In vitro probiotic selection and characterization of *Lactobacillus* spp. isolated from healthy domesticated Turkeys

R. Altarugio,[*,1] I. H. B. Vellano,[*] A. C. I. Moraes,[*] E. L. Milbradt,[*] R. L. Andreatti Filho,[*] P. T. C. Guimarães-Okamoto,[*] C. R. Padovani,[†] and A. S. Okamoto[*]

[*]*Veterinary Clinic Department, FMVZ /UNESP Botucatu – SP/Brazil; and* [†]*Biostatistic Department, IBB/UNESP Botucatu – SP/Brazil*

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SUMMARY

The health of poultry is closely related to how the animals are raised, and the gut microbiota plays a key role in the fulfillment of their productive potential. *Lactobacillus* spp. are bacteria present in the natural microbiota of poultry that, when employed as probiotic agents, should present several characteristics. This study aims at performing the *in vitro* probiotic isolation and characterization of *Lactobacillus* spp. Isolates were obtained from ceca content of healthy turkeys through the isolation and identification of morphological, molecular, and physiologic characteristics. They were identified through Gram staining, catalase and hydrogen peroxide production tests, gas production tests during glucose fermentation, and hydrogen sulfide production during the triple sugar iron test. The samples were identified molecularly through polymerase chain reaction tests and were subjected to genetic sequencing. The assessment of the probiotic potential was conducted through artificial gastric juice tolerance and bile salt tolerance tests. A hydrophobicity test was used as an indirect method of assessing an isolate's probable ability to adhere to the intestinal mucosa. In addition, other analyses were performed, such as multiplication potential tests, *Salmonella* Heildelberg antagonism assays, hydrogen peroxide production tests, and antibiograms, as well as an assessment of the genes responsible for resistance to antimicrobial agents in *Integron C*. In conclusion, we identified, through morphologic, physiologic, pathogen antagonism, and antimicrobial resistance tests, 11 strains of *Lactobacillus* spp. belonging to species *L. reuteri* (9), *L. johnsonii* (1), and *L. frumenti* (1) that showed potential as probiotic candidates for in vivo application.

Key words: poultry, microbiota, probiotic, pathogen, resistance

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DESCRIPTION OF PROBLEM

The Brazilian turkey occupies a strong position in the international market. According to a report [1], Brazil was ranked the second-largest turkey exporter in 2012 and the third-largest turkey producer in 2013.

In 2013, exports of turkey and its derivatives from Brazil to the European Union showed 11 cases of contamination by *Salmonella* spp. Three of these cases were by *S.* Agona, one by *S.* Hadar,

[1]Corresponding author: sakai@fmvz.unesp.br

one by *S.* Typhimurium, one by *S.* Schwarzengrund, and one by *S.* Saint Paul [2].

Salmonella spp. are among the most important pathogens transmitted through food [3]. Over 2,500 serotypes of *Salmonella* spp. are currently known, but only 10% were isolated in poultry, as the serotype distribution for poultry varies according to geography and time [4]. Non-typhoidal serotypes, due to not being specific to birds, can colonize their intestinal tract without causing any clinical signs and, as such, represent a public health hazard by potentially contaminating carcasses and eggs [5]. In the epidemiology of human diseases, there is a preponderance of a few serotypes that are continuously disseminated and others presenting themselves as emergent serotypes, such as *Salmonella* Heidelberg [6]. This pathogen is considered more invasive than other non-typhoidal serotypes, causing diseases with more serious profiles in humans [7]. The antimicrobial resistance of *Salmonella* Heidelberg also has stirred the interest of the scientific community due to the possible prophylactic and therapeutic uses of probiotics [8].

Probiotics are living microorganisms that are beneficial to the host's health when administered in adequate quantities [9]. *Lactobacillus* spp. is one of the most important probiotic genera. The main metabolite it produces is lactate, which reduces infection rates, modulates the gut microbiota, and protects the mucosae against injuries caused by pathogenic microorganisms [10]. According to Lebeer et al. [11], in order to be characterized as a probiotic, the microorganisms must have at least one of the following features: maintain microbial homeostasis and inhibit pathogen proliferation through microbial interactions; promote the functioning of the epithelial barrier; or modulate the immune response.

