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Indigenous *Lactobacillus* strains improve growth performance and high density cholesterol levels in broilers

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Antibiotics have been used extensively in poultry chicken to promote growth rate, increase feed conversion efficiency, and prevent intestinal infections, resulting in an imbalance of the beneficial intestinal flora. The use of lactic acid bacteria as feed additives to substitute antibiotic associated growth stimulators as well as their impact on meat quality, could possibly be the major approach. In this context, here, we studied two *Lactobacillus* cultures *viz*., *L. plantarum* KGL3A and *L. fermentum* KGL4 as an alternative to growth promoters. Broilers were grouped into four different treatments: T1 (control: basal diet + antibiotic growth promoter and immunomodulatory factor), T2 [basal diet without having antibiotic growth promoter and immunomodulatory factor + *L. plantarum* KGL3A (10^8 CFU/mL)], T3 [basal diet without having antibiotic growth promoter and immunomodulatory factor + *L. fermentum* KGL4 (10^8 CFU/mL)] and T4 (basal diet without having antibiotic growth promoter and immunomodulatory factor + combination of T3 and T4 bacterial strains). During the entire study, higher bodyweight was observed among the *Lactobacillus* fed broilers groups (T4: 2433g, T3: 2371 g, T2: 2355 g) as compared to the control group (T1: 2339 g). Lipid profile analysis further confirmed the significant decrease in low-density lipoprotein (LDL) content of T4 (19%) and T3 (16%) groups than the control group while more than 10% increase in high-density lipoprotein HDL content was observed in T4 and T3 groups than the control group. Further, the decrease in coliform and enterococci counts and an increase in *Lactobacillus* counts in treatment groups compared to the control group were found. The results indicate that the potential use of *Lactobacillus* cultures (KGL3A and KGL4) as dietary feed supplements as alternative to the antibiotics as growth promoters in poultry feeds.

Keywords: Antibiotics, Feed supplements, Growth performance, Poultry

India is one of the world's largest producers of chicken meat, producing an average of 4 million tonnes of poultry meat out of 3 billion broilers annually[1]. In the last 3 to 4 decades, the average body weight of broilers has dramatically increased from 1.5 kg to 2.5 kg. The rise in body weight is attributable to supplementation with growth promoter's dependent on antibiotics, immunomodulators, and high-protein basal diet inclusion. The supplementation of antibiotics and vaccines has decreased the economic losses associated with bacterial and viral diseases in the poultry sector *viz*., mycoplasma-based infections, avian cholera, avian influenza, avian bronchitis, Newcastle disease, etc.)[2]. Long-term exposure to antibiotics as part of animal products has created many health issues for customers. Some meat producers have used Clenbuterol to produce less fat (lower cholesterol) and more protein meat to improve meat quality and texture, but resulted with serious adverse effects for young people[3]. Cholesterol is one of the important molecules for regular body fat metabolic function, but an increase in the amount of blood cholesterol is considered a risk factor for cardiovascular disease[4]. In contrast to average blood cholesterol individuals, higher blood cholesterol individuals are 45 per cent more vulnerable to heart attacks[5]. People around the world are therefore searching for new solutions to minimise blood cholesterol by natural means and have diverted to low fat, low sugar diets and daily exercise. With bile salt hydrolysing (BSH) activity and associated cholesterol-lowering potential in the host, few strains of lactic acid bacteria (LAB) were identified[6]. LAB's role in anti-cholesterol, antipathogenic, immune modulator and anticarcinogenic activities has

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increased its demand for the identification of novel LAB strains with health benefits and their inclusion in different industries of food processing[7,8]. *L. plantarum* and *L. fermentum*, the predominant oral and intestinal mucosa species of *Lactobacillus* in humans and animals, have demonstrated the ability to survive the gastrointestinal barrier and colonise after consumption in the intestine[9,10].

