Performance and intestinal microflora response of broilers to a probiotic mixture supplementation

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ABSTRACT

The study evaluated the effects of a *Lactobacillus* strains probiotic mix on performance, carcass traits, organs size, and intestinal microflora in broilers. A total of 200 one-day-old unsexed broilers were allotted into 2 groups with 5 replicates (20 broilers/replicate). During the feeding trial (35 d) the broilers were fed with a control (basal diet) or probiotic (basal diet plus 3% probiotic mix *L. acidophilus* and *L. plantarum*, 1:1 ratio). The probiotic supplementation did not significantly influence the performance, carcass traits and organs size of broilers at slaughter age. No effect of probiotic supplementation on visceral weight or length was observed, except a higher of jejunum ($P=0.007$) and ileum ($P=0.013$) weights, and a tendency to higher the caecum length ($P=0.070$). The probiotic mix had no significant effect on the intestinal pH, but it improved the microflora by decreasing the *Enterobacteria* and *E. coli* counts and increasing the *Lactobacilli* counts and *Lactobacilli*: *E. coli* ratio in the ileum and caecum ($P<0.05$). In conclusion, the probiotic mix (*L. acidophilus* and *L. plantarum* mix, 1:1 ratio) did not significantly affect the productive performance but had a positive effect on broilers' gut microflora.

Keywords: broiler, probiotic, productive performance, gut microflora

INTRODUCTION

Nowadays, in the context of emerging public health concern about antibiotic resistance and food safety, intensive studies have been conducted to investigate natural nutritional alternatives such as probiotics and their role to improve the poultry performance and their products (Al-Shawi, 2020).

FAO/WHO (2002) defined the probiotics as “mono or mixed strains of living microorganisms which confer a desirable health benefit on the host when used adequately”. The species of microorganisms used as probiotics for poultry included *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, *Bacillus*, *Streptococcus*, and fungi e. g. *Saccharomyces* (Hossain et al., 2012; Gadee et al.,

2017). The selection of probiotics is based on tolerance to gastrointestinal (GIT) conditions, the capacity to adhere to the GIT mucosa, and the competitive exclusion of pathogens (Gadee et al., 2017). The probiotics survival rate in manufacture, storage, transport, and their viability and characteristics are important criteria as well (Bajagai et al., 2016).

Probiotics supplementation, either as single or mixed strains, in commercial poultry production, has indicated positive responses on productive performance (Olnood et al., 2015; de Souza et al., 2018; Deng et al., 2020; Lokapirnasari et al., 2020), nutrient absorption and digestibility (Li et al., 2008; Awad et al., 2010), immunity (Awad et al., 2009; Fathi et al., 2017), gut microflora and inhibition of pathogens (Giannenas et al., 2012; Giannenas et al., 2014; Ciurescu et al., 2020; Deng et al., 2020; Gheorghe et al., 2020). It was demonstrated that multiple-strain and multi-species probiotics could have a synergistic effect by acting on different sites and providing various modes of action (Timmerman et al., 2004; Kazemi et al., 2019).

Lactobacillus strains are one of the most used probiotics, that have been revealed to increase the activity of intestinal enzymes and digestion (Awad et al., 2010) and to control GIT pathogenic microbial populations such as *Escherichia coli*, *Salmonella*, and coliforms, improving intestinal health (Hardy et al., 2013; Chen et al., 2017; Attia et al., 2017; De Cesare et al., 2020). Several studies investigated the effects of *L. acidophilus* on performance, metabolic function and gut health in broilers or rurally reared chickens (De Cesare et al., 2017; Forte et al., 2018; Gheorghe et al., 2020). Other researches have been focused on the efficacy of *L. plantarum* in broilers feed (Mountzouris et al., 2007; Hong et al., 2012; Peng et al., 2016). A previous study in pigs (Habeanu et al., 2016) evaluated the relation between sow's milk quality and litter performances and health status as effects of feeding 5% hemp seeds in lactating sows and 2.5% feed additive (*L. acidophilus* 50% and *L. plantarum* 50%) in piglets. The authors noticed that the dietary fatty acids (FA) composition positively affect the colostrum/ milk quality in term of polyunsaturated FA content and piglets performance and health. Moreover, the feed additive addition improved the growth performance response of piglets. Since limited reports are available on the effect of a combined *L. acidophilus* and *L. plantarum* supplementation, this study aimed to investigate the response of broilers to probiotic *L. acidophilus* and *L. plantarum* (1:1 ratio) supplementation on growth performance, carcass parameters, organs size and intestinal microflora.