Due to the lack of probiotic products aimed at turkeys, this study identified strains of *Lactobacillus* spp. according to morphological, molecular, and physiologic characteristics and selected candidates according to probiotic characterization tests.

MATERIALS AND METHODS

Identification of The Genus Lactobacillus

After cervical dislocation of 150 healthy, 80-day old female turkeys of the British United Turkeys of America (**BUTA**) lineage obtained from a commercial poultry farm, both ceca were collected from each turkey, aseptically stored under refrigeration in individual sterile plastic containers, identified by specimen, and immediately processed at the laboratory. The techniques applied in this experiment are in agreement with and were approved by the Ethics Committee on Animal Use (**CEUA**) of the College of Veterinary Medicine and Animal Sciences (**FMVZ**) under protocol number 21/2015. The entire content of the ceca was transferred to sterile test tubes containing 10 mL of DeMan–Rogosa–Sharpe (**MRS**) broth and incubated anaerobically at 37°C for 48 hours. After incubation, the MRS broth was seeded on MRS agar and reincubated at 37°C for 24 hours. Bacterial identification was performed based on morphological and physiological characteristics through catalase production tests, potassium hydroxide production tests, gas production tests during glucose fermentation, Gram staining, and hydrogen sulfide production tests in the triple sugar iron (**TSI**) according to a method described by Barros et al. [12].

After morphological and biochemical identification of *Lactobacillus* spp., we obtained each sample's DNA with an extraction kit [13] and subjected it to molecular identification through polymerase chain reaction (**PCR**) assays, employing the following primer pairs for the conserved region of the 16S gene: *Forw* R16–1 5'-CTT GTA CAC ACC GCC CGT CA- 3' and *Rev* LbLMA1- 5'-CTC AAA ACT AAA CAA AGT TTC -3', aiming at amplifying a product of approximately 250 pb [14].

Each 25 μl reaction consisted of 5 µl of each primer at concentrations of 20 *pmol*, 12.5 μl of Gotaq [15], 2.5 μl of ultrapure water, and 5 μl of DNA. The amplification program was performed at 95°C for 5 min, followed by 20 cycles at 95°C for 30 s (denaturation), 55°C for 30 s (annealing), and 72°C for 30 s (extension), as well as a final extension for 7 min at 72°C [16]. The PCR products underwent electrophoresis in agarose gel at 1.4% [17] and were viewed in an ultraviolet transilluminator [18].

The isolated strains that were identified as belonging to genus *Lactobacillus* underwent a probiotic characterization test.

Probiotic Characterization

In Vitro Gastric Acid and Bile Salt Resistance The in vitro gastric juice resistance test was conducted according to a procedure described by Neumann [19]. *Lactobacillus* spp. cultures, in the stationary growth phase (previous incubation in MRS broth at 37°C/18 h), were diluted 10x in saline solution (0.9% NaCl) at pH 7.0 (control) and in artificial gastric juice (2 g.L^{-1} NaCl, pepsin 3.2 g.L^{-1}) at pH 2.5 (test). The cultures were then incubated at 37°C for 3 h, centrifuged at 13,000 rpm for 1 min [20], suspended in MRS broth, placed in microplates, and incubated at 37°C with 200 μl/well of the control inoculum and of the cultures treated with artificial gastric juice. The optical density (**OD**) in the 7,620 nm spectrum [21] was assessed for 10 h at intervals of 30 minutes. The inhibition rate was calculated according to the formula (1-ASG/ACT) x 100, in which ASG is sample absorbance with the artificial gastric juice, and ACT is sample absorbance with the control isolate. The in vitro bile salt resistance test was conducted according to a procedure described by Walker and Gilliland [22], in which samples of *Lactobacillus* spp. previously incubated in MRS broth at 37°C for 24 h were inoculated (2% v/v) in MRS broth (control) and MRS broth with Oxgall (0.3% v/v) (test) and then incubated at 37°C for 3 hours. The spectrophotometric readings and the inhibition rate were calculated as explained during the artificial gastric juice resistance test.

