Ahmed et al. (2021) to obtain a final concentration of 108 colony-forming units (cfu) per ml of drinking water and were maintained at 4-8 °C for use during the experiment. Prebiotics were purchased locally (commercial name: Biolan B-10; code: WS-00204, Phytobiochem, UK).

The experimental diets were formulated to supply the nutrient requirements of broilers according to Zaghari et al., 2017) during starter (1-15 d), grower (15-28 d), and finisher (28-35 d) periods (Table 2).

Chemical analysis/Proximate analysis

Table 3 illustrates that the feed samples were analyzed for dry matter (Method 934.01), ether extract

(Method 920.39), crude protein (Method 984.13), crude fiber (Method 978.10), and crude ash (Method 942.05), according to the procedure described by AOAC (2006).

Growth performance

The daily feed intake per group was recorded to compute the weekly feed intake. Body weight was recorded at the time of arrival and after every week of age, using an electrical weighing balance. Values of feed intake and weight gain were used to calculate the FCR according to (Zaghari et al., 2020).

Table 1. Experimental design and treatments

Treatments	Groups
T1	Control
T2	Probiotics[a] (10^8 cfu / ml)
T3	50 ppm prebiotics[b]/ ton
T4	Probiotics (10^8 cfu / ml) + 50 ppm prebiotics/ ton
T5	150 ppm prebiotics/ ton
T6	Probiotics + 150 ppm prebiotics
T7	250 ppm prebiotics/ ton
T8	Probiotics (10^8 cfu / ml) + 250 ppm prebiotics
T9	350 ppm prebiotics/ ton
T10	Probiotics (10^8 cfu / ml) + 350 ppm prebiotics
T11	450 ppm prebiotics/ ton
T12	Probiotics (10^8 cfu / ml) + 450 ppm prebiotics
T13	550 ppm prebiotics/ ton
T14	Probiotics (10^8 cfu / ml) + 550 ppm prebiotics

[a] probiotics strains of (*E. faecium, L. acidophilus, B. subtilis and S. cerevisiae*).
[b] Prebiotics β glucan and MOS added for feed.

Table 2. Ingredients and nutrient composition of diets

Ingredients (%)	Starter (1- 15d)	Grower (16- 27d)	Finisher (28- 35d)
Corn	50.74	54.96	58.82
Soybean meal	41.96	37.83	33.73
Corn oil	3.09	3.40	3.96
Dicalcium Phosphate	1.72	1.53	1.35
Calcium carbonate	1.07	0.980	0.900
Salt	0.250	0.250	0.240
Sodium bicarbonate	0.150	0.150	0.160
Premix[1]	0.250	0.250	0.250
Mineral premix[2]	0.250	0.250	0.250
DL-methionine	0.230	0.210	0.180
L-lysine HCl	0.170	0.100	0.100
L-Threonine	0.090	0.050	0.030

[1] Vitamin premix supplied the followings per kg of diet: vitamin A, 9000 IU; vitamin D3, 2000 IU; vitamin E, 36 mg; vitamin K3, 2 mg; vitamin B1, 1.75 mg; vitamin B2, 6.6 mg; vitamin B6, 2.94 mg; vitamin B12, 0.015 mg; nicotinic acid, 29.7 mg; folic acid, 1 mg.
[2] Mineral premix supplied the followings per kg of diet: calcium pantothenate, 9.8 mg; choline chloride, 250 mg; Mn, 99.2 mg; Zn, 84.7 mg; Cu, 10 mg; Fe, 50 mg; Se, 0.2 mg; I, 0.99 mg.

Table 3. Chemical Analysis of diets

Chemical Analysis	Starter (1- 15d)	Grower (16- 27d)	Finisher (28- 35d)
Kcal/Kg	2900.00	3000.00	3100.00
Crude protein %	22.71	20.91	18.93
Dry matter (DM%)	89.40	89.4	89.31
Crude fat %	5.01	5.12	5.65
Crude fiber %	4.21	3.99	3.84

Isolation of RNA, reverse transcription, and real-time PCR

RNA extraction

Total RNA was extracted from tissue samples of the ileum and liver sections using TRIzol Reagent (Ambion, Life Technologies, USA) following the manufacturer's protocol.

RNA assessment

Assessment of both RNA concentration and purity in the extracted samples was carried out using a NanoDrop 1000 spectrophotometer (USA). Absorbance at 260 nanometers (nm) gives a specific measurement of RNA concentration, as do absorbance at 280 nm and 230 nm.

