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ORIGINAL ARTICLE

Probiotics on performance, intestinal morphology and carcass characteristics of broiler chickens raised with lower or higher environmental challenge

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ABSTRACT. This study aimed to evaluate the effect of probiotics on performance, intestinal morphology and carcass characteristics of broiler chickens housed on lower or higher environmental challenge. Three hundred male Cobb chicks were distributed into four groups in completely randomised design with treatments arranged in a 2 × 2 factorial scheme to evaluate effects of two diets (with or without probiotics) and two environmental conditions (lower or higher challenge), totaling four treatments with five replications with 15 birds per box. Probiotics were added on diets and were composed of *Lactobacillus acidophilus*, *Bacillus subtilis*, *Bifidobacterium bifidum* and *Enterococcus faecium.* The environment with lower challenge was made up of new wood shavings used as litter, low bird density (8 birds/m^2) and daily-cleaned bell drinkers. The environment with higher challenge was made up of re-used wood shavings used as litter (after three broods of broilers), bird density of 16 birds/m^2 with bell drinkers cleaned every two days. No significant interaction between diet and environmental challenge was found for any of the variables evaluated. Performance, slaughterhouse variables and chemical carcass composition were not affected by the use of probiotics. Chicks receiving diets without probiotics had lower intestinal crypt depth (267.1 vs. 316.6 µm, *P*=0.0068). Birds raised in the environment with higher challenge decreased feed intake (4,660 vs 5,020 g, *P*=0.0422), weight gain (2,610 vs 2,810 g, *P*= 0.0054), drumstick and thigh yield (21.98 vs 24.14 %, *P*=0.0354), and increased crypt depth (325.2 vs 258.5 µm *P*=0.0009). In conclusion, the probiotic does not promote satisfactory improvements, regardless of the environmental challenge.

Key words: additives, intestinal health, poultry, sanitary challenge.

INTRODUCTION

Growth promoters are antibiotics that have been used to improve the performance of broiler chickens since its discovery near 1950. However, since 2006 the European Union banned the use of antibiotics as growth promoters in animal feed, and the use of alternative additives (non-antibiotics) have been increased (Huyghebaert *et al* 2011).

According to Fuller (1989), probiotics are single or mixed cultures of live microorganisms which beneficially affect the host by improving the balance of their intestinal flora. The use of probiotics stimulates the proliferation of beneficial microorganisms, rather than the proliferation of potentially pathogenic bacteria (Puupponen-Pimiä *et al* 2002). The evaluation of the effect of probiotics on performance, development of the intestinal mucosa and carcass characteristics resulted on divergent outcomes. Comparing probiotics to control group, positive results were obtained on the performance (Ramos *et al* 2014, Marubashi *et al* 2012, Sen *et al* 2012, Patel *et al* 2015), on the development of intestinal mucosa of broilers (Sen *et al* 2012, Fallah *et al* 2013) and on the carcass (Boostani *et al* 2013). However, other results show no significant effect on performance (Souza *et al* 2011, Shargh *et al* 2012, Domingues *et al* 2014) and carcass yield (Souza *et al* 2011, Domingues *et al* 2014).

Studies have reported that when the animals are raised on a low pathogen environment, growth promoters show no effect on performance (Gunal *et al* 2006, Shargh *et al* 2012). In this sense, most experimental conditions do not expose the birds to pathogenic bacteria, making the environment a determining potential of probiotics efficiency, as well as the age of the birds, the route and the time of administration (Timmerman *et al* 2006).

On the other hand, common practice in the commercial poultry breeding such as high density and re-use of litter offer challenges and expose the animals to factors that result in stress and increased proliferation of pathogenic microorganisms. Raising broilers in high density aims to increase productivity and profitability in poultry. Thus, housing a larger number of birds per area results in increased production rate per working area despite lower individual productivity per birds. High densities result in changes indicative of stress parameters such as gait, feather and foot and hock burn scores (Ravindran *et al* 2006) and expression of genes encoding proteins related to stress (Beloor *et al* 2010) and modified intestinal microbiota (Guardia *et al* 2011). Still, the main arguments for re-using poultry litter are cost reduction, reduced withdraw period between flocks and minimised environmental impact (Mendes *et al* 2004). Baracho *et al* (2013) suggested that the creation of broiler chickens in poultry with re-used litter could result in stressful conditions, such as heat with increased temperature. According to Cressman *et al* (2010),

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litter conditions interfere with the intestinal microbiota of broilers. Still, studies report damage to the immune, respiratory and digestive system by re-using litter (Lee *et al* 2011, Malone, 2006, Costa *et al* 2000).