Multiplication Potential The multiplication potential for the *Lactobacillus* spp. isolates was assessed through growth in MRS broth at 37°C and later counted in MRS agar in which 0.1 mL of the sample was diluted in 0.9 mL of phosphate buffered saline (**PBS**) [23], giving us the first dilution (10^{-1}), from which we obtained 8 serial dilutions (1:10). We retrieved 0.1 mL of each solution and transplanted it to MRS agar plates, in duplicate, for counting the colony-forming units (**CFU**) after incubating for 24 h at 37°C. The initial broth was incubated and the count was performed at 0 hours. It was then reincubated at 37°C and the CFU count was repeated 3, 6, 9, 14, and 18 h after the initial count.

Cell Surface Hydrophobicity Cell surface hydrophobicity was assessed according to the methodology described by Pelletier et al. [24] in which cultures of *Lactobacillus* spp. in stationary phase were washed twice with PBS adjusted for 0.4 OD_{400nm} [21] with 0.1 M of KNO_3, pH 6.2 (A_0). A volume of 0.2 mL of hexadecane was added to a 1.2 mL suspension of *Lactobacillus* spp. and, after 10 min of pre-incubation at room temperature, the sample went through homogenization in the vortex for 2 minutes. The aqueous phase was removed after 15 min and its OD_{400nm} [21] was measured (A_1). The microbial adhesion rate to solvents was calculated using the formula $(1\text{-}A_1/A_0) \times 100$. The isolates were classified as follows: high hydrophobicity (66.67 to 100%), medium hydrophobicity (33.37 to 66.66%), and low hydrophobicity (0 to 33.33%), according to a method described by Nader-Macías et al. [25].

Production of Hydrogen Peroxide The hydrogen peroxide synthesis was assessed through the colorimetric method described by Rabe and Hillier [26], in which the stationary phase isolates were inoculated in plates containing Tetramethylbenzidine-plus medium (**TMB**-plus medium) [27], kept for 18 h at 37°C in anaerobiosis, and then exposed to air for 30 minutes. Due to the oxidative nature of the substance produced, the isolates were classified as hydrogen peroxide producers if the inoculum acquired blue or brown tones.

Antimicrobial Resistance The antibiogram used was adapted according to a method described by Charteris et al. [28] and Agência Nacional de Vigilância Sanitária [29]. The samples were cultivated in 5 mL of MRS broth at 37°C for 48 hours. We then transferred 1 mL of the culture to a new MRS broth and incubated it at 37°C until a turbidity compatible with the 0.5 McFarland Standard (1×10^8 UFC/mL) was achieved. We prepared petri dishes with MRS agar, MRS broth with 0.65% agar agar, and 800 μL of each sample. The dishes were kept at room temperature to solidify. After the agar surface dried, we added disks of the following antibiotic agents [30] using sterile tweezers: gentamicin 10 μg, bacitracin 10 μg, colistin sulphate 10 μg, tetracycline 30 μg, ampicillin 25 μg, ciprofloxacin 05 μg, streptomycin 10 μg, sulfametropim 25 μg, erythromycin 15 μg, amoxicillin 10 μg, penicillin 10 μg, and cefalotin 30 μg. The cultures were then incubated at 37°C for 18 hours. The diameter of the halo in each disk was measured with

a caliper to determine if the bacteria in question were sensitive (S), moderately sensitive (MS), or resistant (R) to the antimicrobial agents according to the standard table created by Charteris et al. [28].

After the antimicrobial resistances were assessed, the selected samples underwent an analysis of the Integron C genes, which are related to bacterial resistance to antimicrobial agents. The primers used were Class 1 Integrons 5'-GGCATCCAAGCAGCAA-3' and 5'-GAAGCAGACTTGACCTGA-3' [31–32]. The method employed was the one described by Okamoto et al. [33] and Silva et al. [34], in which bacterial DNA was obtained with an extraction kit [13] and amplified through PCR. Each reaction consisted of 1.25 μL of each primer, 12.5 μL of Gotaq [15], 5 μL of ultrapure water, and 5 μL of the DNA sample, for a total volume of 25 μL. The amplification was conducted with an initial denaturation at 94°C for 10 min followed by 35 cycles at 94°C for 1 min (denaturation), hybridization at 54°C for 1 min, and final extension at 72°C for 10 minutes [16]. The amplified product was detected through electrophoresis in agarose gel (1.0%) [35], stained as described by GelRed™ [17], put through electrophoresis at 100 V for 50 min, and then analyzed under UV light [18].