MATERIALS AND METHODS

The research institute ethical committee (INCDBNA-Balotesti, Romania) approved the procedures used in this trial, following the European legislation (Directive 2010/63/EU).

Probiotic strains

Lactic acid bacteria (LAB) strains (n=5; *L. acidophilus*, *L. brevis*, *L. fermentum*, *L. salivarius* and *L. plantarum*), previously isolated from the GIT of healthy broiler chickens and stored at IBNA collection strains, were evaluated for their probiotic potentials (Dumitru et al., 2019; Dumitru et al., 2020). The overnight culture of each *Lactobacillus* isolates after an incubation at 37°C in anaerobic conditions was evaluated for purity and their viability for three weeks (Shokryazdan et al., 2014). The strains were kept at -80°C in MRS broth medium with 20% glycerol (v/v) as previously described Sorescu et al. (2019).

Due to its ability to survive of several probiotic properties (low pH of the stomach, bile salts, capacity to colonize in the digestive tract, resistance of antibiotics, low temperatures values etc.), only two *Lactobacillus* strains (*L. acidophilus* and *L. plantarum*) were selected as probiotic candidates for broiler feed.

Broilers and experimental design

Two hundred 1-day-old Cobb 500 mixed sex broilers (43.89±1.09 g) were randomly allotted into two groups with five replicates each (20 broilers/replicate) and used for a feeding trial (1 to 35 days of age). Broilers were reared in climate-controlled conditions on floor pens (2 x 1 m) with wood shaving litter. Each pen was equipped with manual feeders and nipple drinker line. A light regime of 23h per day was provided until day 7, and 20h light per day until 35 days. The birds were immunised against Marek's, Newcastle and Gumboro diseases according to veterinary protocol.

Three-phase basal diets were formulated based on the chemical composition of feeds (OJEU, 2009) to meet the Cobb 500 broilers nutrient requirements (Table 1).

Birds were fed with a basal diet (corn-soybean meal) or a probiotic diet (basal diet with 3% probiotic mix added). The two *Lactobacillus* strains (*L. acidophilus* and *L. plantarum*) selected as probiotic candidates were mixed 1:1 ratio and added in a proportion of 3% (concentration of 1×10^9 CFU/mL) into the premix of basal diet for each phase and homogenised.

The feeds were administered in mash form. Water and feed were given *ad libitum* to the broilers.

The performance variables evaluated during the trial were broilers body weight (BW) by individually weighing per each phase and the overall period, and body weight gain (BWG) was calculated. Feed intake (FI) was also recorded to calculate feed conversion ratio (FCR) per each growth phase. Viability was determined as [100 - mortality (%)].

Table 1. Components and nutrients composition of basal diets (as-fed basis)

Ingredients (g/kg)	Starter (1-10 d)	Grower (11-22 d)	Finisher (23-35 d)
Corn	560.0	600.0	657.6
Soybean meal	310.0	269.0	220.0
Corn gluten meal	50.0	46.0	36.0
Sunflower oil	28.0	35.0	40.0
Monocalcium phosphate	18.2	17.0	15.0
Calcium carbonate	14.7	13.9	12.7
Salt	3.0	3.0	3.0
DL-Methionine	1.5	2.0	1.9
L-Lysine HCl	4.0	3.5	3.2
Choline HCl	0.6	0.6	0.6
Vitamin-mineral premix ¹	10.0	10.0	10.0
Probiotic ²	(-)/(+)	(-)/(+)	(-)/(+)
Calculated composition (g/kg)			
Metabolisable energy (MJ/kg) ³	12.61	12.97	13.31
Crude protein	220	200	180
Lysine, total	13.2	11.9	10.5
Lysine, digestible	11.8	10.5	9.5
Methionine + cysteine, total	9.8	8.9	8.2
Methionine + cysteine, digestible	8.8	8.0	7.4
Calcium	9.0	8.4	7.6
Available phosphorus	4.5	4.2	3.9
Crude fibre	35.4	33.1	30.4

