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Poultry blood from slaughterhouses: development of a biopreservation system to improve microbiological quality prior to transforming it into by-products

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Running title: Development of a biopreservation system for poultry blood

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Abstract. 1. The aim of this study was to investigate the use of indigenous lactic acid bacteria (LAB) with specific additives as a Biopreservation System (BS) for poultry blood during its storage in slaughterhouses.

2. The BS consisted of two LAB (*Enterococcus faecalis* DSPV 008SA or *Lactobacillus salivarius* DSPV 032SA) with 4 additives (lactose 2 g/l, yeast extract 0.4 g/l, ammonium citrate 0.4 g/l and NaCl 1 g/l). After 24 h storage at 30°C, lower counts of enterobacteria, coliforms, *Pseudomonas* spp. and *Staphylococcus aureus* were evident in blood treated with the BS than in untreated blood.

3. The ability of LAB to prevent haemolysis was evident. A decrease in pH was associated with control of spoilage microorganisms but it needed to be regulated to prevent coagulation of proteins.

4. On the basis of these results it is worthwhile to supplement blood with a BS to avoid undesirable changes during blood storage before processing.

INTRODUCTION

In Argentina, the number of poultry slaughtered each year is more than 734 million (MinAgri, 2013), producing almost 150 million l of blood which potentially could be collected for industrial applications. Poultry blood is considered to be an important source of high-quality protein, because of its high isoleucine and lysine content (Márquez *et al.*, 2005). Due to excellent nutritional qualities and functional properties, poultry blood has been considered a by-product which can be used as a raw material for both the feed and food industries (Ockerman and Hansen, 1988; Dávila *et al.*, 2006).

The use of animal blood proteins is important considering the high levels of protein deficiencies around the world. However, poultry blood is not collected and handled properly at slaughterhouses and generally is of poor quality as it becomes contaminated by spoilage and pathogenic microorganisms from the feathers, skin and guts of slaughtered animals (Dávila *et al.*, 2006). Also, blood is usually stored without refrigeration and in such conditions foodborne spoilage microorganisms and pathogens readily grow and reduce the opportunities for processing (Dávila, 2006; Zbrun *et al.*, 2013a).

Blood biopreservation using lactic acid bacteria (LAB) could be a valuable biotechnological tool. LAB are used in other areas of food production because of their proven ability to inhibit the growth of undesirable bacteria (De Vuyst and Vandamme, 1994; Signorini *et al.*, 2006; Zbrun *et al.*, 2013a) through a wide variety of mechanisms (Lindgren and Dobrogosz, 1990; Hugas, 1998; O'Sullivan *et al.*, 2002). Dávila *et al.* (2006) demonstrated that supplementation of porcine blood with inulin and inoculation with an active LAB strain could prevent undesirable changes during chill storage, especially useful in preventing the effects of a cold-chain breakdown.

Zbrun *et al.* (2013) identified and isolated two LAB from poultry blood obtained from industrial slaughterhouses, which exhibited ability to grow in blood and inhibit the growth of undesirable microorganisms. The aim of this study was to investigate the use of these

indigenous LAB and other additives as a blood biopreservative system (BS) against spoilage microbiota, and to determine its impact on blood pH and haemolysis during storage at 30°C.

MATERIALS AND METHODS

Confirmation of the Biopreservation System

Ninety-six different LAB, isolated from avian blood obtained in slaughterhouses, were investigated for their antimicrobial activity and ability to grow in blood (Zbrun *et al.*, 2013). *Enterococcus faecalis* DSPV008SA was selected because it produced a substance similar to bacteriocin. It showed antagonistic activity against *Salmonella brandenburg*, *Salmonella* ssp. SA (isolated from slaughterhouse avian blood) and *Pseudomonas aeruginosa* SA grown in agar plates (Zbrun *et al.*, 2013). *Lactobacillus salivarius* DSPV032SA was selected because in earlier studies (Zbrun, 2014) it demonstrated a high ability to grow in blood. Both *Enterococcus faecalis* DSPV008SA and *Lactobacillus salivarius* DSPV032SA were homofermentative and unable to degrade arginine, which are useful characteristics for the biopreservation of blood. Homofermentative bacteria are incapable to produce CO₂, a gas that produce adverse effects during the production. Arginine is a biogenic amine and could cause harmful health effects.

