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ORIGINAL ARTICLE

Identification of lactic acid bacteria with bio-preservative potential isolated from contaminated avian blood obtained at the slaughterhouse

Identificación de bacterias ácido lácticas con potencial biopreservador aisladas a partir de sangre aviar obtenida en mataderos

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RESUMEN

La sangre es uno de los residuos más contaminantes de la industria cárnica, y a la vez es tiene diversas aplicaciones en la industria alimenticia animal. No obstante, dado que la sangre es altamente susceptible a la descomposición microbiana, ella y sus fracciones suelen no estar disponibles para su uso como ingredientes en la industria alimenticia. La biopreservación se presenta como una alternativa para mejorar la calidad de la sangre y de esta forma presentarla como un ingrediente en la elaboración de productos alimenticios. El objetivo de este trabajo fue aislar e identificar bacterias ácido lácticas (BAL) a partir de sangre aviar obtenida en mataderos industriales y evaluar su actividad antimicrobiana. Se aislaron 96 colonias presuntivas de BAL a partir de sangre aviar, las cuales fueron genotipificadas. Se estudiaron 31 BAL obtenidas a partir de diferentes muestras de sangre y se identificaron 11 grupos de bacterias diferentes a partir del análisis de restricción del ADN microbiano. De éstas, 28 produjeron compuestos antimicrobianos como ácidos orgánicos, 11 generaron peróxido de hidrógeno (H_2O_2) y dos fueron productoras de sustancias tipo bacteriocinas. Estas últimas, identificadas como *Lactobacillus salivarius* (DSPV 027SA) y *Enterococcus faecalis* (DSPV 008SA), inhibieron el crecimiento de *Escherichia coli, Pseudomonas aeruginosa* y algunos serotipos de *Salmonella* spp. De esta forma se lograron identificar dos cepas de BAL como potenciales candidatas para ser aplicadas en un sistema de biopreservación de sangre aviar. Esta herramienta biotecnológica es más económica que otras técnicas de sanitización y reduciría el riesgo de transmisión de microorganismos patógenos a lo largo de la cadena agroalimentaria.

Palabras clave: bacterias ácido lácticas, sangre aviar, biopreservación, bacteriocinas.

SUMMARY

Blood is a common by-product of the meat industry, which has several potential applications in the animal feed industry. However, since blood is highly susceptible to microbial spoilage, blood and its fractions are often not suitable ingredients for the feed industry. Biopreservation appears as an alternative for the improvement of blood's quality towards its use as an ingredient in foodstuff. The objective of this work was to isolate and identify Lactic Acid Bacteria (LAB) in avian blood obtained from industrial slaughterhouses and evaluate their antimicrobial activity. Ninety-six LAB were isolated from avian blood and genotyped. Eleven Amplified rDNA Restriction Analysis groups were identified. Between two and five different species were detected in each blood sample (31 strains in all blood samples) which were selected to study antagonistic activity. Twenty-eight of them produced antimicrobial compounds such as organic acids, 11 strains produced hydrogen peroxide (H_2O_2), and two released bacteriocin-like compounds. The latter, identified as *Lactobacillus salivarius* (DSPV 027SA) and *Enterococcus faecalis* (DSPV 008SA), inhibited the growth of *Escherichia coli, Pseudomonas aeruginosa* and some serotypes of *Salmonella* spp. These two LAB strains would be candidates for potential application as a blood biopreservation system. This biotechnological tool is cheaper than others sanitation techniques and could reduce the risk of pathogens transmission thought food chain.

Key words: lactic acid bacteria, avian blood, biopreservation, bacteriocins.

INTRODUCTION

In Argentina, more than 615 million poultries are slaughtered every year[1], producing more than 123 million liters of blood. Taking into account a typical protein content of around 18% (Putnam 1975), this volume of blood is equivalent to a production of 11,500 tons of protein. This blood is considered a by-product that can be used as a raw material in both feed and food industries because of its excellent nutritional value and functional properties (Dávila *et al* 2006). It can be considered as a good food additive (or ingredient) due to its high protein content and even shows an efficacy index larger than casein. Also, blood has high iron content bound to hemog-

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[1] MINAGRI, Ministerio de Agricultura de la República Argentina, http://www.minagri.gob.ar/SAGPyA/ganaderia/aves/index.php (accessed 17 July 2010).

lobin, which is the best bioavailable form (Reizenstein 1980).

However, spoilage and pathogenic microorganisms such as *Salmonella* spp, *E. coli* and *Pseudomona* spp from feathers, skin and gut from slaughtered animals can reach the blood and rapidly grow in this rich medium (Carretero and Parés 2000, Dávila *et al* 2006). This happens when meat industries (mainly avian slaughterhouses) have no suitable facilities to properly collect and handle blood, therefore, contamination is unavoidable even under strict hygienic conditions. Blood is harvested by open system and for this reason, contamination is impossible to prevent.