Note: ¹Provided per kg diet: vitamin A, 4.47 mg; vitamin D3, 0.12 mg; vitamin E, 80 mg; vitamin K3, 4 mg; vitamin B1, 4 mg; vitamin B2, 9 mg; vitamin B6, 4 mg; vitamin B12, 0.020 mg; vitamin B5, 15 mg; vitamin B3, 60 mg; vitamin B9, 2 mg; Mn, 100 mg; Zn, 100 mg; Fe, 40 mg; Cu, 15 mg; I, 1.0 mg; Se, 0.30 mg; Co, 0.25 mg. ²(-), no probiotic; (+), 3% probiotic (*L. acidophilus* and *L. plantarum* mixture, 1:1 ratio). ³calculated using regression equations (NRC, 1994).

Sampling and analyses

At the end of the trial (35 days), eight broilers/group were assigned for carcasses, digestive organs and intestinal segments evaluation. After the fasting period (12h), the birds were slaughtered by cervical dislocation, followed by bleeding, plucked and eviscerated. The gizzard, liver, heart, pancreas, spleen, bursa and abdominal fat were removed and weighed. The intestinal segments (duodenum, jejunum, ileum, and cecum) also were removed and weighed. The results were given as relative weights or lengths (g or cm % of life BW).

The digesta pH from fresh samples was determined in duplicate with a portable pH-meter (WTW ProfiLine pH 3310, Germany) by inserting the pH meter electrode into the distal parts of each intestinal segment.

Digesta from ileum and caecum were sampled in sterile tubes and stored at -20°C until microbial analyses. A conventional technique was used for determination of bacterial populations (total bacteria, LAB, *E. coli* and *Salmonella* spp.), the digesta samples were serially diluted in saline solution and selective agar media were used as previously described Gheorghe et al. (2019). Bacterial counts values were expressed as log-10 colony-forming units (CFU)/ g of intestinal digesta.

Statistical analysis

Experimental data were analysed using one-way ANOVA followed by the Tukey's test to compare the differences between means (SPSS v.20, 2011). Data results are presented as mean and standard error of the mean (SEM). Significant differences were considered at $P < 0.05$.

RESULTS AND DISCUSSION

Growth performance

As shown in Table 2, probiotic supplementation did not significantly affect the productive performance (BW, FI and FCR) during the grower phase and overall experimental period (5-week) compared with control.

Table 2. Effect of probiotic addition on productive performance of broilers¹

Item	Control	Probiotic	SEM	P-value*
Starter (1-10 d)				
BWG (g/bird)	219.21	220.05	4.13	0.937
FI (g/bird)	270.26	274.45	1.26	0.313
FCR (g feed: g gain)	1.23	1.25	0.01	0.646
Grower (11-22 d)				
BWG (g/bird)	675.09	687.61	15.19	0.581
FI (g/bird)	1015.67	1030.41	1.24	0.876
FCR (g feed: g gain)	1.50	1.49	0.10	0.113
Finisher (23-35 d)				
BWG (g/bird)	985.38	999.17	33.25	0.956
FI (g/bird)	2000.15	2050.86	32.52	0.991
FCR (g feed: g gain)	2.03	2.05	0.01	0.090
Overall period (1-35 d)				
BWG (g/bird)	1879.68	1906.83	32.45	0.886
FI (g/bird)	3286.08	3355.72	26.11	0.892
FCR (g feed: g gain)	1.75	1.76	0.03	0.104
Viability (%)	98	99	1.20	0.538

Note: ¹Means of 5 replicate pens (n=20 birds/pen); SEM, standard error of the mean; BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio.

*Means within rows do not differ significantly ($P > 0.05$).

Our results agree with several studies which reported that single or multiple-strains probiotic supplementation have not significantly affected the growth performance of broilers (Khosravi et al., 2010; Waititu et al., 2014; Olnood et al., 2015; Fathi et al., 2017; de Souza et al., 2018; Ding et al., 2019; Gheorghe et al., 2020).

