Scientific Paper

Selection and identification of *Bacillus* spp. isolates from the digestive tract of backyard chicken, with probiotic potential

Fátima Arteaga-Chávez[1], Mario López-Vera[1], Marta Laurencio-Silva[2], Ana Rondón-Castillo[2], Grethel Milián-Florido[2], Vladimir Barrios-González[3] and Ramón Bocourt-Salabarría[4]

[1]*Escuela Superior Politécnica Agropecuaria de Manabí Manuel Felix* **López.**
Calceta, Diez de Agosto No.82 y Granda Centeno.Campus Politécnico
Sitio El Limón, Bolívar, Manabí.Ecuador
[2]*Universidad de Matanzas. Matanzas, Cuba*
[3]*Universidad de León, Facultad de Veterinaria, León, España*
[4]*Instituto de Ciencia Animal, San José de las Lajas, Mayabeque, Cuba*
E-mail: fatimitaespam@yahoo.es

Abstract

In order to obtain *Bacillus* spp. strains with probiotic activity isolates, from the cecum of backyard chicken (*Gallus gallus domesticus*) were selected and identified. For the isolation the method of seriated dilutions and thermal treatment (71 ºC) was used, for eliminating the cells in vegetative phase and obtaining the endospores. The selection included different tests, such as: resistance to acid pH (2) and to biliary salts (1,5 $g.L^{-1}$ of Ox-Bilis), antimicrobial activity against ATCC collection strains and others not belonging to official collections, and resistance to 14 antibiotic types. The data were analyzed according to simple classification design and were processed in the statistical program INFOSTAT. The characterization and identification were developed through biochemical and molecular tests (PCR). Seventy five isolates of *Bacillus* spp. were obtained from which only two (20 BP and 50 BP) survived the action of the acid pH and of biliary salts, showed antimicrobial activity against pathogen strains and were sensitive to 14 antibiotics. These two isolates, identified as *Bacillus subtilis* subsp. *subtilis*, showed potential to be used as probiotics.

Keywords: biliary salts, thermal treatment, viability

Introduction

In the Bolívar de Manabí canton, Ecuador, there is large tradition in the production of backyard (creole) poultry, as it is part of their culinary culture. This rearing is characterized by low investment costs and facility in its management, hence the importance of maintaining such production at small scale. This activity enhances the welfare of farmer families, providing them with products of high nutritional value, such as meat and eggs (Quintero *et al*., 2012). However, the mortality percentage in chicks is high, especially in seasons of high rain and humidity. In that period bacterial infections increase in domestic poultry, and the efficiency with which they digest and absorb feedstuffs decreases (Narváez and Oñate., 2002).

A way to improve the health of poultry is the use of probiotics, because they enhance the balance of the intestinal microbiota, stimulate the immune system, produce substances that inhibit the growth of pathogen microorganisms and increase the productive yields of the animals (Fuller, 1989; Lourenco *et al*., 2012; Menconi *et al*., 2013). The elaboration of these biotechnological biopreparations with autochthonous strains constitutes a challenge at present, mainly for developing countries such as Ecuador, because the products used contain foreign strains which sometimes are not correctly identified or in the indicated doses.

Among the species of higher interest are those of the *Bacillus* genus, which are widely used to obtain probiotic biopreparations. Nevertheless, it is necessary to perform an adequate selection and identification of the isolates, so that the microorganisms used remain viable and in sufficient quantities once they overcome the acid and biliary barriers, show antimicrobial activity and resistance to antibiotics, and are acknowledged as safe (Rosmini *et al*., 2004).

The objective of this study was to obtain *Bacillus* spp. strains with probiotic activity, from the selection and identification of isolates from the cecum of backyard chicken (*Gallus gallus domesticus*).