Reverse transcription

The next step after RNA extraction and quality checks was reverse transcription, and cDNA was synthesized using the extracted RNA as the template. Quantitative Reverse Transcription PCR (RT-qPCR) complementary DNA (cDNA) was synthesized using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (iNtRON Biotechnology, South Korea) following the manufacturer's recommendations. The reverse transcriptase enzyme uses the RNA template and short-sequence primers to direct the synthesis of the first-strand cDNA, which was then used as a template for the qPCR reaction.

Quantitative real-time PCR

The obtained cDNA was diluted to 100 µL of working solution and stored at −20 °C. Each RT-qPCR reaction was performed in two technical replicates. The gene panel included the following genes used to normalize the samples: chicken growth hormone (cGH), insulin-like growth factor (IGF), mucin, and beta-actin reference genes. Primer genes were supplied by Invitrogen (Thermo Fisher Scientific, UK), as described in Table 4. Primers were utilized in a 25µl reaction containing 12.5 µl of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 1 µl of each primer (forward, reverse) of 20 Pico mole concentration, 7.5 µl of water, and 3 µl of cDNA template. The reaction was completed on a real time PCR machine, the Applied Bio systems 7500 Real-Time PCR System (Applied Bio systems, Foster City, California, USA). The amplification conditions were as follows:40 cycles (95 °C for 30 s, 58 °C for 30 s, and 60 °C for 30 s, respectively). Amplification curves and CT values were determined to estimate the variation of gene expression on the RNA of the different samples, and the CT of each sample was compared with the control group, according to the "ΔΔCt" method stated by (Sunkara et al., 2011).

Statistical analysis

The GLM procedure was used to analyze the effects of the treatments on gene expression. The relative expression of the gene in each sample versus a control in comparison to β-actin gene and calculated according to the "ΔΔCt" method stated by (Yuan et al., 2006). Duncan's multiple range test was used to compare the means of ileum & liver gene expression levels. Differences were considered statistically significant at ($P < 0.05$). The resulting values were analyzed using the software (SAS, 2004 version 9.4; SAS Institute, Cary, NC, USA). Differences between means were tested using Duncan's test (1955).

RESULTS

Growth Performance

The data obtained in Table 5 illustrate the values of some performance traits as affected by using different

Table 4. Primer design for genes analyzed by real-time PCR

Gene	Primer sequences	Annealing temperature (^{0}C)	Accession No	Product size (bp)
MUC2	F: CTGTTGTGGATGGGCGGATTG R:CCAAACTTGCTGTCCAGCTCC	60	XM_032444897	157
cGH	F: CACCACAGCTAGAGACCCACATC R: CCCACCGGCTCAAACTGC	62	KY176758	201
IGF1	F: GGTGCTGAGCTGGTTGATGC R:CGTACAGAGCGTGCAGATTTAGGT	58	FJ977570	203
Reference gene				
β actin	F: GAGAAATTGTGCGTGACATCA R: CCTGAACCTCTCATTGCCA	60	L08165	150

F forward primer, R reverse primer, mucin, cGH chicken Growth Hormone, IGF Insulin- like growth factor and β actin beta actin.

concentrations of probiotics and prebiotics separately or in combination with each other (symbiotic). The data revealed that there were significant differences in the growth performance observed during the experiment owing to the main effects of probiotics and prebiotics, which significantly increased body weight gain (P<0.01) and improved FI and FCR of birds in comparison to the control group at whole period.

Data from the same table also showed that the most efficient level of prebiotics was 250 ppm added to the probiotic mix (T8), which had the lowest amount of feed intake (3064 ±26.53) during the whole period, with the highest body weight gain (2007.5 ± 23.88) at the end of the experiment, which was reflected by the best feed conversion ratio (1.52). In addition, T6 and T12 gave the same statistical score for FCR as T8, but with higher feed intake amounts. Collectively, the most effective treatments during the entire experimental period were 250 ppm mannan and β-glucan mixed with probiotics.

Gene expression

Table 6 illustrated the obtained results of estimation of the expression of Muc2, cGH and IGF hormones as affected by the used treatments. The obtained data

Table 5. Growth performance of broilers in the experimental feeding treatments

Treatments	Items		
	Weight gain 1-35 d	FI	FCR
T1	1798.79^{e}	3075.66^{h}	1.70^{a}
T2	1862.23^{ed}	3139.66^{b}	1.69^{a}
T3	1864.18^{ed}	3133.00^{cd}	1.68^{a}
T4	1883.69^{ced}	3137.00^{cb}	1.66^{ab}
T5	1943.98^{abcd}	3110.00^{f}	1.60^{cde}
T6	2000.30^{ab}	3073.00^{h}	1.53^{f}
T7	1991.31^{ab}	3135.00^{cb}	1.57^{def}
T8	2007.50^{a}	3064.00^{i}	1.52^{f}
T9	1954.11^{abc}	3154.00^{a}	1.61^{bcd}
T10	1961.75^{ab}	3136.00^{cb}	1.60^{cde}
T11	1964.99^{ab}	3129.00^{d}	1.55^{ef}
T12	2013.77^{a}	3092.33^{g}	1.53^{f}
T13	1872.33^{ed}	3115.00^{e}	1.66^{b}
T14	1972.16^{ab}	3067.00^{j}	1.55^{ef}
± SE	23.88	26.53	0.010

Data are expressed as mean ± SE (standard error). P values were < 0.05. Means with the same letter are not significantly different.