Carcass traits and organ weights

The probiotic addition did not affect the broilers carcass yield, major cut-up traits and relative organ weights ($P>0.05$; Table 3).

Table 3. Effect of probiotic addition on broiler carcasses and organ weights¹

Item	Control	Probiotic	SEM	P-value*
Carcass weight, g	1433.25	1490.67	19.27	0.149
Carcass yield, %	70.70	70.66	0.42	0.906
Breast, %	24.39	24.98	0.50	0.111
Legs, %	19.60	19.32	0.15	0.311
Abdominal fat, %	1.46	1.37	0.08	0.574
Relative organ weights (% BW)				
Gizzard	1.52	1.44	0.057	0.523
Liver	2.12	2.15	0.052	0.776
Heart	0.47	0.46	0.017	0.885
Spleen	0.10	0.12	0.005	0.608
Pancreas	0.22	0.25	0.013	0.062
Bursa	0.12	0.15	0.009	0.094

Note: ¹n = 8 samples per group; SEM, standard error of the mean; BW, body weight.

*Means within rows do not differ significantly ($P>0.05$).

Similarly, de Souza et al. (2018) investigated multi-strains probiotics (*L. acidophilus*, *Bacillus subtilis*, *Bifidobacterium bifidum* and *Enterococcus faecium*) in Cobb chick's fed and reported no effects on the growth performance, carcasses traits and chemical composition. Other research has also stated that probiotic addition did not influence the lymphoid and digestive organs (Awad et al., 2009; Naseem et al., 2012). Pourakbari et al. (2016), using different doses of commercial multi-strains probiotics, reported variation in the responses of performance and carcass parameters, but no effects on immune organs or immune response. These authors also suggested that the optimum concentration of probiotics in broiler feed differs with the microorganism used in the product composition.

Intestinal measurements and microflora

No effect of probiotic supplementation on visceral weight or length was observed, except a higher of jejunum ($P=0.007$) and ileum ($P=0.013$) weights,

and a tendency to higher the caecum length ($P=0.070$). Pourakbari et al. (2016) also noticed an increase in caecum weight as an effect of multi-strain probiotic supplementation in broilers.

A possible explanation of the enlarged intestinal parts observed in the present work could be the increased length and density of the microvilli of the small intestine and caecum due to the probiotic addition (Yurong et al., 2005).

Table 4. Effect of probiotic addition on relative weight and length of intestinal parts, and digesta pH¹

Item		Control	Probiotic	SEM	P-value
Relative weight and length of intestinal parts (g or cm/100 g BW)					
Duodenum	g	0.66	0.68	0.014	0.752
	cm	1.44	1.43	0.035	0.844
Jejunum	g	1.54 ^b	1.77 ^a	0.051	0.007
	cm	3.87	3.64	0.052	0.019
Ileum	g	1.10 ^b	1.20 ^a	0.040	0.013
	cm	3.75	3.72	0.059	0.818
Caecum	g	0.60	0.63	0.014	0.113
	cm	0.80	0.88	0.023	0.070 ^r
Digesta pH					
Duodenum		5.95	5.84	0.032	0.178
Jejunum		6.27	6.15	0.028	0.184
Ileum		6.48	6.25	0.123	0.389
Caecum		6.84	6.69	0.038	0.054 ^r

Note: ¹n = 8 samples per group; SEM, standard error of the mean; BW, body weight.

^{ab}Means with different superscript within a row differ significantly ($P<0.05$).

Regarding the pH of intestinal digesta (Table 4), our study results showed that the probiotic addition did not affect the pH values ($P>0.05$); even a tendency to decrease the caecum pH was noticed ($P=0.054$) compared to the basal diet. It was stated that the intestinal digesta pH is influenced by the diet composition and also by the fermentation activity of intestinal microbiota (Guardia et al., 2011; Tsiouris et al., 2014). The lactic acid produced from lactose fermentation reduced the pH value of intestinal digesta (Molnar et al., 2018; Jha et al., 2020).