In the present study, the aim was to evaluate the effects of probiotics on performance, duodenal morphometry and carcass characteristics of broiler chickens exposed to lower or higher challenge conditions through different raising environments.

MATERIAL AND METHODS

All procedures were conducted in accordance with the Ethics Committee of Animal Use of University of Western São Paulo (UNOESTE), project protocol number: 192.

EXPERIMENTAL BIRDS, DESIGN AND TREATMENTS

Three hundred one-day-old male Cobb chicks were distributed in a completely randomised design as a 2 × 2 factorial scheme, composed of two diets (with or without probiotics) and two environmental conditions (lower or higher environmental challenge), totaling four treatments with five replications with 15 birds per pen. Water and mash feed were provided *ad libitum*. A 23-h lighting program was employed.

The diets of the starter phase (1 to 21 d) and growth (22 to 42 d) were isocaloric and isonitrogenous, formulated according to Rostagno *et al* (2005). The probiotics used was composed of *Lactobacillus acidophilus* (1×10^9 cfu/g), *Bacillus subtilis* (2.8×10^9 cfu/g), *Bifidobacterium bifidum* (2×10^9 cfu/g) and *Enterococcus faecium* (2×10^9 cfu/g), which was added to the feed during the whole experimental period (table 1).

The environment with lower challenge was made up of new wood shavings used as litter, low stocking density (8 birds/m^2) and daily-cleaned bell drinkers. The environment with higher challenge was made up of re-used (after three broods of broilers) wood shavings as litter, high bird-density (16 birds/m^2) and bell drinkers cleaned every two days. The re-used litter suffered an anaerobic fermentation for 12 consecutive days right after the departure of the previous flock.

EVALUATED CHARACTERISTICS

Performance. The performance was evaluated at 42 d according to the following variables: feed intake, body weight gain, feed conversion ration (feed intake/body weight gain) and production viability [100 - mortality (%)].

Intestinal morphometric parameters. Intestinal morphometric variables were evaluated by light microscopy, where a bird of each replication was sacrificed at 42 d of age after fasting for 12 h. From each bird, a sample from the medial region of the duodenum was collected, opened and immediately fixed in Bouin solution for 24 hours. They were washed in 70% alcohol to remove the Bouin solution and were subsequently dehydrated in ascending series of alcohols, cleared in xylene and embedded in paraffin. Histological sections were made and stained according to the methodology of Giannenas *et al* (2010). Analyses were made by the program Image J® [1]. The variables evaluated were height of the villi, villus width and crypt depth, being held 30 readings per intestinal region.

Carcass and commercial cuts yield. At 42 d, two birds per replication with average weight were selected for determination of carcass yield. The birds were subjected to 12 h of fasting and killed by cervical dislocation. After bleeding by jugular vein, the birds were plucked and eviscerated. For carcass yield was considered the weight of clean and eviscerated carcass in relation to the live weight. For prime cuts, the calculation of yield was performed in relation to the weight of the eviscerated carcass.

Carcass chemical composition. To determine carcass chemical composition, two birds per group were slaughtered by cervical dislocation at 42 d after 12 h of fasting. After slaughter, carcasses were bled, plucked, eviscerated, frozen, crushed and dried in a forced circulation incubator at 55 ± 2°C for 72 h. Then, the carcasses were analysed for content of dry matter, crude protein, ether extract, and mineral matter (Silva and Queiroz 2002).

STATISTICAL ANALYSIS

Data were analised using two-way ANOVA at 5% of probability, using the Statistical Analysis System (SAS 2004) software. The statistical assumption of residual normality was evaluated using the Shapiro-Wilk while Levene's test was used for homogeneity of variances. The F test was used to compare the differences of main effects. The statistical model was:

$$y_{ijk} = \mu + a_i + b_j + (a \times b)_{ij} + e_{ijk}$$

Where: y_{ijk} = response variable of broilers fed or not with probiotics (i) and submitted or not to challenge (j) in replication k. μ = overall mean value for y. a_i = fixed effect of probiotics. b_j = fixed effect of challenge. $(a \times b)_{ij}$ = interaction among probiotics and challenge. e_{ijk} = error term.