Considering the worldwide pressure from consumers, the scientific community and international regulatory agencies, to remove or decrease the use of antibiotics as performance enhancers and the rational use of the therapeutic form in poultry production, maintenance and taking food safety into consideration has been a challenge[11]. The feed supplementing lactic acid bacteria with antimicrobial activity, non-toxic to the host and survival to the intestinal barrier and promoting the host could be an alternative to replace conventional antibiotics as growth promoting substances[12]. Lactobacilli act by competitively excluding the adherence to and invasion of intestinal epithelium by pathogenic bacteria[8] and by enhancing the digestibility of feed[13]. The key field of research is the use of LAB as feed additives to substitute antibiotic-associated growth stimulators and their impact on meat quality. From the traditional rice beverages of Garo tribes of Meghalaya, *L. plantarum* KGL3A and *L. fermentum* KGL4 were isolated. The two strains were both non-toxic in nature and non-haemolytic. In addition to the other *in vitro* studies, cholesterol lowering properties were also found (data not shown). Therefore, in this study, we examined the efficacy of two LAB strains *L. plantarum* KGL3A and *L. fermentum* KGL4 as a growth stimulant and their cholesterol lowering potential in broilers.

Materials and Methods

Preparation of bacterial strains as feed supplements

Two *Lactobacillus* strains viz. *L. plantarum* KGL3A (GI: MF951099) and *L. fermentum* KGL4 (GI: MG722814) were used in this study. Both the strains were isolated from rice beverages of Garo tribes of Meghalaya, India[14]. The cultures were studied primarily for basic safety parameters (Table S1) and probiotic potential parameters (acid tolerance, antimicrobial compound production, susceptible to antibiotics, cholesterol reduction potential) earlier and selected for this study. The cultures were actively grown in de Man Rogosa and Sharpe broth (MRS media) for overnight at 37°C. The active bacterial cells were centrifuged at 6000×g for 7 min at 4℃ and washed thrice with PBS (Phosphate buffer saline), to wash the cells properly. The cells were then suspended in PBS and diluted accordingly to adjust the cell counts (approximately 10^8 CFU/mL) in final volume and stored in below 4℃ for further study.

Experimental design and husbandry

Ninety-six 01-day old broiler chickens (weight: 45-50 g, breed: Cobb 430Y) were purchased from Venky's India Limited. The chickens were wing-banded and randomly assigned to four treatment groups following a completely randomized design. All birds were categorized into 4 groups *viz*., T1 [control: basal diet + antibiotic growth promoter and immunomodulatory factor], T2 [basal diet without having antibiotic growth promoter (BMD-100) and immunomodulatory factor (Immunowall) + *L. plantarum* KGL3A (≈10^8 CFU/mL)], T3 [basal diet without having antibiotic growth promoter and immunomodulatory factor + *L. fermentum* KGL4 (≈10^8 CFU/mL)] and T4 [basal diet without having antibiotic growth promoter and immunomodulatory factor +combination of T3 and T4 bacterial strains]. For the selection of appropriate group and subgroup size, the resource equation approach with 20 degree of freedom of error component considered. And based on it, in each group, 24 birds were divided into 4 replicates, each replicate having 6 birds. The obtained sample size was appropriate for this study. The birds were housed in electrically heated batteries in an environmentally controlled room in the Poultry Department, Anand Agricultural University, Anand, India. The broilers were fed a typical corn-soybean meal as basal diet. The basal diet was formulated to meet the nutrient requirements for starter (from 1 to 21 days) and growth or finishers (from 22 to 42 days). The composition of the experimental diet is presented in Table 1. The experiment was performed in accordance with the Indian animal ethical guidelines and institutional ethical guideline for animals. [Study time line in brief: 1 day to 7 days basal diet of respective groups as mentioned above, 8^{th} day to 15^{th} day probiotic supplementation along with basal diet as described earlier. On 16^{th} day to 42^{nd} day basal diet without probiotics was given as per respective groups].

Growth performance

Broiler performance including body weight, daily feed consumption, and mortality rate were determined up to 42 days[15] during the study. All birds in each

Table 1 — Nutritional composition of basal diet

Ingredient and composition (%)	1 to 21 days Starter (%)	22 to 42 days grower/finisher (%)
Corn	61.0	64.0
Soybean meal	33.27	28.4
Vegetable oil/soybean oil	1.9	3.9
Calcite powder	1.25	1.25
Salt	0.1	0.1
Digestive crude protein	1.0	1.0
Methionine	0.18	0.15
Vitamins	0.05	0.05
Trace Minerals	0.1	0.1
Choline chloride 60%	0.1	0.1
Lysine	0.25	0.16
Phytase	0.01	0.01
Sodium bicarbonate	0.36	0.35
Livertonic	0.1	0.1
Toxin binder	0.1	0.1
Anticoccidal	0.05	0.05
Emulsifier	0.05	0.05
*Immunomodulator (Immunowall / Zist(S))	0.05	0.05
*Growth promoter (BMD-100)	0.02	0.02
Total	100	100

