Journal MVZ Cordoba
2021; January-April. 26(1):e2037.
https://doi.org/10.21897/rmvz.2037

Original

Effect of *Lactobacillus pentosus* LB-31 probiotic additive on broilers

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Received: May 2020; Accepted: August 2020; Published: December 2020.

ABSTRACT

Objective. Determine the biological response of broilers by including the probiotic additive *Lactobacillus pentosus* LB-31 in the diet, which was obtained in an economical culture medium and under different production conditions. **Materials and methods**. A completely randomized design was used with eight animals per treatment. Two experimental groups were established: the first consumed the basal diet without antibiotics (control group) and the second the basal diet with the addition of *Lactobacillus pentosus* LB-31 in a concentration of 10^7 cfu/g of food. Hematological, blood biochemical, morphometric and immunological indicators were determined. **Results**. At 42 days of age of the animals, there was no difference among treatments for hemoglobin and hematocrit. Albumin/globulin relation and albumin, cholinesterase and glutathione levels increased ($p<0.05$) while concentrations of globulins, uric acid, pancreatic amylase, alkaline phosphatase, cholesterol and triglycerides decreased ($p<0.05$) with the inclusion of LB-31 in the diet. In the morphometric indicators, the probiotic only had an effect ($p<0.05$) on relative weight (g/kg of live weight) of small intestine that decreased and the abdominal fat that increased. **Conclusions**. *Lactobacillus pentosus* LB-31, cultivated in a new culture medium and under different production conditions, maintains its probiotic activity in morpho-physiological and blood biochemistry indicators of broilers.

Keywords: Probiotic activity; lactic acid bacteria; animal health (*Source: MeSH, DeCS*).

RESUMEN

Objetivo. Determinar la respuesta biológica de pollos de ceba al incluir en la dieta el aditivo probiótico *Lactobacillus pentosus* LB-31, que se obtuvo en un medio de cultivo económico y diferentes condiciones de producción. **Materiales y métodos**. Se utilizó diseño completamente aleatorizado con ocho animales por tratamiento. Se establecieron dos grupos experimentales: el primero consumió la dieta basal sin antibióticos (grupo control) y el segundo la dieta basal con la adición de *Lactobacillus pentosus* LB-31 en concentración de 10^7 ufc/g de alimento. Se determinaron indicadores hematológicos, de bioquímica sanguínea, morfométricos e inmunológicos. **Resultados**. A los 42 días de edad de los

How to cite (Vancouver).
Sosa-Cossio D, García-Hernández Y, Dustet-Mendoza JC, García-Curbelo Y, Martínez-Pérez M, Sosa-Ceijas A, García-Quiñones D. Effect of *Lactobacillus pentosus* LB-31 probiotic additive on broilers. Rev MVZ Cordoba. 2021; 26(1):e2037. https://doi.org/10.21897/rmvz.2037

ISSNL: 0122-0268

animales no se encontraron diferencias entre tratamientos para la hemoglobina y hematocrito. La relación albúmina/globulinas y los niveles de albúmina, colinesterasa y glutatión aumentaron ($p<0.05$); mientras que las concentraciones de globulinas, ácido úrico, amilasa pancreática, fosfatasa alcalina, colesterol y triglicéridos disminuyeron ($p<0.05$) con la inclusión de LB-31 en la dieta de los animales. En los indicadores morfométricos el probiótico solo tuvo efecto ($p<0.05$) en el peso relativo (g/kg de peso vivo) del intestino delgado que disminuyó y la grasa abdominal que aumentó. **Conclusiones**. *Lactobacillus pentosus* LB-31, cultivado en un nuevo medio de cultivo y diferentes condiciones de obtención, mantiene su actividad probiótica en indicadores morfo-fisiológicos y bioquímica sanguínea de pollos de ceba.

Palabras clave: Actividad probiótica; bacteria ácido láctica; salud animal (*Fuente: MeSH, DeCS*).