RESULTS

There was no significant interaction between the factors for any of the variables studied. No significant effect was observed with dietary supplementation of probiotics on

[1] Rasband WS, Image J. 2004. *National Institutes of Health*. Bathesda, Maryland, USA. Available at http://rsb.info.nih.gov/ij/

Table 1. Composition and calculated values of the experimental diets.

Ingredients, %	Starter (1 to 21 d)		Finisher (22 to 42 d)	
	Control	Probiotics	Control	Probiotics
Maize	57.70	57.70	55.12	55.12
Soybean meal	36.09	36.09	33.49	33.49
Soybean oil	1.35	1.35	6.31	6.31
Dicalcium phosphate	1.75	1.75	1.77	1.77
Limestone	1.03	1.03	1.00	1.00
Sodium chloride	0.49	0.49	0.52	0.52
$_{DL}$-Methionine	0.20	0.20	0.30	0.30
$_{L}$-Lysine	0.18	0.18	0.30	0.30
Probiotics[1]	0.00	1.00	0.00	1.00
Inert	1.04	0.04	1.04	0.04
Premix[2]	0.12	0.12	0.10	0.10
	Composition			
Metabolisable energy (kcal/kg)	2,900	2,900	3,200	3,200
Crude protein (%)	20.68	20.68	19.94	19.94
Calcium (%)	0.93	0.93	0.90	0.90
Available P (%)	0.44	0.44	0.42	0.42
Sodium (%)	0.21	0.21	0.20	0.20
Lysine (%)	1.22	1.22	1.19	1.19
Methionine (%)	0.60	0.60	0.60	0.60
Methionine + cystine (%)	0.90	0.90	0.91	0.91

[1]Probiotics: *Lactobacillus acidophilus* (1×10^9 ufc/g), *Bacillus subtilis* ($2{,}8 \times 10^9$ ufc/g), *Bifidobacterium bifidum* (2×10^9 ufc/g) e *Enterococcus faecium* (2×10^9 ufc/g).
[2]The vitamin premix supplied the following per kilogram of complete feed: vitamin A, 4,500 IU (retinyl acetate); cholecalciferol, 1,000 IU; vitamin E, 25 IU (dl-a-tocopheryl acetate); vitamin B12, 0.02 mg; menadione, 1.5 mg; riboflavin, 3 mg; thiamine, 1.5 mg; pantothenic acid, 5 mg; niacin, 20 mg; choline, 150 mg; folic acid, 0.5 mg; biotin, 0.5 mg; pyridoxine, 2.5 mg; manganese ($MnSO_4 \cdot H_2O$), 60 mg; zinc (ZnO), 40 mg; iron ($FeSO_4 \cdot 7H_2O$), 80 mg; copper ($CuSO_4 \cdot 5H_2O$), 8 mg; selenium (Na_2SeO_3), 0.2 mg; iodine (Iodized NaCl), 0.8 mg; cobalt ($CoCl_2$), 0.4 mg.

feed intake, weight gain, feed conversion and production viability (table 2). Animal raised on environment with higher challenge had reduced feed intake and weight gain compared to those raised under lower environmental challenge.

Birds fed diets with probiotics presented higher crypt depth compared to birds fed diets without probiotics (table 3). In addition, birds submitted to higher environmental challenge conditions showed higher crypt depth. The other morphometric variables had no significant effects.

The probiotics utilisation did not influence the carcass yield and commercial cuts (breast, drumstick + thigh, and wings) (table 4). However, the yield of drumstick + thigh was reduced when chickens were raised in environment with higher challenge. No significant effect was observed with probiotics or environment conditions on the chemical composition of the carcass (table 5).

DISCUSSION

In this study, the addition of probiotics into chicken's feed had no effect on the performance and carcass yield, contrary to previous studies that have showed improvements. Ramos *et al* (2014) reported increased feed intake and weight gain with the probiotics utilization (*Lactobacillus acidophilus*, *Bifidobacterium bifidum* and *Streptococcus faecium*), and attributed these results to the environment with re-used litter. Marubashi *et al* (2012) reported improved feed conversion in broilers fed diets supplemented with probiotic (*Bacillus subtilis*) compared to the control group.

Also, according to Sen *et al* (2012), probiotic based of *Bacillus subtilis* on different levels of inclusion in the diet increased weight gain and improved feed intake and feed conversion. On the other hand, other researchers have not verified the effect of probiotic supplementation on performance (Shargh *et al* 2012, Nosrati *et al* 2017) and on animal carcass traits (Souza *et al* 2011, Domingues *et al* 2014), attributing this effect to experimental environmental conditions.