The quality of blood can be improved by different methods. One possibility is to use lactic acid bacteria (LAB) to control bacterial populations and increase the shelf life of blood. This alternative has been reported in different areas of the food industry, and frequently in meat products (Signorini *et a*l 2006). The mode of action of LAB includes competence for nutrients, adhesion to the substrate and production of antibacterial compounds such as organic acids, diacetyl, hydrogen peroxide, reuterin and bacteriocins. Therefore, LAB strains isolated from the same product in which they will be subsequently used as biopreservative agents (with the subsequent reduction in the use of antibiotics) may have the greatest success in controlling both pathogenic and spoilage microorganisms (Signorini *et al* 2006).

This study aimed to isolate and identify LAB strains in avian blood obtained from industrial slaughterhouses and perform a preliminary evaluation of their antimicrobial activity. The application of biopreservation cultures to blood could reduce the microbiological load, extending its shelf life, and thus avoiding immediate processing after blood collection. Poultry slaughterhouses could thus use blood efficiently, transforming it into a product with high biological value.

MATERIAL AND METHODS

ISOLATION OF LAB

Ten avian blood samples from two slaughterhouses were collected directly from the slaughter line. Samples were kept on ice and immediately transferred to the laboratory for bacterial isolation. Blood samples were serially diluted in sterile Ringer ¼ solution, pour-plated in Petri plates with MRS agar (OXOID) and LAMBAV agar (Hartemink *et al* 1997) as culture media, and then incubated under anaerobic conditions in anaerobic jars with Anaerocult A gas packs (Merck, Darmstadt, Germany), at 37ºC for 48 h.

Colonies from each plate were randomly picked to obtain pure cultures on MRS broth (OXOID) and incubated at 37ºC for 48 h under anaerobic conditions. Gram staining and catalase test were performed as a preliminary screening for LAB. For preservation, the cultures were frozen at – 80ºC with the addition of glycerol 25% v/v.

GENOTYPING OF ISOLATED LAB

DNA from presumptive LAB was isolated following the protocol reported by Marmur (1961) modified by Kurzak *et al* (1998) to amplify the 16S rDNA (1500 bp). To characterize the isolates, an Amplified rDNA Restriction Analysis (ARDRA) was conducted (Soto *et al* 2010). Amplification of the 16S rDNA was performed using 20 to 50 ng of DNA in 25 µL reactions containing 1.5 mM $MgCl_2$, 200 µM each deoxynucleoside triphosphates (Promega), 0.4 µM each of primers 27f and 1492r (Kim and Chun, 2005) and 1U *Taq* polymerase (GoTaq, Promega) in 1 X *Taq* buffer (Promega). The reaction mixtures were incubated in a Thermal Cycler (MJ Research). The amplification conditions were as follows: 94ºC for 5 minutes, 30 cycles of 94ºC for 1 minute, 55ºC for 1 minute and 72ºC for 1 minute, and a final extension step at 72ºC for 7 minute. After cycling, the PCR products were visualized by electrophoresis on a 1% w/v agarose gel (40 minutes, 75V), by staining with Gel Red (1 µL in 10 ml) and visualizing under UV light (DyNA Light UV Transilluminator, LabNet, UV light source wavelength 302 nm).

Then, a 15-µL aliquot of each PCR reaction was incubated for 37ºC for 4 h with 2 μL of 10X incubation buffer, 0.2 μL of bovine serum albumin, 2.5 µL of bidistilled water and 6U of one of the following restriction enzymes: *Hinf* I, *Hae* III, or *Msp* I.

The restriction products were analyzed by electrophoresis in 2% agarose gel. During the analysis of ARDRA patterns, bands with the same gel mobility were considered equivalent, independently of their relative intensity. The results of the separate restriction profiles were combined into a single dataset and analyzed using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm by Treecon for Windows (Version 1.3b, Yves Van de Peer, University of Konstanz, Germany).

Afterwards, isolates with 16S rDNA restriction profiles different for each blood sample were purified with Wizard PCR SV Gel & PCR Clean-Up System kit (Promega) and sequenced. Isolates were then identified by partial sequence using the GenBank BLAST alignment software[2].

ANTAGONISTIC ACTIVITY TESTS

LAB with different ARDRA profiles were inoculated on MRS Broth at 37 ºC for 16 h. Then the cultures were centrifuged and the supernatants were collected (Cell Free Extract, CFE), and one aliquot of this was adjusted to pH 6.00 - 6.50 with 3M NaOH (Cell Free Neutralizing Extract, CFNE). Seven strains origins of DSP (Public Healthy Department, Faculty of Veterinary, University of Litoral, Argentina) Laboratory collection were used as indicator of microorganisms: *Escherichia coli* (GenBank accession number FJ997269*), Salmonella* Dublin (GenBank acces-

[2] http://www.ncbi.nlm.nih.gov/BLAST

sion number FJ997268), *Pseudomonas fluorescens* ATCC DSM50106, *Lactobacillus casei* (GenBank accession number: FJ787305), *Lactobacillus plantarum* (GenBank accession number: FJ751793), *Enterococcus faecium* (Schneider *et al* 2004), and *Lactobacillus acidophilus* (DSP collection). *E. coli*, *S.* Dublin and *P. fluorescens* were selected because they are related to slaughterhouse blood contamination and they present negative effects in the microbiological quality of this product (Zamora-Rodriguez 2003). Also, these bacteria are potential human and animal pathogens.