Antimicrobial Activity against Salmonella Heidelberg Antimicrobial activity was assessed using 5 distinct techniques to verify antagonism between *Lactobacillus* spp. and *Salmonella* Heidelberg (**SH**), which was obtained from the Ornitopathology Laboratory at the Veterinary Medicine College FMVZ Unesp-Botucatu/Brazil, to confirm the antagonistic potential.

The first technique employed was the *Spot on the Lawn* method, which consists of seeding isolates of *Lactobacillus* spp. (**AC**) in MRS broth for 48 h at 37°C. The cultures were added as spots on petri dishes containing MRS agar (10 μL) and, after drying completely, were incubated at 37°C for 18 hours. The sample of SH was seeded in brain-heart infusion (**BHI**) broth and incubated at 37°C for 18 hours. It was then transferred to new BHI tubes with 0.75% agar agar [36]. The previously incubated AC dishes received the inoculum over their surfaces and, after complete solidification, were reincubated at 37°C for 12 hours. The inhibition halo was then measured starting from the edge of the *Lactobacillus* spp. colony.

The second technique employed was a modified *cross-streak* test to assess antimicrobial activity as described by Fang et al. [37], in which we made 1 × 2 cm rectangles with AC (10^6 UFC mL^{-1}) in MRS dishes. After being incubated at 37°C for 24 h, the colonies were removed and we applied chloroform with a swab. The dishes were then incubated for 1 h for drying before we spread 100 μL of SH over them. The dishes were reincubated at 37°C for 24 h, and the antagonism was assessed through the measurement of the inhibition zone around the rectangular growth [38].

The third technique was a *radial streak test* to observe microbial interactions [39]. The AC (10^8 UFC mL^{-1}) was inoculated in a circular area at the center of the MRS dish and, following incubation at 37°C for 48 h, was inoculated with the pathogen (10^8 UFC mL^{-1}) in radial streaks and reincubated at 37°C for 24 hours. The inhibitory activity was assessed using a modified version of the methodology described by Bosch et al. [40], measuring the diameter of the inhibition zone observed between the AC and the pathogen.

The fourth technique was the *agar well diffusion* method to test whether the inhibitory effect of the culture's supernatant is caused by acid pH or by other mechanisms [39]. AC was grown in MRS broth for 24 h, incubated at 37°C, centrifuged [20] at 12,000 x *g* for 20 min, and sterilized through filtration with a 0.20 μm porous membrane. After overnight incubation in stationary phase, the pathogen (10^7–10^8 UFC mL^{-1}) was inoculated into the MRS dishes. We then made 10 mm wells in the dishes and placed 100 μL of the sample's filtrated product in each well. The dishes were incubated at 37°C for 24 h, and the antimicrobial activity (growth-free zones) around the wells were measured in milliliters.

The fifth technique employed was the *liquid co-culture assay* to assess the isolates' antagonistic potential [39]. For the test, we inoculated 100 μL of each AC together with the pathogen (10^8 mL UFC mL^{-1}) in a medium consisting of 2 mL of MRS broth and tryptone soya broth (**TSB**) at a 1:1 proportion and incubated at 37°C for 24 hours. To verify the inhibition, 50 μL

of the suspension was seeded in TSB agar and incubated at 37°C for 48 hours. We compared the growth presented with a positive control and a negative control. Agar dishes that presented growth were categorized as having 25, 50, or 75% inhibitory activity, while dishes that did not present growth were categorized as presenting bactericidal activity (100% inhibition).

Identification of the Species Lactobacillus

Isolates presenting satisfactory results for a probiotic agent in all analyses underwent SANGER sequencing [21]. The results were analyzed with the software Bioedit [22] and Mega7.0 [23], and readings were compared with the database maintained by the National Center for Biotechnology Information.