Our study results have shown that probiotic addition tends to decrease the pH value of caecal digesta (6.69 vs 6.84; $P=0.054$). According to Svihus et al. (2013), caecum represents the primary site for bacterial fermentation in chickens due to its specific habitat. Bacteria metabolize soluble nondigestible carbohydrates into short-chain fatty acids and lactate, which decreases the pH (Rinttilä and Apajalahti, 2013). Several studies have also shown that lowers pH may suppress the development of acid-sensitive bacteria from the

Enterobacteria family (Rinttilä and Apajalahti, 2013; Molnar et al., 2018; Khadem et al., 2018).

Table 5 show the effect on probiotic on the intestinal microflora of broilers at 35 d of age. It was observed that the *Enterobacteria* and *E. coli* counts decrease and *Lactobacilli* counts and *Lactobacilli: E. coli* ratio increase in the ileum and caecum due to the probiotic addition ($P < 0.05$). The samples of intestinal digesta were negative for *Salmonella*.

Table 5. Effect of probiotic addition on intestinal microflora ($\log_{10}$ cfu/g) of broilers¹

Item	Control	Probiotic	SEM	P-value
Ileum				
Enterobacteria	7.212 ^a	7.167 ^b	0.008	0.043
<i>E. coli</i>	5.608 ^a	5.512 ^b	0.020	0.004
<i>Lactobacillus</i> spp.	6.453 ^b	6.482 ^a	0.013	0.029
<i>Lactobacillus: E. coli</i> ratio	1.151 ^b	1.176 ^a	0.006	0.026
Caecum				
Enterobacteria	8.903 ^a	8.783 ^b	0.005	0.003
<i>E. coli</i>	7.762 ^a	7.660 ^b	0.030	0.006
<i>Lactobacillus</i> spp.	9.568 ^b	9.661 ^a	0.005	0.012
<i>Lactobacillus: E. coli</i> ratio	1.232 ^b	1.261 ^a	0.008	0.006

Note: ¹n = 8 samples per group; SEM, standard error of the mean.

^{ab}Means with different superscript within a row differ significantly ($P < 0.05$).

Similarly, Peng et al. (2016) reported that *L. plantarum* in broilers decreased the *Enterobacteriaceae*, and increased the *Lactobacillus* count, small intestinal villus height, and faecal volatile fatty acid concentration.

Our recent work indicated that the dietary addition of *L. acidophilus* in Ross 308 broilers significantly decreased the caecum pH, *E. coli* and *Staphylococcus* spp. counts and increased the *Lactobacillus* spp. and *Lactobacilli: E. coli* ratio (Gheorghe et al., 2020). A study by Olnood et al. (2015) evaluated four *Lactobacillus* strains on Cobb 500 broiler chicks' growth performance and gut microbial profile. These authors reported that the probiotic *Lactobacillus* spp. higher the total anaerobic bacteria count in the ileum and ceca, and the count of LAB and *Lactobacilli* in the ceca. Moreover, the probiotics strains tended to decrease the *Enterobacteria* count in the ileum. It is stated that *Lactobacilli* has the capability to auto- and co-aggregate; usually, the bacteria with a high auto-aggregation capacity have good adhesion to the mucus and the ability to exclude other microorganisms (Jha et al., 2020).

Other studies (Mountzouris et al., 2007; Vicente et al., 2008; Pourakbari et al., 2016; Ding et al., 2019) have also reported the potential of probiotics

to modulate the composition of gut microflora of broiler chickens and to suppress the growth of potentially pathogenic bacteria. Giannenas et al. (2012) found that *Lactobacillus*-based probiotics fed broilers improve the populations of *Lactobacillus* and *Bifidobacterium* in the small intestine.

On the contrary, Cengiz et al. (2015) reported that fed Ross 308 broiler chickens with a multi-strain's probiotics supplemented diet did not significantly affect the gut total aerobic and *Salmonella* populations.

CONCLUSION

The study results indicated that the probiotic mix (*L. acidophilus* and *L. plantarum*, 1:1 ratio) used did not significantly influence the productive performance and carcasses traits. The probiotic mixture positively affected broilers' gut microflora due to improving the beneficial bacteria in detrimental to potentially pathogenic bacteria. Further study is still needed to assess the effective concentration of microencapsulated form to enhance their viability during processing and possible interaction or synergy with other feed bio compounds.

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