Improvements in performance and carcass characteristics with the use of probiotics may occur due to increase in consumption and digestibility of the diet (Shim *et al* 2010). The probiotics could increase digestive enzymes

Table 2. Performance[1] of broiler chickens fed diets containing probiotics without or with challenge.

Effects		Performance, 1 to 42 d			
		Feed intake (g)	Weight gain (g)	Feed:gain (g/g)	Viability (%)
Probiotics	–	4,790	2,740	1.74	99.00
	+	4,880	2,680	1.82	98.63
Challenge	–	5,020	2,810	1.79	99.13
	+	4,660	2,610	1.78	98.50
SD		0.34	0.15	0.12	1.27
Source of variation		Probability			
Probiotics		0.6022	0.3373	0.2571	0.5646
Challenge		0.0422	0.0054	0.9169	0.3430
Probiotics × Challenge		0.8467	0.6666	0.6600	0.1921

[1]Data represent means of 5 replicates (n = 15 per replicate) in a treatment group.

Table 3. Duodenal morphometry[*] of broilers at 42 d.

Effects		Morphometry (μm)			
		Villus height	Crypt depth	Villus width	Villus:crypt
Probiotics	–	1,255	267.1	166.4	4.70
	+	1,271	316.6	164.1	4.01
Challenge	–	1,268	258.5	179.9	4.91
	+	1,258	325.2	150.6	3.87
SD		140.93	61.85	58.05	1.23
Source of variation		Probability			
Probiotics		0.8467	0.0068	0.8768	0.1183
Challenge		0.8914	0.0009	0.0620	0.0971
Probiotics × Challenge		0.5675	0.5022	0.1316	0.7487

[*]Data represent means of 5 replicates (n = 1 per replicate) in a treatment group.

Table 4. Slaughterhouse variables[*] of broiler chickens at 42 d.

Effects		Yield (%)			
		Carcass	Breast	Drumstick + Thigh	Wing
Probiotics	–	71.86	24.40	23.14	8.24
	+	73.27	25.30	22.98	8.00
Challenge	–	74.67	24.51	24.14	8.07
	+	70.47	25.20	21.98	8.18
SD		4.58	3.29	2.19	0.78
Source of variation		Probability			
Probiotics		0.5348	0.5772	0.8574	0.5398
Challenge		0.0817	0.6725	0.0354	0.7892
Probiotics × Challenge		0.9404	0.0785	0.0706	0.1958

[*]Data represent means of 5 replicates (n = 2 per replicate) in a treatment group.

Table 5. Carcass composition* of broilers at 42 d.

Effects		Carcass, %			
		Dry matter	Crude protein	Ether extract	Ash
Probiotics	–	30.30	60.01	35.16	8.22
	+	30.22	60.30	35.63	8.23
Challenge	–	30.38	60.20	35.83	8.20
	+	30.14	60.11	34.97	8.24
SD		0.21	4.42	2.13	0.83
Source of variation		Probability			
Probiotics		0.3205	0.8997	0.6909	0.9873
Challenge		0.4452	0.9677	0.4661	0.9327
Probiotics × Challenge		0.3307	0.2257	0.8126	0.8868

*Data represent means of 5 replicates (n = 2 per replicate) in a treatment group.

activity (Jin *et al* 2000) and promote beneficial intestinal microbiota, improving intestinal health (Sen *et al* 2012).

The type of microorganism used in the probiotics can interfere with product efficiency. *Bacillus subtilis* is a spore resistant to heat and poor storage conditions, and it is considered safe to be used as probiotics (Fuller 1989). Another important point is the feed consumption, since most of the probiotics used in broilers demonstrates efficiency with daily intake of 10^7 to 10^9 cfu (Patterson and Burkholder 2003, Mountzouris *et al* 2010). The dose used in the present test corresponded to the recommended range.

The action of probiotics is based on the mechanism of competitive exclusion, which depends on the oral administration of viable bacteria (Schneitz and Mead 2000). Perhaps, under the conditions of the present study the viability of the microorganisms may have been reduced, resulting in a reduced effect of probiotics.

In addition, the reduction on the feed consumption may have reduced the dose of probiotics that effectively reached the gastrointestinal tract, reducing prebiotics effectiveness. Other studies using different doses of compound probiotics reported variation in the responses of performance and carcass parameters, suggesting that the optimal concentration of probiotics in broiler feed varies with the microorganisms used in the composition of the product (Pourakbari *et al* 2016).