An agar well diffusion assay was used for detection of antagonistic activity (De Vuyst *et al* 1996), MRS or BHI (Brain Heart Infusion) soft agar (0.8 g/100 g agar) plates were used (MRS for *L. casei, L. plantarum, E. faecium,* and *L. acidophilus*, and BHI for *E. coli, S.* Dublin, and *P. fluorescens*). The soft agar was inoculated previously with an overnight culture of each indicator strain. Holes 5 mm in diameter were cut into these agar plates and CFE and CFNE from the isolates was placed (30 µl) into each well. The plates were then incubated under aerobic conditions at 37 ºC for 48 h and subsequently examined for zones of inhibition (2 mm clear or larger zones around the well were scored as positive inhibition).

HYDROGEN PEROXIDE PRODUCTION

To elucidate whether the antimicrobial activity derives from the production of hydrogen peroxide by LAB, each LAB with antimicrobial activity was evaluated with the hydrogen peroxide test (McLean and Rosenstein 2000). MRS agar supplemented with 0.25 mg/ml of tetramethylbenzidine (TMB, Sigma) and 0.01 mg/ml of horseradish peroxidase (HRP, Sigma) was inoculated with LAB and incubated anaerobically for 48 h at 37ºC. The plates were then air exposed for 30 minutes. Colonies that produced hydrogen peroxide were blue due to TMB oxidation.

DETECTION, CHARACTERIZATION OF BACTERIOCINS AND EVALUATION OF ANTAGONIST ACTIVITY AGAINST PATHOGENIC MICROORGANISMS FREQUENTLY ISOLATED FROM THE AVIAN CHAIN

The sensitivity to proteolytic enzymes of the CFNE was investigated adding Proteinase K (Sigma) at a final concentration of 1 mg/mL (Todorov and Dicks 2006). Samples with and without proteases were incubated at 37ºC for 24 h and residual activity was determined. The absence of inhibition zone in the presence of the protease confirmed the polypeptide nature of the antibacterial substances.

To test the influence of pH, the CFNE were adjusted to pH 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 with HCl 3M or NaOH 3M (Sigma), and allowed to stand at room temperature for 30 and 90 minutes. The effect of temperature on CFNE stability was determined by treatment in a water bath at 90ºC and 121ºC for 30 and 15 minutes, respectively (Todorov and Dicks 2006).

The antagonistic activity test was performed against pathogenic microorganisms frequently isolated from the avian chain to study the inhibition spectra, specifically of each LAB producing bacteriocin-like compounds. Sources of these strains are shown in table 1.

The presence of enterocin-encoding genes was studied by PCR amplification with primers for enterococcal bacteriocins (Du Toit *et al* 2000). Total genomic DNA from strains was used. PCR products were analyzed by electrophoresis (85 V for 1 h 5 min) on 2% (w/v) agarose gels in TAE buffer. Gels were stained GelRed (Invitrogen) and observed under UV light. The sizes of the amplified fragments were determined using 100-bp DNA Ladder (Promega) as a molecular weight marker.

RESULTS

This work was structured in two parts. Firstly, LAB from slaughterhouse avian blood was isolated and genotyped and then the antimicrobial activity of the identified LAB was evaluated.

ISOLATION OF LAB

Ninety-six presumptive LAB strains (gram-positive, catalase-negative) were successfully isolated from a total of 10 avian blood samples taken on different days from two industrial slaughterhouses and the average concentration of this population was 4.94 log CFU/ml (SD= 0.80) on MRS Agar and 3.95 log CFU/ml (SD= 0.39) on LAMBAV Agar.

GENOTYPING OF ISOLATED LAB

PCR amplification of the 16S rDNA of the 96 isolates followed by analysis with three different restriction endonucleases was carried out (ARDRA). Each blood sample was analyzed individually with the aim to detect different

Table 1. Sources of pathogenic microorganisms isolated from the avian chain (autochthon indicator bacteria). SA = avian blood.

Fuente de microorganismos patógenos aislados de la cadena aviar (bacterias indicadores autóctonas). SA= Sangre Aviar.