*Included in control group (T1) only, while absent in other groups basal diet. [Broilers of group 1 (T1): Basal diet with growth promoter (BMD-100) and immunomodulators (Immunowall/Zist(s)). All the chickens had free access to water and feed. Group 2 (T2): Basal diet supplemented with 10^8 cells of *L. plantarum* KGL3A from 8 to 15 days of age, all the chickens had free access to water and feed. Group 3 (T3): Basal diet supplemented with 10^8 cells of L. fermentum KGL4 from 8 to 15 days of age, all the chickens had free access to water and feed. Group 4 (T4): Basal diet supplemented with 10^8 cells of *L. plantarum* KGL3A and *L. fermentum* KGL4 mixture from 8 to 15 days of age, all the chickens had free access to water and feed]

group were weighed individually at 0, 1, 2, 3, 4, 5 and 6 weeks of age. The feed offered to each group was also recorded daily using a digital weighing machine (Metis Chicken Weighing Scale). Feed conversion ratio (FCR) and mortalities were also recorded throughout the study.

Biochemical analysis of blood samples after 35 days and 42 days old broiler

Blood serum parameters were examined after 35 and 42 days. Fresh 2.5 mL blood sample was collected from each replicate of four different groups to measure the total cholesterol (TC), triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) and another 2.5 mL blood sample was also collected for haematological parameter (Blood cell counts and haemoglobin) study[16]. The concentrations of TC, TG, HDL-C and LDL-C in blood serum were determined by enzymatic colorimetric methods using commercial kits purchased from Span diagnostics LTD, India, according to manufacturer's instructions.

Histological examination of intestine and liver tissues after 42 days old broiler

For histological examination, liver and intestinal tissues were collected after 42 days from a butcher shop, then dehydrated and embedded in paraffin as per the protocol suggested by Gilani *et al*.[16]. Specific sections (5 µm) were stained with hematoxylin and eosin, and histomorphologically examined under the microscope (Olympus, India).

Enumeration of faecal samples after 42 days old broiler

Eleven grams of faecal sample was collected into to sterile container after 42 days from each group and then serially diluted (from 10^{-2} to 10^{-7}) in the sterile PBS. For *Lactobacillus* counts, the appropriate dilution was plated on MRS agar, for coliforms on Violet Red Bile (VRB) agar and for enterococci on *Streptococcal* agar medium at 37°C for 24-48 h and respective counts were represented as log CFU/g[17].

Statistical analysis

Data were expressed as mean standard error (SEM) from three experiments. The experiments were conducted in triplicate. The analysis of variance was performed using one-way ANOVA and significant differences among the means of samples were analysed by Duncan's test with a 95% confidence level.

Results and Discussion

Effect of *Lactobacillus* feeding on growth parameters of broilers

Lactobacilli are naturally found in the gastrointestinal tract of broilers and have positively correlated with the growth performance of the broilers. Short-chain fatty acids (SCFA) production by *Lactobacillus* cultures could exert a positive role in the broilers. Therefore, 1.0 mL of bacterial culture containing approximately 10^8 CFU/mL *Lactobacillus* cells was orally fed to the broiler chickens in the 2nd week of their initial growth phase and it was continued for seven days. The antibiotics as growth promoters and immunomodulator were not added into the diets for three groups of *Lactobacillus* fed broiler. The effect on growth performance and feed utilization including body weight and FCR were examined to 6 weeks and control group was supplemented with antibiotics as growth promoters and immuno-modulator in their basal diet and body weight and FCR was also examined to 6 weeks. The counts of