INTRODUCTION

Feeding and handling animals are the factors that influence the most on the manipulation of the gastrointestinal ecosystem of poultry (1). The use of probiotics is one of the alternatives of interest to influence this environment because they are dietary additives formed by living microorganisms that have a beneficial effect on the health of the host (2). Generally, the application of these additives in animal production has been related to stabilization and protection of the gastrointestinal ecosystem, improvements in metabolic and digestive processes, as well as modulation of immune system. These effects may increase productive yields and, therefore, the availability and quality of meat and eggs destined for the population (1).

Strains of *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Enterococcus* genera and yeasts (3) are the most used microorganisms as probiotics. Selection of microbial strain(s) is the first step in the design of a probiotic product. These must be Generally Recognized as Safe (GRAS) microorganisms, capable of surviving in the gastrointestinal tract (GIT) and tolerating low pH and high concentrations of bile salts (4). Other required characteristics are the ability of probiotic strains to adhere to the intestinal epithelium for subsequent colonization (5), be genetically stable microorganisms, and possess high growth rates. Furthermore, the selected strain must maintain its viability and probiotic activity during the manufacturing, transport and storage processes (6).

FAO/WHO (2) suggested that probiotics should have a minimum concentration of 10^6-10^7 cells/mL or g of product to assurance it efficacy. However, there is no other methodology or guide that clarifies which should be the doses to be used according to categories or animal species. Specifically, different doses are used in broilers, for example Bai et al (7) evaluated a concentration of 10^7 cfu/g of a *Lactobacillus fermentum* strain, while Kazemi et al (8) used a commercial multi-species probiotic with a minimum concentration of 10^9 cfu/g of product. Likewise, other aspects that may influence the probiotic response should be considered, such as frequency and mode of application of the additive, age and physiological state of the host.

Lactobacillus pentosus LB-31 is a strain of poultry origin, isolated from fermented chicken feces, which showed the highest probiotic potential (1) in *in vitro* tests. LB-31 is a GRAS microorganism that was also shown not to affect animal health through acute toxicity studies in laboratory rats. On the other hand, its beneficial action was confirmed in broilers (1) at a concentration of 10^8 cfu/g of feed. It was also evaluated in rainbow trout (9), growing pigs (10) and pelibuey lambs (11).

Previous research were carried out by cultivating LB-31 in MRS (De Man-Rogosa-Sharpe) broth and small fermentation volumes in laboratory erlenmeyers. The use of this culture medium is not feasible at an industrial level due to the complexity of its composition and its high market prices (12). Therefore, the need arises to use other sources of inexpensive nutrients such as sugar cane molasses for the production of probiotic biomass on productive scales. Furthermore, it is argued that production and manufacturing processes can influence on the properties of probiotic strains and have an impact on their results (13). For these reasons, it is necessary to check the biological response of animals when consuming the additive with LB-31 in each of the scales that are studied for its production at an industrial level. Hence, the objective of the current study was to determine the biological response of broilers by including the probiotic additive *Lactobacillus pentosus* LB-31 in the diet, which was obtained in an economical culture medium and under different production conditions.

MATERIALS AND METHODS

Study area. Experimental work was performed at the poultry unit of the Institute of Animal Science. This center is located at km 47 ½ of the Central Highway, at 22º 53 'north latitude, 82º 02' west longitude and 92 meters above sea level, in San José de las Lajas municipality, Mayabeque province, Cuba.

Animals and basal diet. Sixteen one-day-old male EB-34 hybrid broilers, with an initial mean weight of 40 ± 2 g were used, which were housed in metal cages until 42 days. Animals consumed, at will, water and a diet based on corn and soy. The diet was prepared in the feed factory of the Institute of Animal Science, according to the requirements established by the Poultry Institute of Cuba, and varied in composition for start, growth and finish (Table 1).

Table 1. Composition of the diet for broilers by growth stage.