Materials and Methods

Isolation of *Bacillus* spp. and its endospores from the cecal content of backyard chicken

Biological material. In order to obtain the isolates five adult backyard chicken were slaughtered, by the method of cervical dislocation (Sánchez, 1990). For the isolation of endospores of *Bacillus* spp. 1 g of cecal content was taken and the methodology of seriated dilutions (Stanier, 1996) was followed. Dilutions were made in sterile saline solution (0,9 % of NaCl) to 10^{-12}; the last three dilutions were subject to 71 ºC in thermostated bath (UNITRONIC 320 OR) during five minutes to eliminate the cells in vegetative phase, according to the methodology described by Pérez (2000). Afterwards, 0,5 mL were inoculated in the medium surface, on plates that contained nutrient agar. The incubation was made at 37 ºC during 24 h (TermoScientic incubator).

Seventy five colonies were selected with different morphological characteristics and were grown in nutrient agar wedges. Afterwards, they were purified, according to the planting techniques by stripes or depletion (Stanier, 1996).

Preliminary tests. Gram staining and endospore staining were performed, as well as the catalase test (Harrigan and McCance, 1968). All the isolates which were Gram-positive bacilli with presence of endospores, positive catalase and growth in aerobiosis, were selected.

Survival of the endospores in acid pH. The selected isolates were subject to different levels of acid pH. They were cultivated during 72 h at 37 ºC in nutrient broth at pH 7. Then, they were inoculated in Erlenmeyer flasks that contained 50 mL of nutrient broth, adjusted at pH 2 (with HCl 0,1 $mol.L^{-1}$) and control cultures with pH 7. The cultures were developed in thermostated shaker (UNITRONIC 320 OR) during 3 h at 37 ºC. Afterwards, dilutions were prepared (1:10) and were sown in plates with nutrient agar, which were incubated during 24 h at 37 ºC. To count the resistant endospores samples were taken at hours 0 and 3 of incubation, according to the methodology proposed by Refugio (2002). The percentage of resistance to acid pH was calculated through the equation suggested by Kociubinski *et al*. (1999).

$$\% \text{ R acid pH} = [(CFU.mL^{-1})_{\text{nutrient broth pH2}} \times 100] / (CFU.mL^{-1})_{\text{nutrient broth pH 7}}$$

For each culture three repetitions were made and those isolates with % R acid pH ≥ 60 % were selected.

Survival of the endospores in biliary salts. The isolates that survived acid pH were then subject to the action of biliary salts. The methodology described by Refugio (2002) for the study of resistance to acid pH was followed, with the difference that 1,5 $g.L^{-1}$ of OX-Bilis was added to the cultivation medium. The tolerance to biliary salts was evaluated by counting the viable spores in nutrient agar (NA) at hours 0 and 3. The percentage of resistance to biliary salts (% R BS) was determined through the equation proposed by Kociubinski *et al*. (1999).

$$\% \text{ R BS} = [(CFU.mL^{-1})_{\text{nutrient agar + salts}} \times 100] / (CFU.mL^{-1})_{\text{nutrient agar without salts}}$$

For each isolate three repetitions were made.

Determination of the antimicrobial activity. The technique of diffusion in agar proposed by Chaveerach *et al*. (2004) was used. Five mL were taken from cultures 20BP and 50BP in nutrient broth at 0, 4, 8 and 18 h and were centrifuged at 15 000 rpm at 5 ºC during 10 min in refrigerated centrifuge (P-selecta-Mixtasel). Afterwards, the supernatant was sterilized through filters of cellulose acetate with 0,22-mm pores (Minisart, Sartorius 600 kPa max). The unmodified supernatant was used to inoculate the wells in plates of nutrient agar.

Indicator strains:

Group I. ATCC (American Type Culture Collection) strains: *Staphylococcus aureus* (ATCC-29213), *Enterococcus faecalis* (ATCC-29212), *Escherichia coli* (ATCC-25922).

Group II. Other reference strains: *E. coli, Proteus, Salmonella*, isolated from liver of diseased backyard chicken and identified in the laboratory of microbiology of the Agricultural Higher Polytechnic School of Manabí, Ecuador.