RESULTS AND DISCUSSION

Identification of the Genus Lactobacillus

Of the 170 isolates, 74 presented positive results in phenotype tests with the following standards: positive in the Gram staining test in the form of a non-spore forming Gram-positive bacilli; positive in gas production in the glucose test; negative in the catalase production test, in the potassium hydroxide production test, and in hydrogen sulfide production in the TSI test. These isolates advanced to the DNA extraction stage. However, of these 74 isolates, only 30 were positive for gas production in the glucose test, a finding similar to the one described by Collins and Hartlein [41] in which not all *Lactobacillus* spp. isolates studied were positive for the test.

Isolates that were compatible with the other mentioned requisites, but negative for gas production in the glucose test also were considered part of the genus *Lactobacillus.* All pre-selected isolates underwent confirmation through PCR. Samples presented a profile compatible with the characterization of the control specimen of *Lactobacillus* spp. rather than the one aimed at probiotic analysis.

Probiotic Characterization

To promote beneficial effects, the probiotic agent must be able to persevere and remain viable under the adverse conditions imposed by the host's organism. The first challenge is gastric juice, which has digestive enzymes that remain functional in environments with very low pH. Several bacteria cannot survive in acidic pH [42]. In the case of turkeys, there is a relationship between the protein value in the bird's diet and gastric juice secretion, revealing the gastric phase is in control of secretions. However, when the digested food reaches the proventriculus, the organ stimulates gastric secretion (gastric or mechanic phase). Gastric juice pH varies between 0.5 and 3.0, as a pH level around 2 is ideal for transforming pepsinogen into pepsin. In the small intestine, the pH level of the bird's enteric juice averages 6.7, since the pancreatic and intestinal enzymes are particularly active in this area and function better with pH levels between 6.4 and 7.2 [43–44].

For this study, we conducted 20 readings over a period of 10 hours. However, only the data obtained after incubating the isolates in artificial gastric juice for 3 h were used to define the inhibition rate of an isolate. This is the case because, as described by Macari et al. [43], this time is enough for the food to be under the action of gastric juice in the case of poultry species. The isolates were exposed to 2 types of pH: a control with pH around 7.0 and a pH of 2.5, similar to the one found in the digestive system of turkeys.

Of the isolates analyzed, 3 presented inhibition rates of 23% or higher, revealing that gastric juice had a certain impact over them. One sample did not present any result in the test. The remaining 70 isolates did not present any changes due to the action of the gastric juice and were considered resistant to pH 2.5, which is one of the desirable characteristics for *Lactobacillus* spp. to be used as a probiotic agent. These results support those reached by Morelli [45] and Silva [46], who observed that intestinal bacteria tend to be more resistant to the stomach acids. In their experiment, the number of viable cells decreased 14.33% after 3 h of incubation in pH 2.5. In pH 3.0, the decrease was below 11.35%. In their study, Feng et al. [47] reported that, of the 101 samples tested, 13 presented inhibition rates exceeding 23% when exposed to pH 2.5 for 3 hours.

Another challenge probiotic agents face are the intestinal bile salts, which are molecules

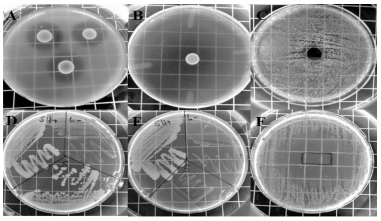

Figure 1. Antagonism testing of *Lactobacillus* spp. (L) against *Salmonella* Heidelberg (SH), (A) *Spot on the Lawn,* (B) *radial streak*, (C) *agar well diffusion*, (D) *liquid coculture assay* without inhibition of SH, (E) *liquid coculture assay* with inhibition of SH, and (F) modified *cross-streak.*

presenting antimicrobial activity and capable of breaching biological membranes [11]. In the bile salt resistance test, we verified that 58.11% of the isolates presented inhibition rates between 25 and 51%, contrasting with the results obtained by Macari [43], Sturkie [44], and Morelli [45], who reported inhibition rates between 25 and 33%. Twenty-three isolates presented inhibition rates below 25%, and 8 isolates were not affected by the bile salts, which is the desirable result for a probiotic isolate. In their study, Feng et al. [47] reported that only 13 isolates (12%) presented bile salt inhibition rates of 21.2% or lower, being considered samples with the best probiotic potential. Higher initial bacterial counts in the sample result in higher absorbance rates during incubation, pointing towards the need to standardize the inoculum, which is not noted in scientific studies. Therefore, the interpretation of results obtained regarding the isolate's capacity to survive in the presence of bile salts may vary from study to study [48].