Ingredients (% on a dry basis)	Start (0-21 d)	Growth (22-35 d)	Finish (36-42 d)
Corn meal	47.00	52.90	58.79
Soy bean meal	41.13	35.87	30.40
Vegetable oil	6.60	6.30	6.00
Calcium carbonate	1.50	1.50	1.40
Monocalcium phosphate	1.80	1.60	1.60
Vitamin-mineral premix*	1.00	1.00	1.00
Choline chloride	0.13	0.13	0.13
Common salt	0.35	0.33	0.33
DL- Methionine	0.31	0.25	0.24
Lysine	0.18	0.12	0.11
Calculated nutrient level			
Crude protein (%)	23.00	20.00	18.50
ME (MJ/kg)	13.00	13.39	13.39
Available phosphorus (%)	0.40	0.45	0.45
Calcium (%)	0.95	0.95	0.95
Methionine+Cystine (%)	0.90	0.85	0.80
Lysine (%)	1.34	1.13	1.01
Threonine (%)	0.99	0.85	0.79
Tryptophan (%)	0.35	0.26	0.23

*Mineral premix per kg of food: selenium (0.1 mg), iron (40 mg), copper (12 mg), zinc (120 mg), magnesium (100 mg), iodine (2.5 mg) and cobalt (0.75 mg) and Vitamin premix per kg of food: vitamin A (10,000 IU), vitamin D3 (2,000 IU), vitamin E (10 mg), vitamin K3 (2 mg), vitamin B1 (thiamine, 1 mg), vitamin B2 (riboflavin , 5 mg), vitamin B6 (pyridoxine, 2 mg), vitamin B12 (cyanocobalamin, 15.4 mg), nicotinic acid (125 mg), calcium pantothenate (10 mg), folic acid (0.25 mg) and biotin (0.02 mg) .

Microorganism and preparation of the probiotic additive. *Lactobacillus pentosus* strain LB-31, belonging to the Bank of Microorganisms for Animal Production (BAMIPA) of the Institute of Animal Science (Mayabeque, Cuba), was used. LB-31 was identified by sequencing of the 16S ribosomal RNA gene and its sequence is deposited at GenBank with accession number: FR717464 (1). For the present research, the probiotic was obtained from a fermentation batch and 20 mL of the activated strain was used in MRS broth (Oxoid, UK) at 37°C and 24 h incubation to obtain the inoculum under these same conditions. An 11 L bioreactor (BIONET, Spain) with an effective volume of 8 L of a culture medium designed with sugar cane molasses, urea and other salts was used in the fermentation process, aspects that are the subject of a possible Cuban patent application. The additive had a concentration of 10^8 cfu/mL and was stored at 4±2ºC in sterile 500 mL flasks throughout the experimental stage.

Treatments. Two experimental groups were established that consumed: 1) basal diet without antibiotics (control group) and 2) basal diet with the addition of *Lactobacillus pentosus* LB-31 in doses of 100 mL/kg of food, equivalent to 10^7 cfu/g of food, as recommended by FAO/WHO (2). The additive was manually mixed, daily, with the basal diet.

Experimental conditions and animal management system. Previously, a sanitization of the area was carried out according to protocols established by the Poultry Institute of Cuba, for the broiler management. Water was offered in nipple drinkers and basal diet was provided in linear feeders, which were adjusted to the size of the broilers during rearing. The lighting of the area during the experiment was 24 hours, 12 hours of natural light and 12 hours of artificial light. Animal vaccination system consisted on a dose of Marek, fowl pox, infectious bronchitis on the first day of hatching, Gumboro at 1, 7 and 21 d and Newcastle vaccine at 14 d.

Experimental procedure for taking and analyzing samples. At 42 days of age, eight animals per treatment were individually weighed and sacrificed by bleeding from the jugular vein. Samples of 1 mL of blood were taken in tubes with and without sodium heparin. Subsequently, the abdominal cavity was opened and accessory organs and digestive tract were removed. The potentialities of this probiotic additive were determined based on hematological, blood biochemistry, morphometric and immunological indicators.

As hematological indicators, hemoglobin was measured by the cyanometahemoglobin method described by Crosby et al. (14) and hematocrit was determined by microcentrifugation at 10000 min^{-1} for 5 min. Blood sediments were read using a Hawkley microhematocrit reader. After subjecting the blood to coagulation at room temperature (26±2ºC), it was centrifuged at 3500 min^{-1} for 15 min in order to obtain the serum to determine indicators of blood biochemistry (total proteins, albumin/globulin ratio, albumin, globulins, uric acid, alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), glucose, pancreatic amylase, alkaline phosphatase, cholinesterase, glutathione, cholesterol and triglycerides) on a Cobas Integra 400 PLUS (Roche Diagnostic Sistem) automated analyzer.