The indicator strains were cultivated during 18 h in nutrient broth at 37 ºC and incubated in thermostated shaker during 18 h at 37 ºC.

Development of the agar diffusion technique. From the cultures of the indicator strains 200 µL were taken, which were inoculated in tubes with 20 mL of nutrient agar (with 10 % of Ion-Agar, Oxoid)). In each plate which contained the indicator strains wells of 5 mm diameter were opened, in which 60 µL of the supernatant of the producing strains were deposited; the plates were maintained at 5 ºC during 4 h for a better diffusion of the substances in the agar. Afterwards, they were incubated during 24 h at 37 ºC until detecting the

growth and appearance of the inhibition halos. The diameter of the halos was measured with a graduated ruler. The diameter of the wells was subtracted from each value (Chaveerach *et al*., 2004).

Determination of the sensitivity to antibiotics. The sensitivity of the isolates 20BP and 50BP to the different antibiotics (NEO-SENSITABS™) was determined through the disc diffusion method (Bauer *et al*., 1966). The sensitivity was determined by the inhibition halos and the test was made in duplicate.

Characterization and identification of the selected *Bacillus* strains

Growth capacity at high temperatures, different pH and saline concentrations. The isolates were inoculated in 50-mL Erlenmeyer flasks, from a culture of 18 h at 27 ºC in nutrient broth, and were incubated at 45, 50 and 65 ºC (Beatle and Willians, 1996). The same procedure was followed to determine the growth under different pH levels, for which the medium was adjusted to pH 6, 7, and 8. The growth capacity in different concentrations of NaCl: 3; 5 and 7 % was also evaluated (Fields, 1978). In all cases the isolates were cultivated for 18 h and then the optical density (OD, $A_{600\ nm}$) was measured in spectrophotometer (Jenway 6305).

Determination of the carbohydrate fermentation profiles. Cultures from the isolates in nutrient broth were inoculated and incubated at 37 ºC during 18 h. Afterwards, they were centrifuged and washed with saline solution at 0,9 %. Since that moment, API 50 CHB Medium and API 50 CH Galleries (BioMérieux, S. A., France) were used. The isolates were identified through the utilization of the Apilab Plus Software, version 3.3.3.

Polymerase chain reaction (PCR). The PCR was made on the isolates selected as candidates for probiotics. For such purpose, the bacteria were cultivated by depletion in nutrient agar at 37 ºC during 24 h. From the obtainment of the pure colonies the following protocol was followed:

DNA extraction. Two well-defined colonies were taken per each fresh culture of 18-24 h, were re-suspended in 100 µL of Lysis buffer and 1 µL of proteinase K (PK) was added at a rate of 0,015 $mg.\mu L^{-1}$; they were incubated at 52 ºC during 60 min in Thermomixer (Eppendorf). Then, lysis and inactivation of PK was carried out by cooking at 98 ºC for 10 min. The Eppendorf were centrifuged during 10 min at 16 000 rpm. With the supernatant the PCR was prepared.

DNA amplification. The PCR was made in a thermocycler (Mastercycler gradient®, Eppendorf, Netheler, Hinz GmbH, Hamburg, Germany) in which the samples were deposited and were subject to the following program: initial denaturation process of 5 min at 95 ºC, followed by 30 cycles of 30 s at 95 ºC, 30 s at 55 ºC and 30 s at 72 ºC. The process ended with 5 min of incubation at 72 ºC (Greisen *et al*., 1994).

A strain of *Bacillus subtilis* ATCC-ER229 was used as positive control and there was a negative control that showed all the PCR components (table 1), but without template DNA.

In order to test the DNA amplification an electrophoresis was made in agarose gel (1,5 %) of the PCR products. Each well was charged with 8 µL of the sample and 2 µL of charge buffer based on bromophenol blue 0,25 % (p/v), glycerol 50 %, EDTA 1 mM and xylene cyanol 0,5 % (p/v). A molecular marker (Gene Ruler™ 100 bp DNA ladder plus, MBI Fermentas) was also used.