Isolates compatible with the genus *Lactobacillus* spp. were initially selected for their resistance or immunity to gastric acid or bile salts, remaining viable in the digestive tract of the bird after evaluating these isolates for their antagonistic potential against the SH pathogen.

Probiotic agents may act through direct antagonism, producing bactericins, organic acids, and hydrogen peroxide with antibacterial action [49]. Of the antagonism tests conducted, the *Spot on the Lawn* test (Figure 1A) revealed antagonism for SH due to the formation of halos in 94.60% of the isolates. We observed that, from the 74 isolates tested for SH with the *radial streak* (Figure 1B), *agar well diffusion* (Figure 1C), *liquid co-culture assay* (Figure 1D), and modified *cross-streak* (Figure 1E) techniques, 31 isolates (41.89%) presented the best possible results only in the modified *cross-streak* test, with 7 isolates presenting inhibition zones $\leq 2.5 \times 3$ cm, classified as good, and 31 isolates presenting inhibition zones $\geq 2.5 \times 3$ cm, classified as excellent. These results support the ones obtained by Coman et al. [39], who reported that the cross-streak test is the most efficient for studying antimicrobial activity using live cells from probiotic strains in comparison with the radial streak and liquid co-culture assays, which also use live cells, and the agar well diffusion test, which uses a supernatant. The agar well diffusion test yielded the worst results, with only 10.81% of the samples showing any reaction, highlighting the fact that more than one antagonistic activity assessment test should be employed to classify a sample as having probiotic potential.

Resilience and multiplication are important properties for a probiotic agent, given that they aid the agent's establishment in the host, which is essential for the activity or composition of the host's microbiota to be altered [50–51]. The probiotic agent must compete with the microbiota for the needed nutrients and find favorable conditions for multiplication [52].

In Brazil, according to the Technological and Scientific Advisory Commission for Functional Food Products and New Food Products, linked to the Brazilian National Sanitary Surveillance Agency, for the final product of a probiotic agent to be approved for daily consumption, it must present a minimal level of viable microorganisms around 10^8 to 10^9 CFU/g. However, lower values are also accepted if the product's effectiveness is demonstrated [53]. In this study, the multiplication rate obtained after 3 h of incubation was 82.43%, showing that the samples multiplied at a fast rate, which means they would keep multiplying in the host's digestive system when administered to the poultry.

Adhesion to the digestive system's epithelial cells is also an important requirement for the colonization of probiotic strains, preventing their immediate elimination through peristalsis and offering a competitive advantage in that environment [49–50]. Auto-aggregation is needed for probiotic strains, promoting the adhesion to the intestinal epithelial cells and the formation of a barrier that prevents

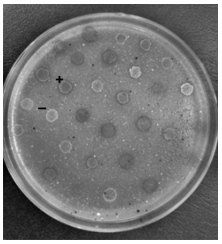

Figure 2. Test for the hydrogen peroxide production of *Lactobacillus* isolates.

colonization by pathogenic microorganisms [54–56]. The physical and chemical characteristics of the cell surface, such as hydrophobicity, may affect auto-aggregation and bacterial adhesion to different surfaces [56–57]. In this study, 32 isolates (41.89%) presented low hydrophobicity, which, according to Pelletier et al. [24], indicates that the samples have a hydrophilic surface and would be easily eliminated through peristalsis. Thirty-four isolates (45.94%) presented negative results. There are few reports of this type of result in the literature, but according to Schmidt et al. [58], increasing concentrations of ammonium sulfate may be used to convert these results and obtain the real hydrophobicity rate. In this study, 4 isolates (5.40%) presented intermediate hydrophobicity, and 4 isolates (5.40%) presented high hydrophobicity. These isolates would likely remain adhered to the gastrointestinal tract for a longer period. In their study, Pelletier et al. [24] reported that most isolated strains, regardless of the animal species, presented low hydrophobicity levels.

