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An In Vivo Pilot Study on Probiotic Potential of Lactic Acid Bacteria Isolated from the Gastrointestinal Tract of Creole Hens (*Gallus gallus domesticus*) Native to Montería, Córdoba, Colombia in Broiler Chickens

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Abstract: The objective of the present study was to characterize the probiotic potential of nine strains of *Lactobacillus* spp. isolated from the gastrointestinal tract of Creole hens through in vitro and in vivo tests. The following parameters were evaluated in vitro: (1) growth at four pH levels, (2) resistance to four bile salt concentrations, (3) tolerance to four NaCl concentrations, (4) growth capacity in the culture medium, and (5) the antimicrobial activity against *Escherichia coli*, *Salmonella*, *Klebsiella*, and *Staphylococcus aureus*. The candidate LP-40 had significantly ($p < 0.05$) increased resistance to pH 3 and 0.3% bile salts and elevated antimicrobial activity in vitro compared to the other strains evaluated. This strain was identified as *Lactobacillus salivarius* by 16S rRNA sequencing. An in vivo experiment was conducted to assess the effect of LP-40 supplementation in the drinking water on 42-day performance parameters in broiler chickens as compared to a non-treated control or dietary treatment with enrofloxacin. The administration of LP-40 in the drinking water significantly ($p < 0.05$) improved body weight, feed conversion compared to the antibiotic-treated control. Thus, the in vitro and in vivo results suggest that *Lactobacillus salivarius* LP-40 demonstrates probiotic potential and, perhaps, could be utilized as an alternative to antibiotic treatment.

Keywords: broiler chickens; Creole hen; lactic acid bacteria; *Lactobacillus salivarius*; probiotics

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1. Introduction

The Colombian poultry industry has increased its production in recent years, with an annual production of 783,546 tons in the first half of 2020 [1]. Similarly, the consumption of chicken products in Colombia has increased significantly to 33.7 kg per capita, almost double that of beef, which is second in terms of preference, with 17.1 kg consumed per person [2].

Commercially reared chickens are exposed to numerous stressors that disrupt intestinal barrier function, alter the microbiota composition, increase susceptibility to disease, and reduce performance parameters [3]. To mitigate this, diets have been traditionally supplemented with antibiotics as growth-promoting additives (APGs), especially in the commercial poultry sector; the European Union banned the use of AGPs in 2006 due to concern regarding the development of resistance to these antimicrobials [4].

The prohibited use of APGs in many countries has increased research efforts to identify antibiotic alternatives to optimize flock health and performance and also satisfy the demands of consumers for antibiotic-free poultry products [5]. Due to concern over APGs use, alternatives to antibiotics, such as probiotics, have shown promise [6]. There is convincing evidence that probiotics could replace in-feed antibiotics in poultry production because they have beneficial effects on growth performance, meat quality, bone health, and eggshell quality in poultry [7]. Probiotic(s) are single or mixed cultures of live microorganisms that, when administered to animals or humans, benefit the host by competitively excluding pathogens or by improving gut-barrier function and intestinal homeostasis [8]. Commensal-type microorganisms have been isolated from the digestive tract of healthy chickens and have been shown to benefit the host [9]. Additionally, isolation of live microorganisms from the digestive tract of poultry that have not received any subtherapeutic or therapeutic antibiotics reduces the risk of obtaining a microorganism that contains resistance plasmids. The present study aimed to evaluate the probiotic potential of *Lactobacillus* spp. strains recovered from the gastrointestinal tract of Creole hens (*Gallus gallus domesticus*) native to Montería, Córdoba.

2. Materials and Methods

2.1. Study and Sampling Location

Lactobacillus spp. were recovered from mucosal scrapings collected from the gizzard, jejunum, and cecum of three Creole hens from the rural area of Monteria. Isolation was carried out at the Biotechnology Laboratory, Faculty of Sciences, GRUBIODEQ group of the University of Córdoba, Montería, Córdoba, Colombia. The in vivo study was conducted according to the standards of the National Institute of Health of Colombia for the care and use of laboratory animals. The experimental design was approved by the Ethics Committee of the University of Córdoba (Resolution No. 1 of January 2021).