Thermal stability of both strains was evaluated to ensure the lethality of the microorganisms after high temperature treatment. In a previous study (Zbrun, 2014) it was demonstrated that treatment at 65°C for 7.5 min was lethal for both strains. This result implies that when slaughterhouse blood is processed in the industry by spray drying at > 300°C the strains of BS will not survive. The selected LAB strains were stored at -80°C with 20% glycerol in 2 ml vials (8 log cfu/ml).

In addition, the blood was enriched with the aim of improving LAB growth. In previous studies (Zbrun, 2014) the effect of different concentrations of additives was evaluated individually for each strain. In this way, the concentration of additives that affected

spoilage bacteria and improved growth of the *Enterococcus faecalis* DSPV008SA and *Lactobacillus salivarius* DSPV032SA was selected for using in the BS. The optimum concentration of each additive was determined: 2 g/l bacteriological lactose, 0.4 g/l yeast extract, 0.4 g/l ammonium citrate and 1 g/l sodium chloride.

Antagonistic effects of the Biopreservation System against spoilage bacteria present in slaughterhouse blood

Experimental design: Blood samples were obtained during the slaughter of poultry in industrial abattoirs. They were collected in sterile containers (250 ml) containing glass beads for defibrination as blood was draining from the animals during bleeding in an open collection system. Containers were maintained at chilled temperatures ($<5^{\circ}\text{C}$ on ice) and immediately transported to the laboratory. At the laboratory, blood samples were divided into two, one to be used as untreated control (non-inoculated) and the other treated with the BS. Five replicates of the experiment were performed.

Blood inoculation

Enterococcus faecalis DSPV 008 SA and *L. salivarius* DSPV 032 SA cultures were grown separately to the stationary phase in MRS broth (Oxoid, United Kingdom) for 24 h at 37°C in an anaerobic atmosphere. After centrifugation at 10,000g for 10 min, the supernatant was discarded and the bacterial cells were re-suspended in slaughterhouse blood in order to obtain a concentration around 10^8 cfu/ml for each bacteria. Then, sterile solutions of bacteriological lactose, yeast extract, ammonium citrate and sodium chloride were added to the suspensions described above.

Control blood and blood with BS treatments were maintained at a constant temperature of 30°C for 48 h. During the storage period, the pH of the samples inoculated with the BS was regulated (pH=6.0) at 6, 12, and 24 h by adding a sterile solution of 2M NaOH. Samples of 5 ml were removed from containers at different times (0, 24 and 48 h of

storage), and used for: microbiological counts, haemolysis measurement and pH measurement.

Microbiological measurements

Microbiological populations were counted in the specific media (Zamora, 2003). Samples were serially diluted in sterile peptone water (peptone 2 g and NaCl 7.5 g/l) and were plated in duplicate. In control and inoculated blood indicator bacteria were determined. The *Enterobacteriaceae* species were determined in violet red bile glucose agar (VRBG, Oxoid, United Kingdom) at 37°C for 24 h. Violet red bile lactose agar (VRBL, Oxoid, United Kingdom) was used for coliform enumeration after incubation at 44°C for 24 h. *Pseudomonas* spp. was determined in *Pseudomonas* cetrimide agar (Oxoid, United Kingdom), and *S. aureus* in mannitol salt agar (Oxoid, United Kingdom), both media being incubated at 37°C for 48 h. MRS agar (Oxoid, United Kingdom) was used for LAB enumeration and was incubated anaerobically for 72 h at 37°C.

pH and haemolysis analysis

The pH of control and inoculated blood was monitored with a pH meter (Altronix, U.S.A.) using a 2-ml samples of blood. Haemolysis was determined because it is considered to be a useful indicator of industrial blood quality. Plasma was obtained by centrifuging (2250 g for 15 min at 4°C) and decanting. The increase in haemolysis was determined by measuring the absorbance at 414 nm using a spectrophotometer (Jenway, United Kingdom) with a 1:5 dilution of the plasma samples (Dávila *et al.*, 2006).