two *Lactobacillus* strains KGL4 and KGL3A individually and or in combination were evaluated for the four groups given in Table 2. T4 group was fed with *Lactobacillus* strains KGL4 and KGL3A in combination and showed maximum (2433 g) (P <0.05) average body weight over 6 weeks, followed by T3 group fed with *L. fermentum* KGL4 (2371 g) alone and T2 group fed with *L. plantarum* KGL3A (2355 g) alone compared toT1 control group (2339 g) respectively. The FCR obtained were 1.88 (T1), 1.87 (T2), 1.85 (T3) and 1.81(T4) respectively for T1, T2, T3, and T4 groups. During the entire experiment, total three mortalities were reported from each of T1 (6th week), T2 (1st week) and T3 (3rd week) group while zero mortality was observed in T4 group. The reported mortality was natural and not associated with the treatment reported in the post-mortem. The growth results obtained were equivalent or higher in comparison to the control group which was supplemented with the antibiotics-growth promoter and immunomodulators (P <0.05). The data signifies the importance of bacterial strains KGL4 and KGL3A individually or in combination as feed additives to the broilers; an alternative to getting rid of antibiotics and antibiotics associated harmful growth promoters to the consumers[3]. Similar results were observed by Fesseha *et al*.[18] who reported that the chickens supplemented with *L. paracasei*, *L. rhamnosus* during the first week of age have shown higher body weight than control. The feed intake of week one of treatment groups *viz*., T2 (supplemented diet with 4 g probiotic/kg feed) and T3 (supplemented diet with 2 g probiotic/kg) were significantly higher than control group. Furthermore, administering single probiotic or combination probiotic feed additions to broiler feed might significantly increase growth performance and lower FCR under normal, stress, disease, and other stressful situations[15]. The strains of *L. casei*, *L. plantarum* and *L. fermentum* fed to broilers resulted in an increase in body weight gain[19].

Table 2 — Growth performance and blood parameters of broilers

Growth Parameters	Treatment Groups				
	T1	T2	T3	T4	S. Em
Body weight (g)	2339	2355	2371	2433*	29.06
FCR	1.88	1.87	1.85	1.81	0.02
WBC (10^3/µL)	239.8	250.8	243.2	262.0	23.88
RBC (10^6/ µL)	3.3125	2.795	2.6625	3.455	0.31
Hb (g/dL)	14.45	11.6	11.6	14.9	1.46
PLT (10^3/ µL)	95.5	70.5	94.25	71	18.84

*differ significantly (P<0.05) [Broilers group 1 (T1): Basal diet with growth promoter (BMD-100) and immunomodulators (Immunowall/Zist(s)). Group 2 (T2): Basal diet supplemented with 10^8 cells of *L. plantarum* KGL3A from 8 to 15 days of age. Group 3 (T3): Basal diet supplemented with 10^8 cells of *L. fermentum* KGL4 from 8 to 15 days of age. Group 4 (T4): Basal diet supplemented with 10^8 cells of *L. plantarum* KGL3A and *L. fermentum* KGL4 mixture from 8 to 15 days of age.]

Biochemical analysis of blood samples of broilers

The feeding of *Lactobacillus* cultures to the broilers could exert a positive impact on haemoglobin due to siderophore activity, and also on white blood cell count (WBC) as an immunomodulator and on lipid profile of the broilers due to their cholesterol reduction potentiality. To study the effect of *Lactobacillus* as feed supplements on blood parameters, they were analysed after the 35th and 42nd day of the feeding and the mean results obtained for blood parameter is given in Table 2. No significant differences (P <0.05) were observed in white blood cell count (WBC), red blood cell count (RBC), haemoglobin (HG) and platelet count among the three treatment groups as well as control groups (Table 2). Serum lipids profile analysis was carried out and found that significant difference (P <0.05) in parameters of lipid profiles (Fig. 1). There was 0.6 to 3% reduction in total cholesterol reported in treatments groups compared to the control group, while a significant increase in HDL in the treatment group was found. Highest HDL was reported in group fed with mixed bacterial cultures (T4) (84.75 mg/dL), followed by group fed with KGL4 (T3) (83.87