2.2. Isolation and Characterization of Lactic Acid Bacteria

For isolation, each mucosal scraping collected from the gizzard, jejnum, or cecum was suspended in 9 mL of saline for 5 min. All samples were enriched with Man, Rogosa and Sharpe (MRS) broth (Hi-media, Mumbai, India), a selective medium for lactic acid bacteria (LAB) isolation, and incubated for 48 h at 37 °C under anaerobic conditions. After 48 h of incubation, the cultures were spread plated onto MRS agar and the plates were incubated at 37 °C for 48 h under microaerobic conditions (5–7% v/v oxygen) in Anaerocult® tanks (Merck, Germany). For identification and characterization, in vitro catalase and oxidase production was assessed and Gram staining was performed for each isolate. The macroscopic and microscopic observations were carried out according to the Bergey's Manual of Systematic Bacteriology for the genus Lactobacillus [10].

2.3. Effect of pH, Bile Salt, or NaCl Concentration on In Vitro Growth of Lactobacillus spp. Isolated from the Gastrointestinal Tract of Creole Hens

Each *Lactobacillus* spp. candidate was individually propagated in vitro in MRS broth and incubated at 37 °C for 24 h. Post-incubation, 10 mL of MRS broth was inoculated with 10% (v/v) of turbid overnight culture for each candidate. All cultures were subjected to the following conditions and incubated anaerobically for 24 h: (1) pH values of 3, 4, 5.6 and 6.5; (2) bile salt concentrations of 0.05, 0.1, 0.15 and 0.3% (Sparks, MD, USA); (3) sodium chloride (NaCl) concentrations of 2, 4, 7 and 10%. Concentration (CFU/mL) was determined by drop plating serial dilutions on MRS agar to evaluate the effect of pH, bile salt, or NaCl concentration on growth. The plates were incubated at 37 °C under anaerobic conditions for 48 h. The viability of *Lactobacillus* spp. at pH 3 (RpH 3) and bile salt concentration of 0.3% (Rsb 0.3%) at 37 °C 3 h post-treatment was calculated as described by Kociubinski et al. [11], where RpH 3 = (Log CFU/mL^{-1} MRS pH: 3)/(Log CFU/mL^{-1} MRS pH: 5.6) × 100 and Rsb 0.3% = (Log CFU/mL^{-1} MRS sb: 0.3%)/(Log CFU/mL^{-1} MRS) × 100. MRS medium

(pH 6.5 and without bile salt inclusion) was used as a control for each isolate. Treatments were performed in triplicates.

2.4. Biochemical Characterization

Carbohydrate fermentation tests were performed for *Lactobacillus*. Strains were cultivated in API® 50 CHL galleries (BioMérieux, Marcy l' Étoile, France), which allowed determination of the fermentation pattern of 50 different carbohydrates. The results were analyzed using APIWEB® software (BioMérieux, France). Additionally, 0.1 mL of each of strain was used to seed 10 mL of MRS broth, which contained a 0.2% (*v*/*v*) bromocresol (0.5%) purple solution, and Durham bells were used for the determination of gas production [12]. Tubes (n = 3/strain) were incubated at 37 °C for 48 h.

2.5. Determination of Antimicrobial Activity against Pathogenic Microorganisms

In vitro antimicrobial activity of the lactobacilli strains was determined by the disk diffusion method [13]. Indicator pathogenic strains included the following: *Escherichia coli* (strain NBRC 102203), *Salmonella enterica serovar* Typhimurium (4,5,12:i:-), *Klebsiella pneumoniae* (ATCC BAA-1705D-5), and *Staphylococcus aureus* (ATCC 29737). Pathogenic strains were obtained from the Microbiology Laboratory of the Biology Program of the University of Córdoba. For the assay, the pathogenic strains were seeded on the surface of Mueller Hinton (MH) agar with a swab in a concentration similar to 0.5 turbidity of the McFarland scale. Disks were soaked with the respective *Lactobacillus* spp. candidate and placed onto the surface of the pathogen-coated MH agar. The plates were then held at 4 °C for 4 h followed by anaerobic incubation at 37 °C for 24 h. The presence of halos around the discs was indicative of antagonistic activity exhibited by the candidate lactobacilli strain [14].