Statistical analysis

The variables (microbiological counts, pH and haemolysis) were analysed using ANOVA. Differences between treatment means for each variable at each time were tested for significance using Student's t-test. The level of significance was set at $P < 0.05$ and the normal distribution and the homogeneity of variances were checked. All statistical analyses

were done using the software package InfoStat (Universidad Nacional de Córdoba, Argentina).

RESULTS

The antagonistic effect of the BS on blood

Counts of *Enterobacteriaceae* and coliforms in blood samples at the beginning of the experiment were 5.5 log cfu/ml and 5.2 log cfu/ml, respectively, while the numbers of *Pseudomonas* spp. and *S. aureus* were 2.5 log cfu/ml and 4.5 log cfu/ml, respectively. Table 1 shows the antagonistic capacity of the BS against spoilage bacteria when the blood was stored at 30°C. The poultry blood treated with the BS had the lowest counts of all spoilage microorganisms throughout the study ($P < 0.05$).

Table 1 near here

Populations of *Enterobacteriaceae*, coliforms, and *Pseudomonas* spp. had similar growth patterns. During the first 24 h of storage the BS was able to inhibit the growth of these spoilage microorganisms. The blood without the BS had higher counts (approximately 3 log cfu/ml) than the blood inoculated with the BS ($P < 0.001$). During the last 24 h of storage at 30°C, the microbial levels of all groups showed an increase. In the treated blood the final levels of *Enterobacteriaceae* and coliforms were not significantly different from those in untreated blood. However, the blood inoculated with the BS showed significantly lower ($P < 0.05$) counts of *Pseudomonas* spp. even after 48 h of storage at 30°C.

The bacterial growth of *S. aureus* followed a different pattern (Table 1). During the first 24 h of storage counts increased approximately 1.5 log cfu/ml in untreated blood. In contrast, the counts of *S. aureus* showed a reduction of 2.3 log cfu/ml at this time in the BS-treated blood. During the last 24 h of storage at 30°C, the levels of these bacteria were similar in both blood treatments ($P = 0.10$).

Regarding the LAB counts, the initial value in untreated blood was 4.99 log cfu/ml, increasing 3.0 log cfu/ml after 24 h storage at 30 °C. This indicates that the blood had native LAB with the ability to grow in the slaughterhouse blood. However, the untreated blood never

reached the LAB count observed in treated blood. This growth of native LAB may be beneficial for preserving blood, but seems not to be effective enough to extend the life of the blood at the slaughterhouse. Furthermore, the treated blood LAB count remained stable throughout the storage period and was higher than untreated blood count ($P < 0.001$). Furthermore, these results indicate that almost all LAB present in the treated blood belonged to the BS. The presence of BS LAB might be beneficial to the blood preservation because these bacteria were studied and previously characterised for this purpose.

Analysis of pH and haemolysis

All through the experiment, the pH was different in the two treatments ($P < 0.001$). The blood treated with the BS had a lower pH level (6.0) at the beginning of the experiment than blood without BS, decreasing to 4.6 after 24 h of incubation at 30°C. At the end of the experiment, the BS-treated blood showed an increase, reaching a value of 6.7. It is important to mention that the pH was adjusted twice (at 12 h and 24 h of storage) to prevent protein coagulation. On the other hand, the untreated blood had a pH of 7.6 at the beginning of the experiment, which decreased by 0.7 after 24 h incubation, reaching a final value of 7.2).

Table 2 near here

Regarding the level of haemolysis, during storage at 30°C the blood stored without the BS exhibited red blood cell rupture with a consequent increase in absorbance (Table 3). By contrast, the blood with BS treatment maintained the same absorbance values throughout storage. At the beginning of the storage period, both blood treatments had similar absorbance values ($P = 0.413$) but after 24 h and 48 h, the absorbance of untreated blood increased three-fold from the initial value ($P = 0.057$ for 24 h and $P = 0.037$ for 48 h) (Table 3).