Table 1. PCR. components

Concentration	Formula Mix (**µ**L)	
Bi-distilled	H_2O	34,1
Pure	Tampón	5
2 mM	$MgCl_2$	2,5
10 mM/mL	DNTPs (0,2 mM)	2
10 mM/mL	Cebadores 16S (0,5 µ M)	2,5
	Cebadores 23S (0,5 µ M)	2,5
50 M	Taq ADN polimerasa (10 U/reacción)	0,4
100-400 ng	ADN (1 µL/reacción)	1
Total volume/reaction		50

DNTP: Deoxyribonucleotide triphosphate

The DNA of each isolate was amplified with the specific primer (Goto *et al*., 2000): 5'-TGT AAA ACG ACG GCC AGT GCC TAA TAC ATG CAA GTC GAG CG-3' and 5'- CAG GAA ACA GCT ATG ACC ACT GCT GCC TCC CGT AGG AGT- 3'.

Purification and sequencing. The amplified DNA was purified from a strip that was extracted from the agarose gel of 600 pairs of bases. Subsequently, a new PCR was made and the product of the reaction, after the purification and amplification, was subject to a new electrophoresis to test its quality. The sequencing of the samples was conducted at the University of León, Spain, with an automatic sequencer ABI PRISM 310 Genetic Analizer (Applied Biosystem).

An amplified DNA fragment was taken for PCR from the genomic DNA of the evaluated isolates. The sequences were compared with the international database of the collection of non-redundant nucleotides (nr/nt) of the GenBank+EMBL+DDBJ+ PDB sequences, through the software BLASTN 2.2.19 (Stephen *et al*., 1997) which is in the webpage of the NCBI (National Center of Biological Information).

Statistical analysis. A simple classification design was used and the statistical program INFOSTAT, version 1.0 (Balzarini *et al*., 2001) was used. In the cases it was necessary, Duncan's (1955) test was used for mean comparison. The data represented the mean values of three repetitions per experiment.

Results and Discussion

Seventy five isolates of cecum from backyard chicken were obtained. From them, 27 showed *Bacillus* characteristics such as: Gram-positive bacilli, positive catalase and growth in aerobiosis. The presence of endospores was also observed, which coincides with the description made by Mayea *et al*. (1997) and Juárez-Caratechea *et al*. (2010) for this genus.

The bacteria of the *Bacillus* genus reach naturally the gastrointestinal tract of birds through feedstuffs (Calvo and Doris, 2010). Besides, in nature they are found in the soil, water and other environments, including man's intestine (Barrios, 2013).

Table 2 shows the performance of the 27 evaluated strains in the presence of acid pH and biliary salts. All the isolates survived the acid pH; however, only nine exceeded 60 % of resistance, which were selected.

Studies conducted by Milián (2009) showed that these sporulated microorganisms are found in counts from 10^9 to 10^{10} CFU.g^{-1} of cecal content in fattening chicken. Also Yegani (2010) and Aguavil (2012) islated *Bacillus* spp. strains from the ileum and cecum content of Broiler Ross-308 chicken, which were used as probiotics with good results.

Table 3 shows the percentages of resistance of the isolates against the action of biliary salts. This condition negatively influenced cell viability, because only two isolates (20BP and50 BP) showed capacity of resistance to this gastric barrier.

Table 2. Isolates resistant to pH 2 during three hours.