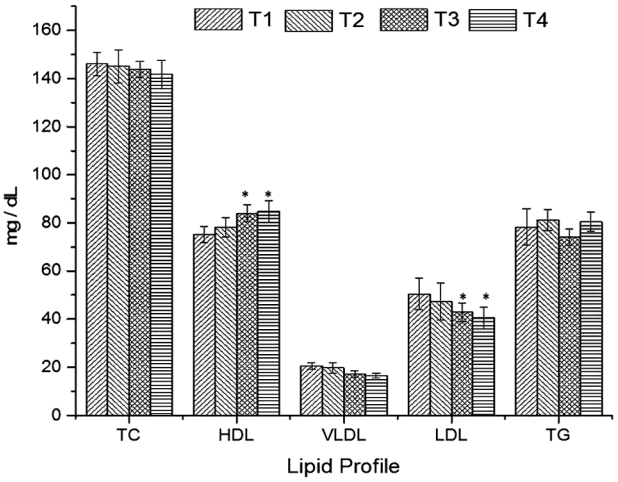

Fig. 1 — Lipid profiles of Blood. Broilers group 1 (T1): Basal diet with growth promoter (BMD-100) and immunomodulators [Immunowall/Zist(s)]. Group 2 (T2): Basal diet supplemented with 10^8 cells of *L. plantarum* KGL3A from 8 to 15 days of age. Group 3 (T3): Basal diet supplemented with 10^8 cells of *L. fermentum* KGL4 from 8 to 15 days of age. Group 4 (T4): Basal diet supplemented with 10^8 cells of *L. plantarum* KGL3A and *L. fermentum* KGL4 mixture from 8 to 15 days of age. *differ significantly (P <0.05)

mg/dL) and KGL3A (T2) (78.15 mg/dL) than control group (75.05 mg/dL). Similarly, the decrease in LDL and VLDL contents were also observed in broilers fed with bacterial cultures. T4, T3, and T2 showed 1915and 6% reduction in LDL and 1916 and 4% reduction in VLDL, respectively, compared to the control group. However, the increase in triglyceride in T4 and T2 group while the decrease in T3 group was also found during the study.

When compared to the control, plasma triglycerides, low density lipoprotein (LDL), and total cholesterol levels decreased, whereas high density lipoprotein (HDL) cholesterol levels increased in lactic acid bacteria supplemented broiler groups[20]. When additional forms of lactobacilli probiotics were used in the diet of broiler chickens, similar cholesterol-lowering effects were reported in other studies as well[17,21]. Cholesterol reduction potential of *Lactobacillus* is well reported, however, the exact mechanism behind it is yet to be understood. It is assumed that bile salt hydrolysing potential of *Lactobacillus* strains responsible for possible cholesterol reduction in the host[21]. The reduction in cholesterol is considered to be positive as to quality meat with health benefits to the consumers. Probiotic *Lactobacillus* fed broilers meat showed good organoleptic scores in overall acceptability, appearance, texture, and juiciness compared to the basal diet fed broilers meat[22]. Moreover, probiotic fed broilers were reported with better meat and protein ratio compared to the basal diet group[23].

Histological examination of intestine and liver tissues after 42 days old broiler

Histological methods indicate the effect of *Lactobacillus* feeding on the internal organs of broilers, particularly, liver and intestinal tissues. Liver and intestinal sections were examined to study the effect of bacterial feeding on broilers. Upon histopathological examinations in the study, there were no significant differences observed amongst the *Lactobacillus* fed broiler's liver and intestinal tissues compared to control group. The obtained result indicates the safe nature of *Lactobacillus* cultures to be used as potential lactic acid bacteria supplements along with basal diets without employing growth promoters for broilers. The fine macroscopically examined intestinal tissues suggested the well-organized epithelial lining and villi structure in *Lactobacillus* fed broiler groups (T2, T3, T4) and control group (T4) (Fig. 2). The results are in