Table 6. Effects of different dietary prebiotics and symbiotic levels on the expression of some growth genes

Treatments	MUC2	cGH	IGF
T1	3.04^{i}	2.40^{i}	2.03^{m}
T2	3.15^{i}	3.54^{h}	3.58^{j}
T3	4.05^{f}	5.91^{f}	3.53^{j}
T4	3.24^{i}	7.29^{c}	5.80^{d}
T5	3.28^{h}	3.99^{h}	5.14^{e}
T6	7.32^{b}	5.54^{g}	7.48^{b}
T7	6.77^{c}	6.39^{e}	7.26^{c}
T8	8.70^{a}	8.66^{a}	7.60^{a}
T9	4.11^{f}	6.78^{d}	4.62^{g}
T10	7.18^{b}	7.99^{b}	4.54^{g}
T11	3.57^{g}	6.32^{e}	3.82^{i}
T12	6.13^{e}	8.46^{a}	4.22^{h}
T13	4.13^{f}	5.82^{f}	2.89^{k}
T14	6.61^{d}	8.65^{a}	4.88^{f}
±SE	1.29	1.38	1.33

Data are expressed as mean ± SE (standard error). P values were < 0.05. Means with the same letter are not significantly different.

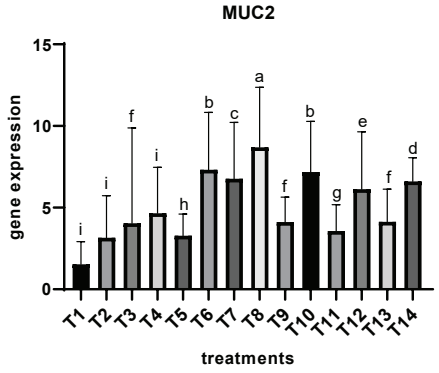

Fig. 1 Relative expression of intestinal muc2 gene

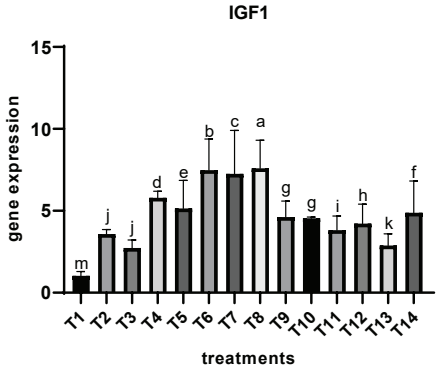

Fig. 2 Relative expression of hepatic IGF-1 gene

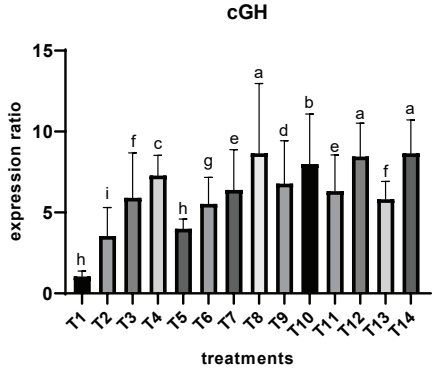

Fig. 3 Relative expression of hepatic cGH gene

revealed that all treatments except T2 and T4 had a better effect on the muc2 gene than the control group. T8, which had the highest expression, was the most effective treatment. From the same data, it was clear that increasing the concentration of prebiotics alone by more than 250 ppm was inversely proportional to the amount of mRNA expressed by muc2 (figure 1).

The same trend was observed for the effect of treatments on the expression of cGH (figure 2) and IGF (figure 3) genes. The most effective treatment, with the highest amount of expressed mRNA, was T8. In addition, lower expression was obtained by increasing the concentrations of the prebiotics and symbiotics.