Isolate	% resistance to pH 2	SD %	VC %
2 BP	32,45	0,56	0,46
3 BP	33,71	0,96	1,94
4 BP	30,22	0,78	1,26
5 BP	45,52	0,65	0,86
6 BP	59,24	0,67	0,72
7 BP	49,78	0,68	0,51
8 BP	51,45	0,62	0,48
10 BP	57,78	0,25	0,07
12 BP	33,28	0,29	0,10
15 BP	39,58	0,58	0,52
19 BP	51,33	0,48	0,53
20 BP.	99,92	0,51	0,63
23 BP	48,23	0,32	0,15
27 BP	52,84	0,84	0,96
28 BP	55,23	1,29	1,95
29 BP	44,88	0,61	0,88
36 BP	97,51	0,79	1,47
41 BP	42,58	0,53	0,65
44 BP	98,76	0,39	0,34
45 BP	30,93	1,10	1,71
48 BP	98,71	0,49	0,58
50 BP	99,33	0,59	0,73
52 BP	97,52	0,51	0,59
53 BP	98,13	0,73	1,23
54 BP	97,78	1,02	2,09
55 BP	97,53	0,80	1,45
56 BP	49,56	0,55	0,44

The endospores of some *Bacillus* strains have the capacity to stand acid pH and bile discharges in their passage through the gastrointestinal tract of the animals (Milián, 2009). The microorganisms that were selected with these purposes should survive the gastric barrier so that they can reach the low parts of the tract where they are going to develop their probiotic action. Overland *et al.* (2003) referred that the microorganisms used must stand low pH levels, because they are subject to the action of stomach acids during their transit through the digestive tract, where the pH can reach very low values.

Milián (2009), when evaluating *Bacillus* spp. strains isolated from different environments, obtained that 33 % showed capacity to stand acid pH

Table 3. Resistance of the isolates to the action of biliary salts.

Isolate	% resistance to biliary salts	SD %	VC %
20 BP	97,30	0,62	0,95
36 BP	-	-	-
44 BP	-	-	-
48 BP	-	-	-
50 BP	96,78	0,53	0,31
52 BP	-	-	-
53 BP	-	-	-
54 BP	-	-	-
55 BP	-	-	-

and only two strains C34 and C31 survived the presence of biliary salts in this same concentration. Nevertheless, Barbosa *et al*. (2005) isolated 259 *Bacillus* strains from the gastrointestinal tract of chicken and only 7 % of them withstood the acid barrier under laboratory conditions. Other works refer that the endospore germination is affected by the presence of biliary salts in 20-25 % (Duc *et al*., 2005).

It is known that at pH 2 many strains substantially decrease their viability (Milián *et al*., 2014), for which in the isolates that were evaluated at this pH an extremely drastic survival obstacle for the bacterial cells appeared. Nevertheless, it constitutes an important selection criterion if the fact that the gastric juice of chicken and ducks can reach pH values as low as 0,5-2 is taken into consideration (Lara and Burgos, 2012).

Biliary salts act as detergent and destabilize the lipids present in the cytoplasmic membrane. This situation causes the formation of pores that disturb the integrity and physiology of the cells, which can provoke death (Lara and Burgos, 2012). From these results it is inferred that the evaluated isolates can be considered potentially probiotic, because they could pass through the digestive tract of the birds and would thus facilitate the colonization of this ecosystem.

The two isolates that survived the concentration of biliary salts (20BP and 50BP) turned out to be Gram-positive bacilli, with the presence of oval and centrally-arranged endospores. The colonies showed irregular shape, cream color, undulated edges, opaque internal structure and convex elevation.

Between 0 and 4 h no antimicrobial activity was observed (table 4) against the indicator strains; however, from 8 to 18 h inhibition halos appeared in all the strains and differences were manifested ($p <$

Table 4. Antimicrobial activity of the isolates.