Strains	Origin
Escherichia coli (SA)	Avian blood of slaughterhouse
Salmonella spp (SA)	Avian blood of slaughterhouse
Pseudomonas aeruginosa (SA)	Avian blood of slaughterhouse
Bacillus sp (SA)	Avian blood of slaughterhouse
Staphylococcus aureus (SA)	Avian blood of slaughterhouse
Salmonella muenchen	Avian egg
Salmonella gallinarum	Avian egg
Salmonella agona	Avian egg
Salmonella brandenburg	Avian egg
Salmonella enteritidis	Avian egg
Salmonella typhimurium	Avian egg

species of LAB in samples taken on different days. In this way, between two and five different species were detected in each sample (31 strains in all blood samples).

ARDRA revealed different profiles having three to six fragments ranging in size from 60 to 1000 bp for different isolates and grouped all isolates into 11 species groups using Treecon® Software (figure 1). Restriction digestion of the amplified product with different enzymes revealed that *Msp* I was more discriminatory.

The most frequently detected species in the slaughterhouse blood samples were *Lactobacillus salivarius* and *Enterococcus faecalis* (7 out of 10 samples had these species). Finally, isolates with different ARDRA profiles into blood samples were sequenced and submitted to GenBank (table 2).

ANTAGONISTIC ACTIVITY TESTS

Ninety percent (28/31) of the identified and tested LAB strains showed antimicrobial activity against one or more indicator strain from DSP Laboratory collection by agar well diffusion assay, and only three strains showed no antagonistic activity (table 3). Eleven of the strains with antimicrobial activity showed activity in the CFNE. Regarding the indicator strains, *L. casei* was not inhibited by any CFE, whereas *L. acidophilus* was affected by 10% of CFE and not affected by any CFNE. Regarding the other LAB, *L. plantarum* was inhibited by 16% of CFE and 10% of CFNE, whereas *E. faecium* was inhibited by 48% CFE and 3% CFNE. With regards to pathogens, *E. coli* and *Salmonella* Dublin were inhibited by 68% and 77% of CFE, respectively. However, only one CFNE inhibited *S.* Dublin. In contrast, *P. fluorescens* was inhibited by 55% of CFE and 23% of CFNE.

HYDROGEN PEROXIDE PRODUCTION

Eleven strains with antimicrobial activity in CFNE (gray in table 3) were analyzed for H_2O_2 production. Eighty percent of these strains were positive before 10 minutes of air exposure. Only *L. salivarius* (DSPV 027SA) and *E. faecalis* (DSPV 008SA) were negative to H_2O_2 production. Therefore, antimicrobial activities of these two strains might be due to the production of bacteriocin-like compounds.

DETECTION, CHARACTERIZATION OF BACTERIOCINS AND EVALUATION OF ANTAGONIST ACTIVITY AGAINST PATHOGENIC MICROORGANISMS FREQUENTLY ISOLATED FROM THE AVIAN CHAIN

To study the inhibitory compounds of *L. salivarius* (DSPV 027SA) and *E. faecalis* (DSPV 008SA), CFNE were incubated with proteinase K. Results showed that antimicrobial effect disappeared after this assay, confirming proteinaceus bacteriocin-like substances. Then,