The production of hydrogen peroxide was initially employed to select catalase-positive bacteria during the identification of the *Lactobacillus* spp. samples, but it was assessed again at this stage in a modified medium (TMB-Plus), which allowed us to quantify the intensity of the hydrogen peroxide production (Figure 2). The isolates were classified as having no production when no color alterations were noted after incubation, low production when the samples acquired a light blue tone, intermediate production when the isolates acquired blue tones, and high production when the isolates acquired dark blue and/or brown tones, as described by Feng et al. [47]. In the study conducted by Charteris et al. [28], the results obtained for growth and color change were equal or better in TMB-Plus than in TMB agar for 98% of the isolates. In general, 70% of the isolates tested presented better growth and 47% acquired more intense colors. Correlating the antagonism tests and the hydrogen peroxide production tests conducted for this study, 14 samples (18.92%) did not produce hydrogen peroxide but presented antagonism for SH in one of the tests, indicating that the antagonistic activity may be related to the production of other substances. Sixty samples (81.08%) presented high hydrogen peroxide production and acquired brown tones. These isolates presented antagonism for SH in at least one of the antagonism tests, pointing towards the molecule's bactericidal activity.

The problem of bacterial resistance to antimicrobial agents has been widely discussed in the human and veterinary medicine fields, but one thing is certain: the resistance may be transferred among bacteria through their genes. Therefore, further research regarding the genetics of probiotic bacteria is needed to prevent a possible source of antimicrobial resistance genes. Resistance to antimicrobial drugs is defined as the ability of an organism to resist a chemotherapeutic agent to which it was previously susceptible. The resistance genes are transferred through genetic exchange with other microorganisms and, as such, the isolated bacteria should undergo tests to determine their sensitivity to antimicrobial agents, such as the agar diffusion test [59].

This study tested 12 antimicrobial agents. In the study conducted by Charteris et al. [28], most of the samples presented resistance to penicillins and cephalosporins, differing from our results, which saw ampicillin and cefalotin with the lowest resistance rates (12.16% for penicillin and 29.73% for cefalotin) and bacitracin with one of the highest rates at 90.54%. The same authors reported that all samples were resistant to aminoglycosides, supporting the results obtained by this study, in which 100% of the samples were resistant to this category of antibacterial agents (gentamicin and streptomycin). The strains tested in this study were also sensitive to tetracycline (91.89%) and ciprofloxacin (98.65%). Most of the strains were also sensitive to amoxicillin (44.59%), cefalotin (29.73%), and ampicillin (12.16%). The aforementioned authors also reported that their samples were resistant to sulfadiazine/trimethoprim and colistin

Table 1 Characterization of selected probiotic strains in their ability to inhibit *Salmonella* Heidelberg.

	Isolates										
Analyzed	*L. frumenti*	*L. reuteri*	*L. reuteri*	*L. reuteri*	*L. reuteri*	*L. reuteri*	*L. reuteri*	*L. reuteri*	*L. reuteri*	*L. johnsonii*	*L. reuteri*
Gastric acid[1]	**15.12**	**4.51**	**0**	**2.06**	**1.31**	**0**	**0**	**13.76**	**0**	**1.33**	**8.81**
Bile salts[1]	**22.01**	**11.60**	**16.69**	**13.57**	**0**	**23.80**	**22.03**	**18.58**	**35.19**	**45.37**	**0**
Spot on the Lawn[2]	**0.60**	**0.53**	**0.70**	**0.60**	**0.60**	**0.60**	**0.60**	**0.50**	**0.27**	**0.47**	**0.50**
Radial streak[2]	**2.30**	**1.80**	**2.80**	**2.53**	**0.53**	**2.85**	**0.45**	**2.53**	**2**	**0.50**	**3.07**
Agar well diffusion[2]	**0**	**2.5**	**2.2**	**0**	**0**	**0**	**0**	**1.8**	**0**	**0**	**1.7**
Liquid coculture assay[1]	**50**	**50**	**25**	**0**	**25**	**75**	**10**	**50**	**75**	**25**	**50**
Cross streak[1]	**75**	**100**	**75**	**75**	**75**	**75**	**25**	**100**	**25**	**100**	**100**
Multiplication potential[3]	**8.0×10^6**	**8.6×10^4**	**9.5×10^6**	**8.0×10^8**	**6.5×10^8**	**6.2×10^6**	**5.5×10^7**	**7.3×10^7**	**9.5×10^7**	**8.0×10^7**	**6.5×10^7**
Hydrophobicity[1]	**0**	**0**	**7.40**	**0**	**11.13**	**5.88**	**3.40**	**0**	**0**	**0**	**0**
Hydrogen peroxide production	**high**	**high**	**high**	**high**	**high**	**high**	**high**	**high**	**high**	**little**	**high**