Indicator strain	Isolates (inhibition halo, mm)	
	20BP	50BP
8 h		
S. aureus ATCC-29213	4,43^{a}	6,27^{a}
Enterococcus faecalis ATCC-29212	4,33^{a}	5,23^{a}
E. coli ATCC-25922	8,43^{b}	9,20^{b}
E. coli spp.	11,40^{e}	11,17^{c}
Proteus spp.	9,40^{c}	9,53^{b}
Salmonella spp.	10,33^{d}	10,37^{bc}
p	< 0,001	< 0,001
SE	0,04	0,41
18 h		
S. aureus ATCC-29213	9,76^{b}	9,66^{a}
Enterococcus faecalis ATCC-29212	7,52^{a}	8,28^{a}
E. coli ATCC-25922	10,66^{bc}	10,10^{ab}
E. coli spp.	13,76^{e}	13,59^{c}
Proteus spp.	11,60^{cd}	11,40a^{bc}
Salmonella spp.	12,13^{d}	12,84^{bc}
p	< 0,001	< 0,05
SE	0,45	1,01

p: probability, SE: standard error. a, b, c, d , e: means with different letters within each column differ for $p < 0,001$ (Duncan, 1955).

0,001) in their diameter, because of the incidence of antimicrobial substances. The highest inhibition halo in the indicator ATCC strains occurred in the *E. coli* strain at 8 and 18 h, followed by *E. faecalis* and *S. aureus*. Also coincidently among the reference strains, the *E. coli* culture showed the highest inhibition halo followed by *Salmonella* and *Proteus*.

It is known that during the first weeks of life of farm and backyard poultry the main causative agent of diarrheic diseases is *E. coli*, and approximately 70 % of respiratory mycoses in chicks are accompanied by enterobacterial infections, in which the presence of this germ stands out as the most commonly isolated microorganism (Pérez *et al*., 2011).

Milián (2009) evaluated three *Bacillus* strains (C-31, C-34 and E-44), and they were capable of producing antimicrobial substances which totally inhibited such indicator strains as: *Aerobacter, Staphylococcus, Klebsiella, Proteus, Listeria inocua, L. monocytogenes, S. aureus* 29737, *Klebsiella* 130300, *S. epidermidis* 12228 and *P. vulgaris* 13315.

Laurencio (2010) reported that a mixture of competitive exclusion based on *Bacillus* spp. and *Lactobacillus* against enteropathogens inhibited *E. coli* between 12 and 24 h. Such results are similar to the ones in this study, which shows that the production of antimicrobial substances occurred in the logarithmic phase of the culture growth.

The isolates 20BP and 50BP were sensitive to the 14 evaluated antibiotics (table 5). These results are similar to the ones reported by by Milián (2009), who evaluated three *Bacillus* strains with 25 antibiotics and all of them were sensitive. This indicates that these strains could not show resistance plasmids; hence the importance of utilizing backyard chicken for the isolation of the strains, because these animals are not usually treated with antibiotics. There is the belief that the starter cultures for the elaboration of foodstuffs, as well as the bacteria that are used with probiotic purposes, serve as hosts of antibiotic resistance genes, which could be transferred to the pathogen bacteria of men and animals (Danielsen and Wind, 2003; Alvarado *et al*., 2007).

Table 5. Resistance to different antibiotics

Antibiotic (µg)	Strain	
	20BP	50BP
Kanamycin 100	s	s
Amikacin 300	s	s
Ampicillin 33	s	s
Azithromycin 15	s	s
Erythromycin 200	s	s
Amoxicillin 30	s	s
Ciprofloxacin 0,5	s	s
Streptomycin 100	s	s
Vancomycin 5	s	s
Apramycin 40	s	s
Bacitracin 40	s	s
Neomycin 120	s	s
Lincomycin 19	s	s
Spectinomycin 200	s	s

s: sensitive

Table 6 shows the growth of the isolates under adverse conditions. It was proven that these isolates had the capacity to grow in that pH range and at high temperatures. It was equally shown that they were tolerant to the different concentrations of NaCl, and there were differences among them.

The growth of the isolates at high temperatures, besides having taxonomic value, proved the capacity of these microorganisms to grow under extreme conditions. The bacteria that show these characteristics demonstrate higher growth capacity and can proliferate in the gastrointestinal tract, where temperature is higher than 37 ºC (Salminen *et al*., 1996). The fact that they grow at temperatures higher than 50 ºC means that they are thermophilous, for which it can be inferred that the isolates should be thermostable and remain viable without negative physiological affectations. This facilitates their utilization in technological procedures in which high temperatures are applied.