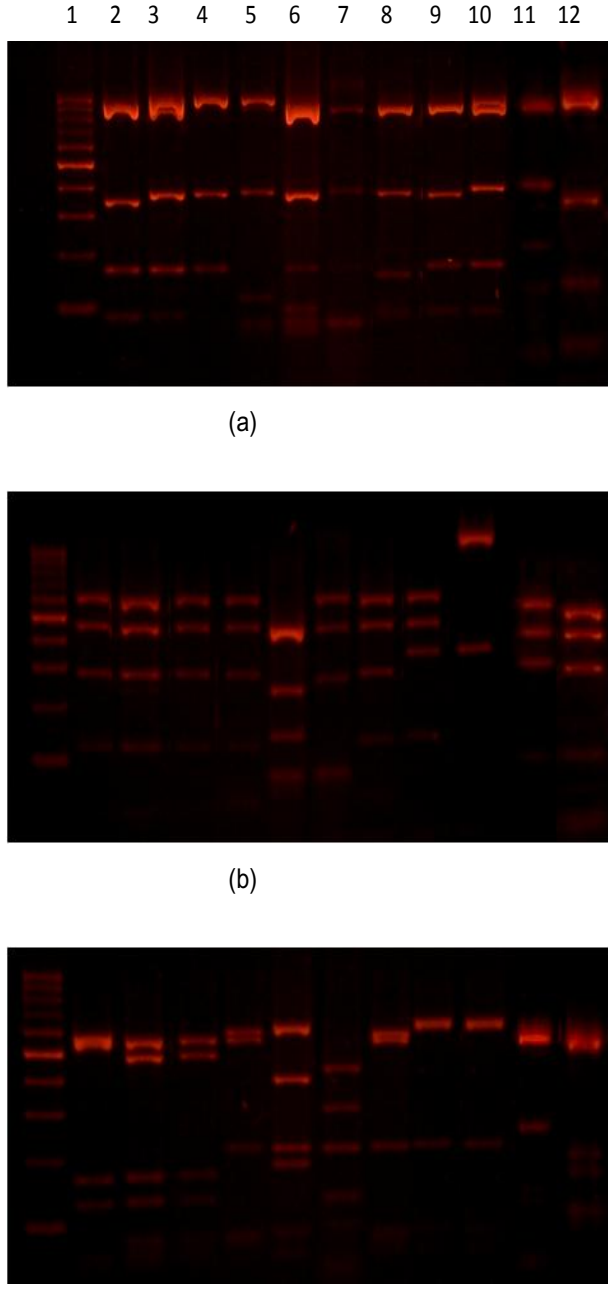

Figure 1. LAB isolated from slaughterhouse avian blood: agarose gel with different groups of ARDRA.

BAL aisladas de sangre de matadero aviar: gel de agarosa con los diferentes grupos de ARDRA.

References: Line 1 MW ladder (100 bp); line 2, ARDRA group 1 (*E. faecalis*); line 3, ARDRA group 2 (*E. faecium*); line 4, ARDRA group 3 (*E. durans*); line 5, ARDRA group 4 (*L. brevis*); line 6, ARDRA group 5 (*L. crispatus*); line 7, ARDRA group 6 (*L. reuteri*); line 8, ARDRA group 7 (*L. salivarius*); line 9, ARDRA group 8 (*P. acidilactici*); line 10, ARDRA group 9 (*W. paramesenteroides*); line 11, ARDRA group 10 (*P.pentosaceus*); line 12, ARDRA group 11 (*L. plantarum*). Restriction fragments obtained with each enzyme: (a) Hinf I, (b) Hae III, (c) Msp I.

Table 2. List of bacteria isolated in this study and their closest affiliation according to the 16S rDNA sequencing (1500 bp) or by belonging to the same ARDRA group.

Listado de bacterias aisladas en el estudio y su afiliación de acuerdo a la secuenciación del gen 16rADN (1500 pb) o en función del mismo grupo según ARDRA.

*Strain*s	Species group	ARDRA Group	Identity Value	Blood Samples	Accession number
DSPV 001SA	*Enterococcus durans*	1	94%	4	JQ322216
DSPV 002SA	*Enterococcus faecalis*	2	97%	8	JQ322217
DSPV 003SA	*Enterococcus faecalis*	2	99%	2	JQ322220
DSPV 004SA	*Enterococcus faecalis*	2	99%	1	JQ322221
DSPV 005SA	*Enterococcus faecalis*	2	99%	3	JQ322222
DSPV 006SA	*Enterococcus faecalis*	2	97%	4	JQ322212
DSPV 007SA	*Enterococcus faecalis*	2	98%	10	JQ322214
DSPV 008SA	*Enterococcus faecalis*	2	100%	5	JQ322228
DSPV 009SA	*Enterococcus faecium*	3	99%	6	JQ322238
DSPV 010SA	*Enterococcus faecium*	3	99%	4	JQ322235
DSPV 011SA	*Enterococcus faecium*	3	95%	7	JQ322213
DSPV 012SA	*Lactobacillus brevis*	4	94%	4	JQ322211
DSPV 013SA	*Lactobacillus crispatus*	5	98%	7	JQ322240
DSPV 014SA	*Lactobacillus plantarum*	6	99%	3	JQ322237
DSPV 016SA	*Lactobacillus reuteri*	7	99%	9	JQ322218
DSPV 017SA	*Lactobacillus reuteri*	7	99%	10	JQ322219
DSPV 018SA	*Lactobacillus reuteri*	7	99%	8	JQ322226
DSPV 019SA	*Lactobacillus reuteri*	7	99%	5	JQ322227
DSPV 020SA	*Lactobacillus reuteri*	7	99%	6	JQ322209
DSPV 021SA	*Lactobacillus salivarius*	8	100%	2	JQ322239
DSPV 022SA	*Lactobacillus salivarius*	8	98%	4	JQ322236
DSPV 023SA	*Lactobacillus salivarius*	8	99%	9	JQ322241
DSPV 024SA	*Lactobacillus salivarius*	8	99%	10	JQ322242
DSPV 025SA	*Lactobacillus salivarius*	8	99%	1	JQ322225
DSPV 026SA	*Lactobacillus salivarius*	8	99%	5	JQ322229
DSPV 027SA	*Lactobacillus salivarius*	8	99%	6	JQ322208
DSPV 015SA	*Pediococcus acidilactici*	9	99%	3	JQ322207
DSPV 028SA	*Pediococcus acidilactici*	9	99%	4	JQ322215
DSPV 029SA	*Pediococcus pentosaceus*	10	99%	1	JQ322223
DSPV 030SA	*Pediococcus pentosaceus*	10	99%	10	JQ322224
DSPV 031SA	*Weissella paramesenteroides*	11	96%	6	JQ322210

Table 3. Antimicrobial effects of the supernatants of lactic acid bacteria isolated from slaughterhouse blood before (Cell Free Extract, CFE) and after neutralization (Cell Free Neutralizing Extract, CFNE) against strains of DSP Laboratory collection (DSP collection indicator bacteria). Grey color indicates strains with CFNE diameter halo was over 2 mm.