[1]Percentage.

[2]Centimeters.

[3]Colony-forming units/mL.

sulfate, results supported by this study (81.08 and 100% respectively).

The variable regions from class 1 Integrons have been associated with antibiotic resistance [60–61]. According to data collected by the World Organization for Animal Health [62], antimicrobial agents reduce the gastrointestinal tract microbiota and increase colonization by *Salmonella* spp. The gene that expresses resistance to antimicrobial agents (class 1 Integron) was not observed in any of the isolates selected for PCR analysis. The study conducted by Okamoto et al. [33], which analyzed positive isolates with an antibiogram, also had no samples with the Integron resistance genes, supporting the notion that there is no correlation with the antimicrobial resistance gene (Class 1 Integron). In this study, samples presented over 50% resistance to the antimicrobial agents tested, but did not present any antimicrobial resistance genes in the technique that assesses the Integron C genes. This shows that Integrons are not directly related with resistance to multiple antimicrobial agents in *Lactobacillus* spp.

Identifying the Species of Selected Samples

The identification of lactic acid bacteria through phenotypical characteristics is often inconclusive and requires complimentary tests to precisely determine the species being studied.

The 11 isolates that showed satisfactory results for a probiotic agent by presenting resistance to gastric juice and bile salts, high potential for multiplication and/or high hydrophobicity, production of hydrogen peroxide, and the ability to inhibit SH (Table 1) were identified by sequencing one *Lactobacillus frumenti*, 9 *Lactobacillus reuteri*, and one *Lactobacillus johnsonii*. According to Barros et al. [12], in commercial probiotic products available for birds, pigs, cows, sheep, horses, dogs, and cats, the most common species of *Lactobacillus* spp. are: *L. bulgaricus, L. acidophilus, L. casei, L. lactis, L. salivarius, L. plantarum, L. reuteri*, and *L. johnsonii.* These microorganisms are non-pathogenic and are derived from the normal microbiota of the species the product is aimed at [63].

Turkey Production and Selection of Probiotic Bacteria

The growing use of probiotics in broiler production is a well-known fact and it is not different in the case of turkey production. Improving performance mainly through the complex and dynamic process that occurs at the intestinal level by selecting the microbioma promotes benefits in the digestion process, the absorption of nutrients, and the prevention of undesirable agents such as SH. Moreover, the international pressure for quality in exported products free of chemical residues demands more health controls. Therefore, a careful selection of bacteria with probiotic potential has a fundamental importance, as was shown with *Lactobacillus* spp. in this project.

CONCLUSIONS AND APPLICATIONS

1. In this experiment 11 isolates of *Lactobacillus* spp. were identified and selected with probiotic characteristics.
2. For antagonism tests directed against SH, the modified *cross-streak* test proved the most effective.
3. The *Lactobacillus* spp. isolates that were tested in this study demonstrated resistance to the majority of the antimicrobials tested but did not present Integron C resistance genes, thus reducing the possibility of transferring resistance genes to other bacteria. Therefore, these isolates are possible candidates for making a product with probiotic potential for further evaluation through in vivo efficiency studies.

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