The carbohydrate fermentation profiles are shown in table 7; the isolates showed equal fermentation profiles against the different sugars.

The bacteria of this species are considered "generally recognized as safe" (GRAS) microorganisms, according to the criteria of the Association of American Feed Control Officials and the Food and Drug Administration (Anon, 1998). At present, this species, along with the bacteria of the *Lactobacillus* genus, are the most widely used in the obtainment of probiotic preparations, with beneficial effects on the production of monogastric animals (Rondón, 2009).

The alignments of the sequence of the rDNA 16S fragment of both isolates with sequences deposited in the GenBank are shown in table 8. The identification by molecular techniques allowed to confirm the results of the carbohydrate fermentation profiles. By showing a profile similar to and coinciding with the international database of the collection of non-redundant nucleotides, these isolates (20BP and 50BP) were reaffirmed as belonging to *Bacillus subtilis* in 99 %. This confirms the biological

Table 6. Growth capacity of the isolates under adverse conditions.

Indicator		Growth (OD)						p
	20BP	SD	VC	50BP	50BP	SD	VC	
pH	6	1,65	0,10	0,15	1,78	0,10	0,13	
	7	1,89	0,09	0,12	1,93	0,07	0,06	
	8	1,43	0,07	0,07	1,31	0,19	0,46	
Temperature (ºC)	45	1,87	0,12	0,24	1,86	0,16	0,34	
	50	1,77	0,04	0,03	1,75	0,10	0,17	≥ 0,05
	65	1,79	0,09	0,13	1,80	0,13	0,29	
NaCl (%)	3	1,33	0,10	0,15	1,31	0,21	0,73	
	5	1,27	0,07	0,08	1,53	0,12	0,21	
	7	1,12	0,08	0,13	1,15	0,26	1,09	

SD: standard deviation, VC: variation coefficient.

Table 7. Carbohydrate fermentation profiles.

Sugars	Isolate	
	20 BP	50 BP
Control	-	-
Glycerol	+	+
Erythritol	-	-
D-arabinose	-	-
L-arabinose	+	+
Ribose	+	+
D-xylose	+	+
L-xylose	-	-
Adonitol	-	-
a-methyl-D-xyloside	-	-
Galactose	-	-
D-glucose	+	+
D-fructose	+	+
D-manose	+	+
L-sorbose	-	-
Rhamnose	-	-
Dulcitol	-	-
Inositol	+	+
Manitol	+	+
Sorbitol	+	+
α-methyl-D-mannoside	-	-
α -methyl-D-glucoside	+	+
N-acetylglucosamine	-	-
Amygdalin	+	+
Arbutin	+	+
Esculin	+	+
Salicin	+	+
Cellobiose	+	+
Maltose	+	+
Lactose	+	+
Melibiose	+	+
Sucrose	+	+
Trehalose	+	+
Inulin	-	-
Melezitose	-	-
D-raffinose	+	+
Starch	+	+
Glycogen	+	+
Xylitol	-	-
a-gentiobiose	-	-
D-turanose	-	-
D-lyxose	-	-
D-tagatose	-	-
D-fucose	-	-
L-fucose	-	-
D-arabitol	-	-
L-arabitol	-	-

+: positive reaction, -: negative reaction

Table 8. Alignments of the strains.