Efecto antimicrobiano del sobrenadante de las bacterias ácido lácticas aisladas a partir de sangre aviar de mataderos antes (Extracto Libre de Células, ELC) y después de la neutralización (Extracto Libre de Células Neutralizado, ELCN) contra cepas de la colección del Laboratorio del DSP (bacterias indicadores de colección). El color gris indica halos de inhibición de CFNE con diámetros mayores a 2 mm.

		L. casei		*L plantarum*		*E. faecium*		*L. acidophilus*		*E. coli*		*Salmonella dublin*		*P. fluorescens*	
Strain	Species	CFE	CFNE	CFE	CFNE	CFE	CFNE	CFE	CFNE	CFE	CFNE	CFE	CFNE	CFE	CFNE
DSPV 001SA	*Enterococcus durans*	-	-	-	-	+	-	-	-	+	-	+	-	-	-
DSPV 002SA	*Enterococcus faecalis*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DSPV 003SA	*Enterococcus faecalis*	-	-	-	-	-	-	-	-	-	-	+	-	+	+
DSPV 004SA	*Enterococcus faecalis*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DSPV 005SA	*Enterococcus faecalis*	-	-	-	-	+	-	-	-	+	-	-	-	-	-
DSPV 006SA	*Enterococcus faecalis*	-	-	-	-	-	-	-	-	+	-	-	-	-	-
DSPV 007SA	*Enterococcus faecalis*	-	-	-	-	-	-	-	-	+	-	+	-	+	-
DSPV 008SA	*Enterococcus faecalis*	-	-	-	-	-	-	-	-	-	-	-	-	+	+
DSPV 009SA	*Enterococcus faecium*	-	-	-	-	+	-	-	-	+	-	+	-	+	-
DSPV 010SA	*Enterococcus faecium*	-	-	-	-	-	-	-	-	-	-	+	-	+	+
DSPV 011SA	*Enterococcus faecium*	-	-	-	-	-	-	-	-	+	-	+	-	+	+
DSPV 012SA	*Lactobacillus brevis*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DSPV 013SA	*Lactobacillus crispatus*	-	-	-	-	-	-	-	-	-	-	-	-	+	+
DSPV 014SA	*Lactobacillus plantarum*	-	-	-	-	+	+	-	-	-	-	+	-	-	-
DSPV 015SA	*Lactobacillus reuteri*	-	-	+	+	-	-	-	-	+	-	+	-	-	-
DSPV 016SA	*Lactobacillus reuteri*	-	-	+	-	-	-	-	-	+	-	+	-	-	-
DSPV 017SA	*Lactobacillus reuteri*	-	-	-	-	+	-	-	-	+	-	+	-	+	-
DSPV 018SA	*Lactobacillus reuteri*	-	-	+	-	+	-	+	-	+	-	+	-	+	-
DSPV 019SA	*Lactobacillus reuteri*	-	-	-	-	+	-	+	-	+	-	+	-	+	+
DSPV 020SA	*Lactobacillus reuteri*	-	-	+	+	+	-	-	-	+	-	+	+	+	-
DSPV 021SA	*Lactobacillus salivarius*	-	-	-	-	-	-	-	-	-	-	+	-	-	-
DSPV 022SA	*Lactobacillus salivarius*	-	-	-	-	+	-	-	-	+	-	+	-	+	-
DSPV 023SA	*Lactobacillus salivarius*	-	-	-	-	+	-	-	-	+	-	+	-	+	+
DSPV 024SA	*Lactobacillus salivarius*	-	-	-	-	+	-	-	-	+	-	+	-	+	-
DSPV 025SA	*Lactobacillus salivarius*	-	-	-	-	+	-	-	-	+	-	+	-	-	-
DSPV 026SA	*Lactobacillus salivarius*	-	-	-	-	+	-	+	-	+	-	+	-	+	-
DSPV 027SA	*Lactobacillus salivarius*	-	-	+	+	+	-	-	-	+	-	+	-	-	-
DSPV 028SA	*Pediococcus acidilactici*	-	-	-	-	-	-	-	-	-	-	+	-	-	-
DSPV 029SA	*Pediococcus pentosaceus*	-	-	-	-	+	-	-	-	+	-	+	-	+	-
DSPV 030SA	*Pediococcus pentosaceus*	-	-	-	-	-	-	-	-	+	-	+	-	+	-
DSPV 031SA	*Weissella paramesenteroides*	-	-	-	-	-	-	-	-	+	-	+	-	-	-

CFNE from *L. salivarius* DSPV 027SA were not affected by different pH. On the other hand, *E. faecalis* DSPV 008SA was affected by low pH (pH = 4.00 and 5.00). CFNE from both strains remained active after heat treatment. Like most bacteriocins (Nettles and Barefoot 1993), all bacteriocin-like substances included in this study are resistant to high temperature. This biochemical characteristic is important if this substance is applied in process of feed or food industries.