Regarding the environmental conditions used in this study, birds raised in an environment with higher challenge had lower feed intake, less weight gain and lower yield of drumstick + thigh, showing that environmental conditions used were detrimental to such variables. However, the results only show main effects of diet and environment, without interactions, suggesting that the higher density impacted the performance of birds but it was not enough to significantly change the probiotic effect. Forbes and Park (1959) suggested that the sanitary challenge must be sufficient to produce growth-promoting effects of additive utilisation in the animal performance.

Various researchers showed the negative effects of high animal density on the performance or broiler carcass (Dozier *et al* 2005, Dozier *et al* 2006, Jankowski *et al* 2014). However, regarding the re-used litter, there is disagreement between results. Some researchers have showed a negative effect of re-used litter on bird immunity (Lee *et al* 2011), and ammonia emissions in the environment (Malone 2006).

In contrast, using proper sanitary management, re-used litter may present microbiological quality equal to or greater then new litter (Hess *et al* 2008, Muniz *et al* 2014), not resulting in increased environmental pollution (Chinivasagam *et al* 2010, Roll *et al* 2011). The reuse of litter in three previous flocks could not be enough to see detrimental effects. In addition, anaerobic fermentation of litter probably was effective in reducing the microbial load of the litter.

In this study, probiotics caused no effect on the villus height, on the villus width and on the relation villous:crypt. However, birds that have received diets with probiotics had lower crypt depth in the duodenum. Several studies show that the use of the *Bacillus subtilis* caused increased height of the intestinal villi (Sen *et al* 2012, Samanya and Yamauchi 2002) without changing the depth of the crypts. However, Mountzouris *et al* (2010) also did not found changes in height of the villi in the small intestine when using *Lactobacillus reuteri*, *Enterococcus faecium*, *Bifidobacterium animalis*, *Pediococcus acidilactici* and *Lactobacillus salivarius* combined, while no change occurred in the depth of the crypts.

Gunal *et al* (2006) found higher villus height in the jejunum and ileum of birds fed with combined probiotics (*Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Aspergillus orizea*, *Bifidobacterium bifidum*, *Enterococcus faecium*, and *Candida pintolepesii*) and attributed this effect to the production of short-chain fatty acids by probiotics. The probiotics used in this study consists of *Lactobacillus*

acidophilus, *Bacillus subtilis*, *Bifidobacterium bifidum*, and *Enterococcus faecium*. Microorganisms used as probiotics appear to cause different effects on the intestinal mucosa, suggesting that further investigations are needed on the action of different probiotics on the intestinal morphology of birds. Physiologically, cell renewal and proliferation are constant and occur as a result of mitotic divisions of stem cells located in the intestinal crypts (Uni *et al* 1998, Applegate *et al* 1999).

In the presence of some stimulus or invader, the intestine responds with imbalance in cell turnover (Maiorka *et al* 2002). The results of this experiment suggest a greater villi extrusion rate that may occur in birds raised in an environment with higher challenge. According to Furlan *et al* (2004), an increase in extrusion rate of the villi promotes cell proliferation in intestinal crypt epithelial, as an attempt to recover the loss of the apex of the villi and, as a result, there is an increase in the depth of intestinal crypts.

The present study demonstrated that yield of drumstick + thigh was reduced when chickens were raised in an environment with higher challenge, these results were expected, feed intake and weight gain were also affected by challenge, high density of birds impair litter quality, and negatively affect broiler performance (Garcia *et al* 2002, Oliveira *et al* 2004), consequently meat yield decreased.

The addition of probiotics in the diets did not affected chemical composition of the carcasses, similary Zhou *et al* (2010) observed no significant differences in the contents of moisture, ash, crude protein and ether extract.

However, Payard and Mahmoudi (2008) observed higher dry matter, crude protein and ether extract percentage in the carcasses using *S. cerevisiae* as probiotics, concluding that the effects on the characteristics of poultry carcasses are quite variable, demonstrating that specific studies for this point are still necessary.

In conclusion, the probiotics used do not influence the performance and the carcass characteristics, regardless of the environmental challenge, requiring further research using other types of challenges to investigate the effects of this additive in broiler production. The higher environment challenge decreased the feed intake, weight gain, drumstick + thigh yield and increased crypt depth of the broilers.

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