Morphometric and immunological indicators were expressed, for their statistical analysis, as relative to live weight (g/kg of live weight). In the first case, empty crop, proventriculus, gizzard, small intestine, caeca and empty colon-rectum, liver, pancreas and abdominal fat were weighed. While in the second, organs related to the immune system were weighed: bursa of Fabricius and thymus. For this, a Sartorius BL 1 500 technical balance was used.

Experimental design and statistical analysis. A completely randomized design was used, where animals were individually distributed in metabolism cages. Eight animals were used per treatment and each constituted an experimental unit. The experimental data was processed with Infostat statistical package (15).

RESULTS

In the current study, the inclusion of *Lactobacillus pentosus* LB-31 in the diet of broilers had no effect on hematological indicators. Hemoglobin values of 10.46 and 10.50 g/dL (SE±0.13; p=0.8302) and hematocrit of 31.38 and 31.50% (SE±0.04; p=0.8281) were obtained for the control and probiotic treatment, respectively.

Table 2 shows the results of blood biochemistry indicators. Albumin/globulin ratio and concentrations of albumin, cholinesterase and glutathione were found to increase (p<0.05), while levels of globulins, uric acid, pancreatic amylase, alkaline phosphatase, cholesterol and triglycerides decreased (p<0.05) when animals consumed *L. pentosus* LB-31 in the diet. The rest of indicators (total proteins, ALAT and ASAT enzymes and glucose) did not show differences among treatments (p>0.05).

Table 2. Effect of LB-31 additive on blood indicators of broilers at 42 days of age

Indicator	Treatments		±SE p-value
	Control	LB-31 probiotic	
Total proteins (g/L)	29.55	28.84	1.29 p=0.7029
Albumin/Globulin	0.60	0.83	0.03 p<0.0001
Albumin (g/L)	11.05	12.93	0.23 p=0.0001
Globulin (g/L)	20.37	15.93	0.88 p=0.0032
Uric acid (mmol/L)	535.17	335.33	25.76 p=0.0001
ALAT (u/L)	3.75	3.63	0.35 p=0.8018
ASAT (u/L)	225.63	220.50	6.74 p=0.5993
Glucose (mmol/L)	11.74	12.39	0.42 p=0.2943
Pancreatic amylase (u/L)	593.00	498.33	20.81 p=0.0062
Alkaline phosphatase (u/L)	3831.17	1762.00	193.33 p<0.0001
Cholinesterase (u/L)	1704.67	1972.33	46.67 p=0.0012
Glutathione (u/L)	16.50	21.67	0.36 p<0.0001
Cholesterol (mmol/L)	3.53	3.05	0.12 p=0.0152
Triglycerides (mmol/L)	1.49	1.05	0.07 p=0.0004

Table 3 demonstrates the results of the morphometric indicators of broilers at 42 days of age for the treatments. No differences were found for live weight, relative weight of crop, proventriculus, gizzard, liver, pancreas, caeca and colon (p>0.05). However, the small intestine decreased (p<0.05) and abdominal fat increased (p<0.05) with the inclusion of *L. pentosus* LB-31 in the diet of animals.

As for the organs related to the immune system, LB-31 had no effect on relative weight of the bursa of Fabricius and thymus. In the first case, values of 2.22 and 2.25 g/kg live weight (SE±0.13; p=0.8523) were obtained and, in the second, 3.30 and 2.81 g/kg live weight (SE±0.31; p=0.2774) for treatment control and probiotic, respectively.

Table 3. Effect of the LB-31 additive on the gastrointestinal tract and accessory organs of broilers at 42 days of age.