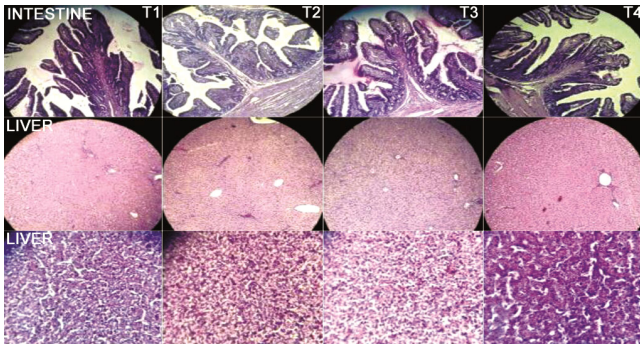

Fig. 2 — Histological images of intestine and liver tissue of broilers. Broilers group 1 (T1): Basal diet with growth promoter (BMD-100) and immunomodulators [Immunowall/Zist(s)]. Group 2 (T2): Basal diet supplemented with 10^8 cells of *L. plantarum* KGL3A from 8 to 15 days of age. Group 3 (T3): Basal diet supplemented with 10^8 cells of *L. fermentum* KGL4 from 8 to 15 days of age. Group 4 (T4): Basal diet supplemented with 10^8 cells of *L. plantarum* KGL3A and *L. fermentum* KGL4 mixture from 8 to 15 days of age.

agreement with the other studies in which intestinal morphology of *Lactobacillus* fed broilers was examined[24,25]. Hussein *et al*.[25] reported an increase in intestinal villi height, the number of mucilage cells, intraepithelial immune cells and antibody precursor cells. Furthermore, positive impact on broilers growth performance and microflora count was also depicted. From the histological analysis of liver and intestinal tissues, the beneficiary effects of *Lactobacillus* cultures as the feed supplement to the poultry birds were reported.

Faecal samples (Lactobacilli, enterococci and coliform counts) analysis of broilers

Increase in *Lactobacillus* number in the intestinal tract of broilers could help in the improvement of growth performance as well as inhibiting the growth of pathogens and other harmful bacteria. Feeding of *Lactobacillus* cultures to the broilers had a significant change in the faecal count, that was observed during the study (Fig. 3). The *Lactobacillus* counts increased significantly (P <0.05) in *Lactobacillus* fed broiler faecal samples compared to the control group in the 2nd week of faecal samples (Fig. 3A). The 2% higher *Lactobacillus* faecal count was observed in the 2nd week of treatment groups compared to the control group. However gradual (1 to 3%) decrease in *Lactobacillus* faecal count every week, after feeding week was observed in treatment groups. Although after 2nd week onwards, *Lactobacillus* counts were found higher throughout the study among the treatment groups (T2, T3 & T4) compared to the control group (T1). In T2 (*L. plantarum* fed), the