<table>
<tr><td>16S rDNA Bacillus subtilis subsp subtilis 20BP</td></tr>
<tr><td>CTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGT-
GAGCCGTTACCT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGT-
GAGCCGTTACCT

CACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCCGAAGCCACCTTTTATGTTTGAAC-
CATGCGGTTCAAA
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCCGAAGCCACCTTTTATGTTTGAAC-
CATGCGGTTCAAA

CAACCATCCGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGT-
TACTCACCCGTCCG
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CAACCATCCGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGT-
TACTCACCCGTCCG</td></tr>
<tr><td>16S rDNA Bacillus subtilis subsp subtilis 50BP</td></tr>
<tr><td>GGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGT-
GAGCCGTTACCTCA
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGT-
GAGCCGTTACCTCA

CCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCCGAAGCCACCTTTTATGTTTGAAC-
CATGCGGTTCAAACA
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCCGAAGCCACCTTTTATGTTTGAAC-
CATGCGGTTCAAACA

ACCATCCGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTTACT-
CACCCGTCCGCC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ACCATCCGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTTACT-
CACCCGTCCGCC</td></tr>
</table>

safety of the isolates that were identified for their possible use in the elaboration of this type of additive.

Figure 1 shows the phylogenetic trees of the two isolates, where the homology established among the obtained sequences with others deposited in the database, is confirmed.

The results of this study coincide with those obtained by Calvo and Doris (2010), who isolated *Bacillus* strains from fattening chicken and characterized the microbiota belonging to this genus. They also coincide with the ones reported by Milián *et al.* (2014) when evaluating 48 *Bacillus* spp. strains.

Conclusions

Isolates 20BP and 50BP, from the digestive tract of backyard chicken and which were identified as *B. subtilis* subspecies *subtilis*, showed potential characteristics to be used as probiotic candidates.

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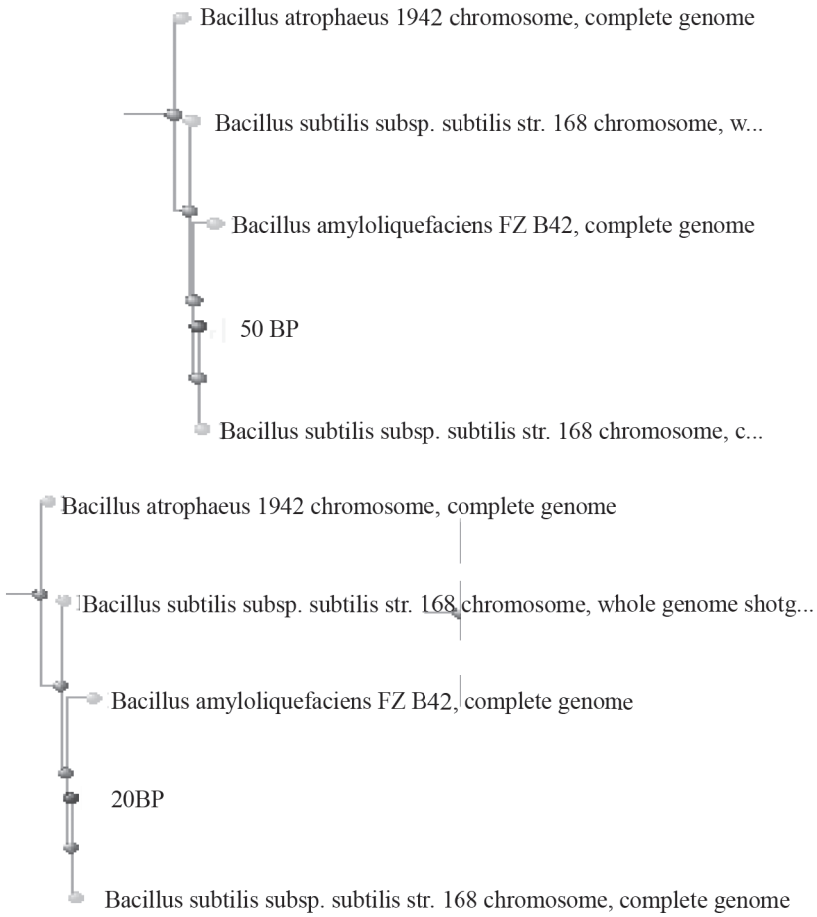

Figure 1. Phylogenetic trees of isolates 20BP and 50BP.

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