Relative weight (g/kg live weight)	Treatments		±SE p-value
	Control	LB-31 probiotic	
Live weight (kg)	2.51	2.60	0.08 p=0.4299
Crop	5.40	6.40	0.64 p=0.2895
Proventriculus	4.05	4.29	0.20 p=0.4261
Gizzard	15.31	16.51	0.61 p=0.1846
Small intestine	28.01	25.42	0.71 p=0.0226
Caeca	4.36	4.00	0.31 p=0.4365
Colon	2.05	2.45	0.27 p=0.3117
Liver	24.09	22.90	1.43 p=0.5668
Pancreas	2.51	2.37	0.11 p=0.3847
Abdominal fat	7.56	12.92	0.91 p=0.0010

DISCUSSION

Results of hematological indicators (hemoglobin and hematocrit) are within the range of values reported by Avilez et al (16) and Gutiérrez and Corredor (17) as normal for broilers (8.7-10.1 g/dL and 22-35%, respectively). Furthermore, they are similar to those obtained by García et al. (1) when they evaluated *L. pentosus* LB-31 strain in this same animal category, but cultivated in MRS medium at 37ºC, under static conditions and 18-24 h of incubation.

Within total proteins, as indicators of protein metabolism, albumins and globulins are quantified. Albumin is synthesized in the liver and represents the highest protein fraction in poultry. A low concentration of this protein is associated with liver and kidney diseases. The increase in the albumin/globulin proportion shows that the animals consuming the probiotic showed better use efficiency of protein fraction and, therefore, better nutritional status. In this sense, Gutiérrez and Corredor (17) stated that positive effects of probiotics on digestive processes of broilers can be evidenced through blood indicators. On the other hand, the decrease of globulin levels indicated that there were no infectious processes and that the production of antibodies did not increase during the time of consumption of the additive. This result shows that LB-31 improves modulation of the immune system and, therefore, animals that consumed it demonstrated a better health state.

Regarding uric acid, Hashemzadeh et al (18) obtained similar results to those of the present study when they used, in diet for broilers, probiotic strains of *Lactobacillus rhamnosus* and *Berevibacillus laterosporus*. The same authors pointed out that uric acid is the final product of protein metabolism (parts of DNA and RNA) and that a decrease in its concentration levels demonstrates a positive effect of the probiotic on kidney function. In turn, they argued that some probiotic microorganisms can use urea, uric acid, creatinine and other toxins as nutrients for their growth.

ALAT and ASAT enzymes are used for detecting liver damage, obesity, muscle diseases and metabolic syndrome (19,20). Not detecting differences among treatments for each case, indicates that these pathologies did not exist in animals, which is beneficial for the use of LB-31 as a probiotic in the health of broilers.

Regarding indicators of carbohydrate metabolism, results for pancreatic amylase indicate that there were no problems with inflammation or damage to pancreas. While glucose levels are consistent with those reported by Abdel-Hafeez et al (21) and were within the normal range for broilers (11.1-22.2 mmol / L), according to Díaz et al (22).

Alkaline phosphatase is an enzyme found in almost all body tissues, but its greatest presence is in the liver, bile ducts, and bones. Its high levels can indicate liver and bone lesions. Studies by Aluwong et al. (23) and Wu et al. (24) showed similar results to the present study since they also obtained a decrease of alkaline phosphatase with the inclusion of probiotic strains of yeast and *Lactobacillus plantarum* 16, respectively, in the diet of broilers. The authors stated that these results indicated improvements in liver function, although they do not describe the exact mechanism by which this effect occurs, aspect that should be explored in subsequent studies.

In the consulted scientific literature, there is no evidence of the effect of probiotics on cholinesterase in broilers. However, it is known to be associated with two compounds that catalyze the hydrolysis of the neurotransmitter acetylcholine in choline and acetic acid, a necessary reaction to avoid neuronal damage. In general, it could be argued that the additive

with *L. pentosus* LB-31 has a positive effect on this indicator, so it would be of interest in future research.

The increase of glutathione levels in animals that consumed LB-31, demonstrated that the probiotic additive could have antioxidant capacity by acting as a protector of cells against oxidative stress. In this sense, Kullisaar et al (25) demonstrated that *Lactobacillus fermentum* was able to synthesize glutathione, even with a complete system of synthesis, absorption and redox rotation capacity. Likewise, Capcarova et al (26) found that probiotic strains of *L. fermentum* and *Enterococcus faecium* could be beneficial in oxidation resistance by eliminating free radicals. Meanwhile, Cortez et al (27) and Wu et al (24) evidenced the antioxidant capacity of probiotic strains of *Lactobacillus* spp.