2.6. Propagation of Top Performing Candidates (LP-10. LP-40, LP-50) In Vitro

Lactobacillus spp. strains with increased resistance to bile salts (0.3%) and pH of 3.0 were cultured in 100 mL of MRS broth at 37 °C for 24 h. Concentration (CFU/mL) of all cultures were determined by drop plating serial dilutions made with peptone water (1% *w*/*v*) onto MRS agar at 0 h and 24 h post-incubation [12].

2.7. Identification of Lactobacillus spp. LP-40 Probiotic Candidate

LP-40 was identified by 16S rRNA gene sequencing. Genomic DNA was extracted from colonies obtained from MRS agar using the commercial kit "Power Soil DNA Isolation Kit" (QIAGEN, Germantown, MD, USA) according to the manufacturer's instructions. The 16S rRNA ribosomal gene was amplified by PCR using the universal primers F27(5′-AGAGTTTGAT CMTGGCTCAG-3′) and R1492(5′-TACGGYTACCTTGTTACGACTT-3′). PCR products were purified by the method described by Sanger et al. [15] and sequenced (Macrogen Inc., 2017, Seoul, Korea). Sequences were deposited into GenBank database and compared using the BLAST program (Basic Local Alignment Search Tool) available on the National Center for Biotechnology Information (NCBI) website. An identity greater than 99% in the 16S rRNA gene sequence was used as an identification criterion. The phylogenetic trees were constructed using the Tamura-Nei genetic distance model (TN93) and the "Neighbor-Joining" method with 100 repetitions [16].

2.8. Evaluation of the Effect of Lactobacillus salivarius LP-40 on 42-Day Performance in Broiler Chickens

2.8.1. Probiotic Preparation

Based on in vitro results, *Lactobacillus salivarius* LP-40 was used for the in vivo study. LP-40 was cultured in CEACF-4 medium [17]. Concentration (CFU/mL) was determined after anaerobic incubation at 37 °C for 48 h by plating on MRS agar. Post-incubation, the biopreparation was stored in 400 mL glass bottles at 4 °C for one week or until administration.

2.8.2. Experimental Design

Day-of-hatch Cobb 500 male broiler chicks (n = 300 total) were randomly assigned to T0, T1, or T2. Each treatment group had 4 replicate pens (n = 25 chicks/pen). T0, or the non-treated control, received a basal diet without antibiotics or additives. T1, or the antibiotic treated group, received enrofloxacin in the diet (enrofloxacin: Bayer, Tokyo, Japan: 1 $kg.t^{-1}$). T2, or the probiotic treated group, received a basal diet without antibiotics or additives, but received the probiotic preparation (*Lactobacillus salivarius* LP-40) in the drinking water for the 42-day duration of the study at a final concentration of 10^9 CFU/mL. Feed rations were offered to all animals equally. Diets and water were provided *ad libitum.* Diets were formulated as recommended by the NRC [18] to meet the nutritional requirements (Table 1). Body weight (BW) was normalized at day-of-hatch across all groups. BW was recorded weekly to evaluate changes in live weight across treatment groups until termination. Cumulative feed intake and mortality were also recorded to determine feed conversion ratio (FCR).

Table 1. Composition of starter, grower, and finisher experimental diets.

Ingredients (%)	Starter (0–14 d)	Grower (15–28 d)	Finisher (29–42 d)
Corn flour	42.43	54.32	60.27
Soybean meal	43.88	33.68	28.58
Sunflower oil	8.80	7.28	6.52
Calcium phosphate	2.57	2.45	2.39
Calcium carbonate	0.74	0.72	0.25
Common salt	0.25	0.25	0.25
Methionine DL	0.33	0.30	0.29
Vitamin-mineral premix *	1.00	1.00	1.00
Nutritional contributions (%)			
Crude protein	23.00	20.00	18.80
Metabolizable energy (ME/kg)	13.38	13.38	13.38
Calcium	0.95	0.95	0.95
Available phosphorus	0.42	0.42	0.42
Methionine + cysteine	0.92	0.87	0.82