On the other hand, *L. salivarius* DSPV 027SA and *E. faecalis* DSPV 008SA showed an important antimicrobial activity against autochthon indicator bacteria (table 1) such as *Salmonella* Agona*, S.* Brandenburg, *Salmonella* spp. (SA) and *Pseuodomonas aeruginosa* (SA). Only *L. salivarius* DSPV 027SA had antagonistic activity against *S.* Typhimurium when the test was performed. Regarding the other microorganisms tested, *L. salivarius* DSPV 027SA and *E. faecalis* DSPV 008SA showed no antimicrobial activity. The analyses of structural enterocins genes in the DNA of *E. faecalis* DSPV 008SA by PCR reactions was also done and revealed that the *entA* gen was amplified. The size of the fragment observed was 137bp.

DISCUSSION

The slaughterhouse blood microbiota includes predominantly bacteria from gastrointestinal tracts (GIT) of animals and, in a lower percentage, environmental microorganisms. Therefore, it is important and useful studying the microbiota diversity existing in slaughterhouse blood because this product could contain beneficial microorganisms with preservation potential.

The importance of LAB in biopreservation has increased. Many LAB genera like *Lactobacillus*, *Enterococcus*, and *Bacillus* sp. are used as probiotics or in biopreservation in products for humans and animals (Chukeatirote 2003, Kolozyn-Krajewska and Dolatowski 2012, Delavenne *et al* 2013). In this study, LAB population from blood slaughterhouses was isolated and counted. LAB were in average 4.94 log CFU/ml on MRS Agar and 3.95 log CFU/ml on LAMVAB. This indicates that *Lactobacillus* spp count on LAMVAB plates represent a major part of all LAB that growth on MRS agar. Lactobacillus is one of the LAB genus that generally occur in traditional fermented foods, this factor must be taken into consideration when evaluating the use as biopreservative (Holzapfel 1997).

Various species of LAB, reported as being predominant in chicken GIT, can contaminate the slaughterhouse in the evisceration process. ARDRA has been used to compare bacterial isolates within a wide range of microbial communities. The advantages of ARDRA are that it is rapid, reproducible, relates to microbial diversity, and is invaluable in analyzing a large number of samples together (Ziemer *et al* 2004).

In this work, ARDRA was useful to detect 11 different species of LAB. Our results are in agreement with those of other studies where ARDRA is accurate enough to identify species level (Guan *et al* 2003, Soto *et al* 2010). However, the bacteria most frequently detected in our experiment were *L. salivarius and E. faecalis* in contrast with earlier studies where the strains most frequently isolated from the chicken GIT microbiota were *Lactobacillus crispatus*, *Lactobacillus gallinarum*, *Lactobacillus johnsonii*, and *Lactobacillus reuteri* (Abbas *et al* 2007, Bjerrum *et al* 2006, Guan *et al* 2003). Bacterial diversity and the composition of the microbiota are related to age, rearing environment, production system and diet of chickens (Knarreborg *et al* 2002, Lu *et al* 2003). Blood samples were taken during different days in this study because every day, poultry flocks from different farms are slaughtered and it could be an important source of bacterial diversity.

The biopreservation properties of microorganisms are characteristic of each strain, for example some strains have the capacity to produce antimicrobial compounds including organic acids, hydrogen peroxide and bacteriocins (Juárez Tomás *et al* 2004, Espeche *et al* 2009), which can inhibit the growth of enteric pathogens. Several studies have reported the antagonistic properties of LAB against many common gastroenteric pathogens, e.g. *Salmonella* spp, *Escherichia coli* O157:H7, *Clostridium perfringens, Campylobacter jejuni, Listeria monocytogenes* and *Helicobacter pylori* (Mukai *et al* 2002, Brashears *et al* 2003, Casey *et al* 2004, Chaveerach *et al* 2004, Kim and Chun 2005).

We detected 31 strains from 10 blood samples corresponding to 11 LAB species with different ARDRA profiles, however, the isolates belonging to the same species had a different spectrum of inhibition, suggesting that they were a different strain. Four LAB and three pathogens (DSP collection indicator bacteria, table 3) were used to detect antagonistic activity by 31 LAB isolated from slaughterhouse avian blood. Regarding organic acid production (CFE), only one strain was not affected (*L. casei*). In contrast, pathogens were highly affected by this substance because these microorganisms are commonly sensitive to low pH. Organic acid production is not always beneficial for food conservation because a decrease in pH could be harmful regarding the maintenance of the organoleptic characteristics of food or the integrity of plasmatic protein.