Regarding the indicators of lipid metabolism, several authors reported the hypocholesterolemic effect of probiotics. They noted that these additives can contribute to the regulation of serum cholesterol concentrations by deconjugating bile acids, preventing them from acting as precursors in cholesterol synthesis. Deconjugated bile acids are less soluble at low pH and are less absorbed in the intestine and are more likely to be excreted through feces. Another mechanism by which serum cholesterol can be reduced is that probiotic microorganisms produce short chain fatty acids such as propionic acid, inhibiting hydroxymethyl-glutaryl-coenzyme A reductase, an enzyme involved in the cholesterol synthesis pathway (17,28). On the other hand, Ashayerizadeh et al (28) obtained results similar to those of the current study for the concentration of triglycerides when they supplied, in the diet of broilers, the commercial probiotic Primalac composed of a mixture of strains of *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bifidobacterium thermophilum* and *Enterococcus faecium*. These authors indicated that the reduction of triglyceride level may be related to the increase of lactic acid bacteria in the GIT, since these microorganisms could decrease the activity of acetyl coenzyme A carboxylase that limits synthesis speed of fatty acids.

Probiotic microorganisms, when established in the gastrointestinal tract as an ecological niche, perform functions that contribute to creating a beneficial state for the entire organism. In this sense, the results obtained for the small intestine coincide with the studies by García et al (1), who suggested that the decrease of relative weight of this organ could be related to the antimicrobial activity of lactobacilli. They also pointed out that this is because *Lactobacillus* strains have the ability to colonize the GIT, which reduces the adhesion sites of potentially pathogenic bacteria that produce toxic substances and thicken the intestinal mucosa. In addition, probiotic strains can produce antimicrobial substances such as organic acids or bacteriocins, decreasing these populations of enteropathogenic bacteria and, thus, influence on the reduction of organ weight and improve intestinal health.

There are different results from those obtained in the present research regarding the effect caused by probiotics on the abdominal fat of broilers. Some authors such as Kalavathy et al (29) and Wang et al (30) affirmed that probiotics influence on abdominal fat reduction. These differences could be associated with the genus or species of the used strain. The increase of abdominal fat deposition may be due to increased metabolic efficiency in the use of energy. However, this is an aspect that should be verified in subsequent studies.

Bursa of Fabricius and thymus are organs responsible for immunity in poultry. Some research indicates that the addition of probiotics stimulates the development of these lymphoid organs (31), allowing animals to have a better immune response to pathogen attack. However, García et al (1) obtained similar results to the current study, because they did not find effects on the relative weight of the bursa with the inclusion of *L. pentosus* LB-31 (cultivated in MRS) in the diet of broilers. These differences in results are mainly because the action of probiotic microorganisms is specific to species and strain to be used. They also depend, fundamentally, on administration dose, methods preparation, animal age, diet composition and hygiene state (32,33).

In general, Aragón et al (34) stated that the processes for obtaining microbial biomass must ensure that strains maintain their probiotic characteristics. The aspects that influence the most are culture medium composition and growth conditions such as stirrer speed, pH, temperature and concentration of dissolved oxygen. In this sense, the results of studied indicators ratify the probiotic characteristics of *Lactobacillus pentosus* LB-31 obtained in previous studies and it is demonstrated that the new culture conditions of the strain did not affect its potential.

In conclusion, results of the present study allow to suggest that *Lactobacillus pentosus* LB-31, grown in a new culture medium and under different production conditions, maintains its probiotic activity in morpho-physiological and blood biochemistry indicators of boilers. These results are encouraging for their production and subsequent introduction into livestock systems.

Conflict of interests

The authors declare that there is no conflict of interest with this publication.

Acknowledgments

The authors acknowledge the work of the technical staff and workers of the Central Laboratory Unit and of the Department of Monogastric Animals of the Institute of Animal Science, Mayabeque, Cuba.

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