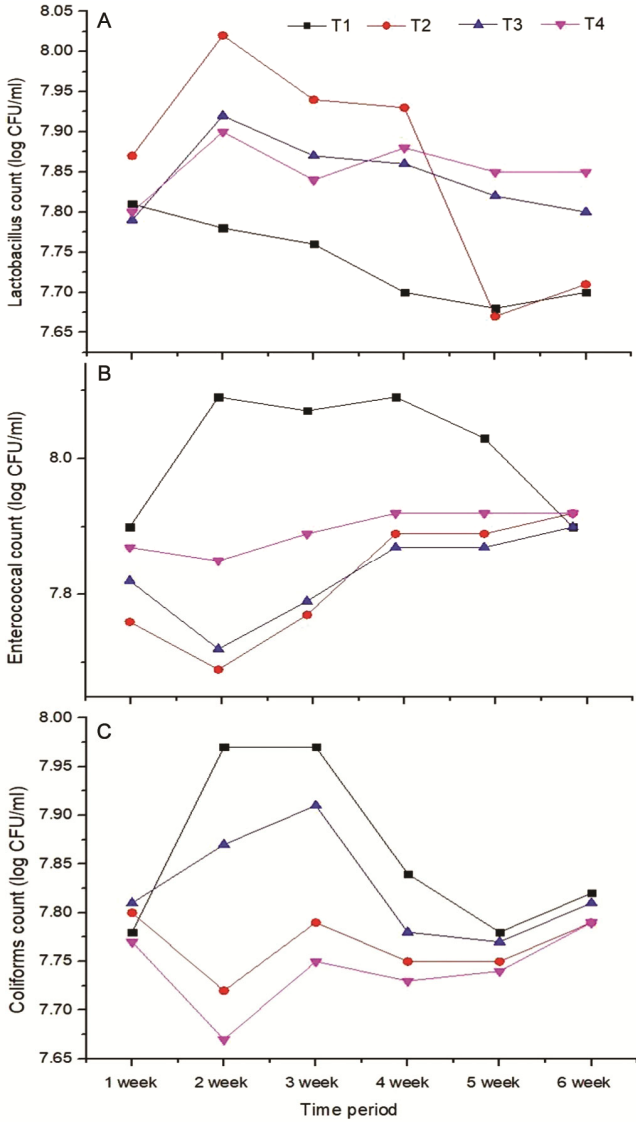

Fig. 3 — Faecal counts of Lactobacilli, enterococci and Coliforms. Broilers group 1 (T1): Basal diet with growth promoter (BMD-100) and immunomodulators [Immunowall/Zist(s)]. Group 2 (T2): Basal diet supplemented with 10^8 cells of *L. plantarum* KGL3A from 8 to 15 days of age. Group 3 (T3): Basal diet supplemented with 10^8 cells of *L. fermentum* KGL4 from 8 to 15 days of age. Group 4 (T4): Basal diet supplemented with 10^8 cells of *L. plantarum* KGL3A and *L. fermentum* KGL4 mixture from 8 to 15 days of age. (A) *Lactobacillus* faecal count of respective treatments; (B) Enterococci faecal count of respective treatments; and (C) Coliforms faecal count of respective treatments.

significant (P <0.05) decrease in *Lactobacillus* count were observed as time increases during the study. In the case of T3 and T4, a gradual decrease (non-significant) in *Lactobacillus* count was also found during the study period. From the faecal analysis, it could be said that T3 and T4 showed a higher intestinal survival rate of Lactobacillus cultures compared to T2 treatment.

Schokker *et al*.[26] reported similar results in their respective experiments of broilers fed with *Lactobacillus* cultures. Furthermore, the effect of *Lactobacillus* feeding on the gram-positive cocci (enterococci) and coliforms counts was also observed. In T2, T3 & T4, the lesser number of enterococci counts reported compared to the control (T1) group (Fig. 3B). During *Lactobacillus* feeding (2nd week), a significant decrease (P >0.05) in enterococci counts were observed. But thereafter in the following weeks, a gradual increase (P <0.05) in enterococcal counts were also observed. Incorporation of probiotics in the diet has led to an increase in lactobacilli and enterococci count compared to the control groupreported[27].

Similarly, significant (P <0.05) decrease in coliforms count in T2, T3 and T4 treatment groups were also reported, compared to the control (T1) group (Fig. 3C). In the 2nd week of treatment, the highest decrease of coliforms in the faecal count was reported in *Lactobacillus* fed groups and in the following weeks, lesser faecal counts compared to the control group was further reported. The results were in agreement to the study conducted by Jha *et al*.[28] and Abd El-Hack *et al*.[29], who reported the feeding of *Lactobacillus* could reduce the coliforms count and increase in *Lactobacillus* faecal counts in broilers. The obtained results advocate the possible bactericidal properties of the fed cultures due to either lactic acid or other short-chain fatty acids or due to bacteriocin-like compound production in the gut[30]. Wang *et al*.[31] also have noticed an improvement in the intestinal microbial community in broilers administered probiotics, and the findings in the study were consistent with their results.

Conclusion

The oral feeding of *L. plantarum* KGL3A and *L. fermentum* KGL4 individually or in combination along with basal diet without adding growth promoter as antibiotics to the broiler chickens improved the growth performance (FCR; T4- 1.81, T3-1.85, T2- 1.87, T1- 1.88) and increased the *Lactobacillus* counts and decrease in coliforms and enterococci counts in faecal samples. Further, the gain in bodyweight in *Lactobacillus* (without antibiotic growth promoter) fed broilers (T4: 2433 g, T3: 2371 g, T2: 2355 g) were higher or at par with the control group (feed supplemented with antibiotics as a growth promoter and immunomodulator, T1: 2339 g). The

good cholesterol to bad cholesterol ratio significantly improved over the study than the control group. The lipid profile analysis indicated the LDL content of the T4 (19%) and T3 (16%) groups was slightly lower than that of the control group (T1), while HDL content was more than 10% higher than that of the control group. However, incorporation of *L. plantarum* KGL3A and *L. fermentum* KGL4 cultures as a feed supplement to the broilers had overall positive effects on broilers performance. Further, more clinical trials are required to validate the claim for the above-mentioned *Lactobacillus* cultures *viz*., KGL4 and KGL3A.

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Conflict of interest

The authors declare no competing interests.

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