Eleven out of these 31 strains showed CFNE with antimicrobial activity. This indicates that the inhibition is due to H_2O_2 or bacteriocin production. Regarding H_2O_2 production, nine isolates showed positive activity against indicator strains. The release of this substance was positive (inhibition halo > 2 mm of diameter) and caused inhibition mainly against *P. fluorescens*. The growth inhibition of one bacterial species by the H_2O_2 generated by another species is a well-recognised mechanism of bacterial anta-

gonism (Thompson and Johnston 1950, Dahiya and Speck 1968, Juárez Tómas *et al* 2004, Patterson *et al* 2008).

Strains used in this work, such as *P. fluorescens*, *E. coli* and *Salmonella dublin*, are catalase-positive, so the level of H_2O_2 should be high to inhibit these pathogens (Zamora Rodríguez 2003). Nevertheless, LAB strains with capacity to inhibit catalase-positive pathogens have been described previously (Gilliland and Speck 1972). However, in the same way as organic acid production, high levels of H_2O_2 in food may generate undesirable effects. Peroxides affect lipids and vitamins, causing oxidation, thus decreasing nutritional food quality (Sanders 1994).

On the other hand, only two strains showed antagonistic activity in CFNE but did not produce H_2O_2. These were *L. salivarius* DSPV 027SA and *E. faecalis* DSPV 008SA. The inhibitory substances generated by these strains were characterised and evaluated to know their inhibition spectrum. The proteinaceous nature of the inhibitory compounds was confirmed because the antibacterial effect did not remain after treatment with proteinase K. Later, detection of *Ent*A gen in *E. faecalis* DSPV 008SA was confirmed by PCR. Encoding genes were almost studied for enterocins (Cintas *et al* 1998, Du Toit *et al* 2000) but the case of salivaricin is more complicated because there is not enough information about bacteriocin types and the genes envolved in their expression. Although production of bacteriocins by *L. salivarius* has been previously reported (Pilasombut *et al* 2006, Busarcevic *et al* 2008) the mode of action of these antimicrobial peptides has not totally elucidated (Pingitore *et al* 2009).

Then, the antagonistic activity of these substances was tested against other pathogens and spoilage microorganisms probably present in avian blood from slaughterhouse (autochthon indicator bacteria). Results were encouraging because these antimicrobial compounds were able to inhibit many serotypes of *Salmonella* such as *S.* Agona*, S.* Brandenburg, *Salmonella* spp (SA) and *Pseudomonas aeruginosa* (SA). It is known that *Salmonella* spp are very important in avian production because they affect avian health and cause a high impact in human health. On the other hand, *Pseudomonas* spp. could cause damage in meat products for human consumption and particularly P. aeruginosa is implied in foodborne illness.

L. salivarius showed good characteristics to be used in biopreservation, because it belongs to obligatory homolactic lactobacilli that produce only lactic acid during glucose fermentation. Some clones of *L. salivarius* are screened for the presence of potential probiotic properties (Martín *et al* 2006, Hermans *et al* 2011). Bacteriocin production by mucosal-associated *L. salivarius* strains has been described previously (Ocaña *et al* 1999, Flynn *et al* 2002). *L. salivarius* is considered as a potential probiotic bacterium and can be found in many commercial probiotic products. In a previous research (Barros *et al* 2009) *L. salivarius* isolated from chicken showed inhibitory activity against several *Salmonella* spp. The results in this study demonstrate that *L. salivarius* DSPV 027SA can inhibit this important pathogen of the avian chain. The probiotic potential of LAB has been related with some evidence of the host-specificity of some members of the indigenous microbiota to colonize specific hosts (Zoetendal *et al* 2006). The results obtained in this study suggest that *L. salivarius* DSPV 027SA is a good candidate for slaughterhouse blood biopreservation.

E. faecalis DSPV 008SA belongs to the genus Enterococcus, an important group of the LAB generally recognised as safe (GRAS). Different types of enterocins exhibit bactericidal activity against a wide variety of Gram-positive bacteria, including food spoilage and pathogenic bacteria such as *Bacillus cereus, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Staphylococcus aureus* and *Listeria monocytogenes* (Mendoza *et al* 1999, Abriouel *et al* 2002). *E. faecalis* also shows activity against some Gram-negative species.

The present study shows that LAB microbiota from slaughterhouse avian blood is diverse and composed of many different species with probiotics or biopreservation potential. Two strains that released antimicrobial compounds were selected (*L. salivarius* DSPV-027SA and *E. faecalis*-DSPV 008SA) and they could be suitable for application as biopreservation for avian blood.

However, this potential biopresevation system should be included into the slaughterhouses quality systems (GMP, SSOS, etc.) with the aim to use the avian blood as food or feed additive. This preliminary study allows initiating researches where slaughterhouse avian blood can be used as a functional ingredient in food formulations with technological and health benefits.

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