* Composition per 1 kg of feed: 10,000 IU of vitamin A, 2000 IU of vitamin D, 10 mg of vitamin E, 2 mg of vitamin K, 1 mg of thiamine, 5 mg of riboflavin, and 2 mg of pyridoxine, 15.4 mg of vitamin B, 125 mg of nicotinic acid, 10 mg of pantothenic acid, 0.25 mg of folic acid, and 0.02 mg of biotin. Mineral supplements: 0.1 mg of selenium, 40 mg of iron, 12 mg of copper, 120 mg of zinc, 100 mg of magnesium, 2.5 mg of iodine, and 0.75 mg of cobalt. T0 and T1: received basal starter, grower, and finisher diets. T2: received enrofloxacin (1 kg/ton) treated starter, grower, and finisher diets.

2.9. Statistical Analysis

All in vitro assays were performed in triplicate followed by one in vivo study. The data were analyzed according to a completely randomized design using analysis of variance (ANOVA). Duncan's multiple comparison test was used to separate means using the statistical software SPSS version 21 [19]. Mortality was analyzed using the chi-squared test of independence [19,20], testing all possible combinations to determine the significance ($p < 0.05$).

3. Results

Nine presumptive *Lactobacillus* spp. candidates were obtained from presumably different colonies. The macroscopic characteristics of the colonies on MRS agar and microscopic characteristics of the cells are presented in Table 2. All strains were confirmed to be Gram-positive, rod-shaped, and negative for catalase and oxidase production, which is consistent with lactic acid bacteria. Table 3 summarizes the growth of the nine *Lactobacillus* spp. probiotic candidates isolated from Creole hens when subjected to different pH, bile salt concentrations, and NaCl concentrations in vitro. In the present study, there was an

inverse relationship with growth and pH, where a lower pH reduced proliferation in vitro. However, all nine strains grew similarly at a pH of 3 and had the ability to withstand the acidic conditions. There were no significant ($p > 0.05$) differences in growth across all nine strains at each pH level evaluated. Bile salt concentrations of 0.05 or 0.10% did not affect growth of any strain evaluated. Growth of LP-10, LP-40, and LP-50 was significantly ($p = 0.031$) higher at bile-salt concentrations of 0.15% compared to the other strains evaluated. However, the growth of LP-40 was markedly higher ($p < 0.05$) than both L-P10 and LP-50 isolates at 0.30%. Although NaCl inclusion at 2, 4, 7, or 10% reduced growth of all strains with increasing concentrations, there was no significant effect on growth between evaluated strains when assessing by level of NaCl inclusion.

Table 2. Morphological evaluation and preliminary assessment of nine presumptive *Lactobacillus* spp. isolated from Creole hens.

Isolate	Macroscopic Observation	Microscopic Observation	Gram Stain	Oxidase	Catalase
LP-10	White, convex colonies with regular borders	bacilli	+	−	−
LP-20	Cream colonies, transparent with irregular borders	bacilli	+	−	−
LP-30	White colonies, flat with irregular borders	bacilli	+	−	−
LP-40	Yellow colonies, convex surfaces with regular edges	bacilli	+	−	−
LP-50	Cream colonies, convex with defined borders	bacilli	+	−	−
LP-60	Flat white colonies with convex surface and regular edges	bacilli	+	−	−
LP-70	White colonies, flat surface with regular edges	bacilli	+	−	−
LP-80	Cream colonies, flat surface with regular edges	bacilli	+	−	−
LP-90	Transparent yellow colonies with defined borders	bacilli	+	−	−

+ positive; − negative.

Table 3. Effect of pH, bile salt, or NaCl concentration (%) on in vitro growth of nine *Lactobacillus* spp. strains isolated from Creole hens.