DISCUSSION

The use of probiotics, prebiotics, and symbiotics as safe, effective, and cost-effective alternatives to antimicrobial growth promoters is gaining popularity in poultry nutrition. This can lead to an increase in the integrity of the digestive and immune systems by increasing the number and type of microflora. There is no doubt that gut health is a major factor in animal performance because of its importance in food digestion and metabolism, the incidence of intestinal diseases, and immune responses (Hamasalim 2016). Many studies have confirmed that probiotics play an important role in improving the growth performance and enhancing the symbiotic microbes in the gut of broiler chickens (Latorre et al., 2017; Rhayata et al., 2017). In the present study, the beneficial effects of a symbiotic on broiler performance parameters, including BWG, FCR, and FI, were in agreement with previous studies (Kabir et al., 2004; Mountzouris et al., 2007; Samli et al., 2007). The results of feed intake were in agreement with those (Abdel-Raheem and Abd-Allah (2011), who found that feed intake was improved by supplementation with probiotics and prebiotics. The increase in BWG with lower feed intake in supplemented broilers is believed to be a cumulative effect of prebiotic and probiotic foods, which promote beneficial bacteria, intestinal function, and disease resistance (Awad et al., 2008). Similarly, Nikpiran et al. (2013) reported improved FCR with probiotics and prebiotics. Fallah et al. (2014) concluded that FCR was improved by symbiotics in broiler chicks. FCR may be due to the maintenance of normal microbiota

and better ileal digestibility by the addition of probiotics and prebiotics. Feed intake was reduced, whereas feed conversion was improved significantly. This means that the birds consumed the least amount of feed to increase their weight and conversion factor. Dietary probiotics and prebiotics influenced the expression of muc2, cGH and mRNA IGF1 in the ileum and liver. An increase in this expression reflects the growth performance of birds. Many studies have suggested that the effectiveness of probiotics and prebiotics for bird growth stimulation is the result of an improved gastrointestinal ecosystem, resulting in an improved intestinal environment, intestinal mucosal barrier integrity, digestive and immune function, and broiler health (Tellez et al., 2006; Mountzouris et al., 2010).

Changes in mucin dynamics affect gut function, and may increase nutrient absorption. Previous studies have shown that the gastrointestinal microbiota can influence mucin dynamics (Dharmani et al., 2008). It has been reported that bacterial colonization of the gut can regulate mucin production by activating various signaling cascades and secretory chemical factors. Some researchers have suggested that Lactobacillus may bind to specific receptor sites on intestinal cells and induce myosin up-regulation (Mack et al., 1999; Mattar et al., 2002). The dependence of nutritional and growth hormones on hepatic IGF-1 production has been demonstrated (Beckman 2011). Moreover, among the genes influencing growth, IGF1 has been demonstrated to be an indicator of growth rate in chickens by several authors (Beccavin et al., 2001). The pituitary releases growth hormones, which stimulate the hepatic production of IGF-1 through the action of GH-activated GH receptors. However, the overall nutritional status of the animal modulates the ability of the hepatic tissue to respond to GH (Beckman 2011). It has been shown that the gut microbiota can dynamically modulate circulating IGF-1 in the host by producing short-chain fatty acids (SCFAs), which act directly on the liver and adipose tissue to induce circulating IGF-1 levels and promote growth and skeletal development. The dependence of nutritional and growth hormones on hepatic IGF-1 production has been demonstrated (Kareem et al., 2016). The current study found an increase in IGF-1 gene expression in the liver and improved growth performance in broilers fed probiotics and prebiotics.

CONCLUSION

This study demonstrated that the addition of probiotics, prebiotics, and symbiotics had beneficial effects on total BWG, feed efficiency, and expression of IGF1, cGH, and muc2 mRNA in broiler chickens. However, birds fed T8: prebiotics (250 ppm with probiotics) had the best result of total BWG, FCR, and FI, with higher gene expression of the previous genes than the other treatments. These additives could be used as substitutes for antibiotics in broiler diets to improve the growth and gut health of broiler chickens.

Abbreviations

E. faecium: Enterococcus faecium; L. acidophilus: Lactobacillus acidophilus; B. subtilis: Bacillus subtilis; S. cerevisiae: Saccharomyces cerevisiae; MOS: Mannan oligosaccharides; *β*- glucan: beta glucan; BWG: Body weight gain; FCR: Feed conversion ratio; FI: Feed intake; AR: Antibiotic resistance; cfu: colony-forming unit; ppm: parts per million; IU: International Unit; PCR: Polymerase chain reaction; RNA: Ribonucleic acid; cDNA: complementary Deoxyribonucleic acid; nm: nano meter; µL: micro liter; cGH: chicken Growth Hormone; IGF: Insulin- like growth factor; *β* actin: beta actin; Ct: cycle threshold; bp: base pair; SAS: Statistical Analysis System; GLM: generalized linear model; SD: standard deviation; p-value: probability value; mRNA: messenger RNA; SCFAs: short-chain fatty acids

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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