Isolate	pH				Bile Salts (%)				NaCl (%)			
	3	4	5.6	6.5	0.05	0.10	0.15	0.30	2	4	7	10
LP-10	2.80	5.30	9.30	11.23	10.66	10.32	10.44 ^{a}	8.76 ^{b}	10.33	8.44	6.17	5.16
LP-20	2.33	5.30	9.48	10.22	10.42	10.44	8.24 ^{c}	6.58 ^{d}	10.53	8.23	6.18	5.21
LP-30	2.45	5.80	9.56	10.34	10.34	10.28	9.66 ^{b}	6.74 ^{d}	10.22	8.12	6.22	5.16
LP-40	2.20	5.20	9.66	11.46	10.30	10.22	10.88 ^{a}	9.48 ^{a}	10.20	8.58	6.45	5.45
LP-50	2.33	5.66	9.45	11.24	10.26	10.11	10.73 ^{a}	8.46 ^{b}	10.12	8.49	6.12	5.32
LP-60	2.42	5.88	9.68	10.86	10.62	10.53	8.54 ^{c}	7.78 ^{c}	10.22	8.33	6.35	5.12
LP-70	2.52	5.48	9.64	10.23	10.14	10.48	8.46 ^{c}	7.88 ^{c}	10.04	8.65	6.18	5.18
LP-80	2.40	5.33	9.34	10.33	10.22	10.36	8.33 ^{c}	7.56 ^{c}	10.11	8.27	6.21	5.22
LP-90	2.54	5.46	9.48	10.48	10.34	10.25	8.12 ^{c}	7.66 ^{c}	10.22	8.51	6.19	5.34
SEM	0.154	0.130	0.090	0.092	0.126	0.932	0.863	0.546	0.179	0.812	0.953	0.122
p-value	0.479	0.315	0.489	0.769	0.423	0.061	0.031	0.006	0.100	0.121	0.063	0.078

$^{a–d}$ Differing superscripts indicate that means within columns are significant at $p < 0.05$. Log_{10} CFU/mL reported. SEM: standard error of the mean. $n = 3$ replicates.

Table 4 shows the biochemical characterization of the LP-10, LP-40, and LP-50 using API CHL. This test revealed two *Lactobacillus* spp. had identical fermentation patterns,

presumably belonging to *Lactobacillus fermentum* (LP-10 and LP-40: 98.7%), and the strain LP-50 was identified as *Lactobacillus delbrueckii* subsp. bulgaricus (62.8%). However, speciation for LP-40 (top performing candidate) was confirmed using 16S rRNA sequencing. The phylogenetic tree confirmed the homology of *Lactobacillus salivarius* (100%) with the sequence LP-40. The nucleotide sequences were deposited in the GenBank database with accession number OK310568 [21].

Table 4. Carbohydrate fermentation profile from API 50 CHL (BioMerieux, S.A.).

Carbohydrates	LP-10	LP-40	LP-50
0. Control	−	−	−
1. Glycerol	−	−	−
2. Erythrol	−	−	−
3. D-arabinose	−	−	−
4. L-arabinose	+	+	+
5. Ribose	+	+	−
6. D-xylose	+	+	−
7. L-xylose	−	−	−
8. Adonitol	−	−	−
9. α-Methyl-D-xyloside	−	−	−
10. Galactose	+	+	−
11. Glucose	+	+	−
12. Fructose	+	+	−
13. Mannose	+	+	−
14. Sorbose	−	−	−
15. Rhamnose	−	−	−
16. Dulcitol	−	−	−
17. Inositol	−	−	−
18. Mannitol	−	−	−
19. Sorbitol	+	+	−
20. α-Metil-D-mannoside	−	−	−
21. α-Methyl-D-glucoside	−	−	−
22. N-Acetyl-glucosamine	+	+	−
23. Amygdaline	+	+	−
24. Arbutin	−	−	−
25. Esculin	−	−	+
26. Salicin	+	−	−
27. Cellobiose	−	+	+
28. Maltose	+	+	−
29. Lactose	−	+	+
30. Melibiose	+	+	−
31. Sucrose	+	+	+
32. Trehalose	+	+	−
33. Inulin	−	−	−
34. Melezitose	−	−	−
35. Raffinose	−	−	−
36. Starch	−	−	−
37. Glycogen	−	−	−
38. Xylitol	−	+	−
39. α-Gentiobiose	+	+	−
40. D-turanose	−	−	−
41. D-xylose	−	−	−
42. D-tagatose	−	−	−
43. D-fucose	−	−	−
44. L-fucose	−	−	−
45. D-arabitol	−	−	−
46. L-arabitol	−	−	−
47. Gluconate	+	+	−
48. 2-Ketogluconate	−	−	−
49. 5-Ketogluconate	−	−	−

+ positive reaction, − no reaction.

The level of resistance (%) to pH 3 and 0.3% bile-salt concentration 3 h post-incubation, in vitro growth in MRS broth at 24 h., and antimicrobial activity of *Lactobacillus* spp. (LP-10, LP-40, LP-50) isolated from Creole hens native to Monteria is presented in Table 5. LP-40 appeared to be markedly ($p < 0.05$) more resistant to pH 3 and a bile salt concentration of 0.3% after a 3 h incubation period. All three strains showed antagonistic activity against the evaluated pathogens, except for LP-50, which did not inhibit *Salmonella enterica* Typhimurium (4,5,12: I:-).

Table 5. Evaluation of the level of resistance (%) to pH 3 and 0.3% bile salt concentration 3 h post-incubation, in vitro growth in MRS broth at 24 h, and antimicrobial activity of select *Lactobacillus* spp. (LP-10, LP-40, LP-50) isolated from Creole hens.

	LP-10	LP-40	LP-50	SEM	*p*-Value
Indicator			**Level of resistance (%) [1]**		
%R pH (3)	54 [b]	60 [a]	52 [b]	1.342	0.012
bile salts (0.3%)	17 [b]	47 [a]	23 [b]	1.254	0.004
In vitro growth			**Log_{10} CFU/mL**		
%R pH (3)	11.3 [b]	12.4 [a]	11.2 [b]	1.420	0.001
Pathogen, strain			**Inhibition halo [1] (mm)**		
Escherichia coli, NBRC 102203	8.4 [b]	12.3 [a]	5.3 [b]	0.320	0.007
Salmonella Typhimurium, 4,5,12:i:-	6.7 [b]	13.7 [a]	0.0 [c]	0.373	0.001
Klebsiella pneumoniae, ATCC® BAA-1705D-5 ™	2.6	4.3	3.9	0.221	0.674
Staphylococcus aureus, ATCC® 29737	3.8	4.3	5.0	0.378	0.886

[1] %R calculated as described by Kociubinski et al. [11]. [a–c] Differing superscripts indicate that means within columns are significant at $p < 0.05$. SEM: standard error of the mean. $n = 3$ replicates.

In vitro proliferation of LP-10, LP-40, and LP-50. The growth of the LP-40 strain was higher than the other strains evaluated (Table 5). Specifically, LP-40 had significantly higher antimicrobial effects ($p < 0.05$) against *Escherichia coli* and *Salmonella enterica* serovar Typhimurium as compared to LP-10 and LP-50. However, there were no differences observed between strains for *Klebsiella pneumoniae* or *Staphylococcus aureus*. All three strains did have the ability to ferment glucose without gas production 24 and 48 h of incubation (Table 4).

Based on the in vitro results, the LP-40 strain was selected for the in vivo study since a commercially applicable probiotic candidate must tolerate extreme conditions of the gastrointestinal tract (pH, bile salt, NaCl), exhibit antimicrobial properties, and must propagate efficiently in vitro. Performance data and mortality for the in vivo study is presented in Table 6. BW (g) was significantly ($p < 0.05$) improved for the LP-40 group at all time points evaluated compared to the non-treated control and enrofloxacin-treated group. Feed conversion ratio (d0–42) for the LP-40 group was markedly ($p < 0.05$) lower than the other groups. There were no differences in mortality across all groups.

Table 6. Effect of *Lactobacillus salivarius* (LP-40) administered in the drinking water on 42-day performance compared to non-treated or enrofloxacin-treated group.

Parameters	T0	T1	T2	SEM	*p*-Value
Live weight (g)					
0	48.50	48.50	48.50	0.43	**0.12**
7	178.75 ^{b}	184.00 ^{a}	185.75 ^{a}	0.13	**0.04**
14	464.25 ^{b}	471.25 ^{b}	492.75 ^{a}	0.31	**0.02**
21	941.75 ^{b}	955.75 ^{b}	981.50 ^{a}	0.23	**0.04**
28	1531.25 ^{b}	1517.50 ^{b}	1620.75 ^{a}	0.27	**0.02**
35	2185.00 ^{b}	2242.50 ^{b}	2356.50 ^{a}	0.42	**0.03**
42	2857.50 ^{b}	2871.25 ^{b}	3002.50 ^{a}	0.54	**0.01**
Feed intake (g)					
0–42 d	4758.00	4774.00	4831.00	1.89	**0.426**
Feed conversion ratio					
0–42 d	1.66 ^{b}	1.66 ^{b}	1.61 ^{a}	0.42	**0.013**
Cumulative mortality					
0–42 d	9/100 (9%)	7/100 (7%)	4/100 (4%)	-	**ns**

^{a,b} Means with different letters in the same row differ at $p < 0.05$. Mortality expressed as number of mortalities/total number of chickens placed (%). ns indicates no significant differences. T0: non-treated control; T1: enrofloxacin; T2: ~10^{9} CFU/mL of *Lactobacillus salivarius* LP-40.

4. Discussion

There is evidence that increased osmolarity in culture medium inhibits growth of certain *Lactobacillus* spp. [22]. However, in the present study, all of the *Lactobacillus* spp. strains appeared to be osmotolerant, considering growth was achieved at even the highest concentration (10% NaCl) evaluated. Additionally, strains LP-10, LP-40 and LP-50 were deemed as the top candidates due to higher resistance to low pH and bile salts as compared to the other strains evaluated. To be efficacious in vivo, the beneficial bacteria must survive transit through the gastrointestinal by tolerating the unfavorable conditions without suffering extensive damage [23]. Bile salts act as detergents and destabilize the lipids present in the cytoplasmic membrane [24]. This situation results from the formation of pores that disturb the integrity and physiology of the cells, leading to cell death. Perhaps, some of the cells are moderately affected by the bile salts, briefly, until synthesis of the bile-salt hydrolase (BSH) enzyme is induced, which will subsequently act in the hydrolysis of conjugated bile salts (CBS). Synthesis of BSH by the *Lactobacillus* spp. leads to the reduction of CBS concentration in the medium, promoting replication in vitro [12]. The concentration of bile salts in the gastrointestinal tract is approximately 0.2% to 0.3% and can reach up to 2% (*w*/*v*) depending on the host and the type and quantity of feed ingested [25]. In the present study, the LP-40 exhibited resistance to a pH is 3.0 and bile-salt concentration is 0.3%, which suggests LP-40 would withstand passage through the harsh digestive tract environment. While in the intestinal tract of birds little is known about this parameter, the intestinal concentration of bile acids in the human tract is approximately 0.3% [26]. While in the present study, we did not evaluate the BSH activity, in a recent study, it was demonstrated that BSH is responsible for the bile-salt resistance in *Lactobacillus gasseri* JCM1131T strain, supporting the importance of the typical lactic acid bacterium as probiotics [27]. The growth of LP-40 at high concentrations of bile salts could be associated with the ability of *Lactobacillus salivarius* to produce the BSH enzyme intracellularly [28].

Based on all these results, LP-40 was selected as the best candidate since the strain exhibited resistance to gastric barriers, high NaCl conditions, and the ability to inhibit replication of pathogenic microorganisms. Specifically, LP-40 had an enhanced level of resistance to the highest bile-salt concentration evaluated (0.3%) and lowest pH (pH 3) compared to LP-10 and LP-50. As a result, molecular identification of LP-40 was conducted. Molecular identification was presumed to be more accurate than biochemical identification using API-50CHL. For example, García-Hernández et al. [29] isolated LAB from chicken feces, which inaccurately identified as *Pediococcus pentosaceus*, *Lactococcus lactis*, *Leuconostoc*

spp., *Lactobacillus* spp., *L. crispatus*, *L. delbrueckii* and *L. plantarum* using biochemical methods. However, using molecular techniques from the genetic sequence of the 16S rRNA of those isolation LAB, the isolates were identified as *L. pentosaceus* and *L. crispatus*. Due to the discrepancy regarding speciation in previous studies, we analyzed the 16S rRNA of strain LP-40 against the database RefSeq of the NCB (National Center for Biotechnology Information) and identified LP-40 as *L. salivarius*.

Studies have shown that *L. salivarius* is a ubiquitous bacterium in the digestive tract of chickens [30,31]. For instance, enteric colonization by *L. salivarius* IBB3154 72 h post-hatch was confirmed post-in ovo application during embryogenesis suggesting that this LAB can survive and persist in the neonatal gastrointestinal tract [32], which is critical for pioneer colonization by beneficial microbes. When *L. salivarius* colonized the intestine and produced organic acids, such as lactic acid, the decreased pH inhibited colonization and replication of pathogenic bacteria [33], reduced enteric inflammation, and improved performance parameters [34,35]. The antimicrobial activity results observed in the present study were similar to those obtained by Sobrino et al. [36], who evaluated antimicrobial activity of *L. salivarius* MP100 against *E. coli*, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus*, and *Klebsiella pneumoniae*. The antimicrobial effects of *L. salivarius* could be related to the colonization efficiency of the gastrointestinal tract, the production of organic acids that decrease intestinal pH [37], and that production of other antimicrobial substances, such as hydrogen peroxide [38]) and bacteriocins [39]. Other investigators, such as Seo et al. [40], showed that *L. salivarius* inhibited *Salmonella* spp., *Campylobacter jejuni* and *Staphylococcus pseudintermedius*. Pineda-Quiroga et al. [41] suggested that probiotic application alters the gut microbiota composition as well as the function of the digestive tract in avian species. *E. coli*, *Salmonella enterica* serovar Typhimurium, and *Clostridium perfringens* can be inhibited by high levels of lactic and acetic acid produced by LAB when fermenting dietary carbohydrates [42].

In addition to the resistance to the gastric environment and antimicrobial activity of *Lactobacillus salivarius*, dietary inclusion of this probiotic strain has shown improvement in performance parameters in broiler chickens [9].

L. salivarius alters the microbiota composition in the gastrointestinal tract, which improves digestion and uptake of nutrients as reflected by improved body weight and reduced feed conversion ratio [43]. Rondón (2009) [30] also reported improved performance parameters post-treatment with *L. salivarius* C65 strain. Furthermore, reduced mortality (2.1%) in a replacement pullet flock was observed in the *Lactobacillus* spp.-treated group compared to the non-treated control group. In another study, application of three strains of *Lactobacillus salivarius* isolated (CI1, CI2, and CI3) from chicken intestines improved performance parameters, while reducing total cholesterol, LDL cholesterol, and triglycerides [44]. The cecal microbiome population was shifted as a result of LAB treatment and an improvement in intestinal histomorphology was observed [41]. Similar findings were reported by Sureshkumar et al. [45].

5. Conclusions

In the present study, nine *Lactobacillus* spp. strains isolated from Creole hens were evaluated for resistance to harsh conditions of the gastrointestinal tract and antimicrobial activity, which was simulated in vitro. The top performing candidate, LP-40, was selected for the in vivo study. While the number of replicates per treatment of the present study is low (n = 4), the preliminary results of this pilot study suggest that the supplementation with LP-40 bio-prepared in drinking water may improve growth performance and feed conversion ratio in broiler chicken compared to the other groups evaluated, providing promising results as alternatives for antibiotic growth promoters in broiler chickens. Further studies with an increased number of replicates are needed to confirm the results of this study,; it should include a digestibility analysis of nutrients.

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Institutional Review Board Statement: The in vivo study was conducted according to the standards of the National Institute of Health of Colombia for the care and use of laboratory animals. The experimental design was approved by the Ethics Committee of the University of Córdoba (Resolution No. 1 of January 2021).

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets analyzed for this study can be found in the GenBank database under the accession number OK310568.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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