Table 3 near here

DISCUSSION

The ability of LAB to inhibit or at least counteract the negative effects of food spoilage bacteria has been much studied (Holzapfel *et al.*, 1995; Ross *et al.*, 2002), but not evaluated using slaughterhouse by-products. In this study the development of a novel biopreservative system was investigated. This system included two LAB and 4 additives and aimed to preserve poultry blood obtained from slaughterhouses and stored at 30°C (simulating a high environmental temperature).

The results suggest that the BS was able to prevent the growth of spoilage bacteria during the first 24 h of storage at 30°C. Also, the level of haemolysis in blood with the BS remained unchanged during 48 h, indicating that the BS prevented the rupture of red blood cells.

One of the possible mechanisms that could explain the biopreservative effect is the lactic acid production by LAB. The homolactic fermentation by LAB produces lactic acid almost exclusively. This lowers pH and it has bacteriostatic action which inhibits most pathogenic bacteria (Schillinger and Lucke, 1990). In this way, the decrease in intracellular pH alters the membrane structure and leads to a decrease in the activity of several enzymes that are pH-sensitive (Shelef, 1994; Cotter and Hill, 2003; Signorini *et al.*, 2006).

However, the control of the pH level in the blood during storage is extremely important because it is necessary to maintain the inhibitory action on spoilage bacteria but not too low to generate coagulation of the proteins. In this way, the pH regulation at 12 h allowed optimum LAB growth without affecting protein coagulation.

Another mechanism associated with LAB as biopreservative agents is related to the growth rates and the competitiveness for nutrients, which produces a selective advantage over slower growing competitors (Bredholt *et al.*, 1999). Also, the inhibition of spoilage bacteria by LAB may be due to numerous mechanisms such as the production of hydrogen peroxide or

other antimicrobial substances such as bacteriocins (Schillinger and Lucke, 1990; Daeschel, 1992; Skytta *et al.*, 1993; Vandenberg, 1993).

Also, the biopreservative properties could be associated with the addition of other inhibitory substances such as sodium chloride and ammonium citrate. Sodium chloride is one of the oldest and most common agents used for food preservation. The salt concentration in fermented products inhibits the growth of many undesirable microorganisms and promotes the growth of more salt tolerant lactic acid bacteria (Kołozyn-Krajewskaa and Dolatowski, 2012). The potential of LAB to tolerate salt is one of the challenges for the fermentation process. In this way, LAB of BS have been tested against different concentrations of ammonium citrate and sodium chloride previously (Zbrun, 2014). Furthermore, the results of several studies show that the viability of bacteria in a fermented product matrix is strain-dependent (Cogan *et al.*, 2007; Santivarangkna *et al.*, 2008). In the same way, ammonium citrate has a recognised capacity for inhibiting gram-negative bacteria and is used in several culture media. For that reason, this substance could be related to the limited growth of spoilage bacteria.

All of the possible inhibitory mechanisms mentioned above could directly affect the spoilage bacteria being studied here. However, *S. aureus* has special characteristics as it is resistant in low pH and halotolerant media. Studies showed a direct correlation between medium acidification and the level of *S. aureus* inhibition (impact on growth rate or survival) (Barber and Deibel, 1972; Metaxopoulos *et al.*, 1981), although this inhibitory effect can be modified according to media composition and totally abolished if culture conditions are changed (Daly *et al.*, 1972). On the whole, in the current study, the data showed that LAB activity can have different impacts on *S. aureus* growth depending on the media considered. The effect of inoculation with LAB on blood haemolysis was also evaluated. The haemoglobin content may be responsible for off-flavours and discolouration of slaughterhouse blood (Lynn Knipe, 1988). The haemoglobin content in the plasma fraction increased in blood without BS, while in samples inoculated with BS the haemolysis value

remained constant. Therefore, no loss in this parameter of plasma quality could be observed in the blood with the BS. The results agree with those at refrigeration temperatures reported by Dávila *et al.* (2006).

On the basis of these results, supplementing blood with a BS to avoid undesirable changes during storage prior to processing is recommended that. The BS provides a useful protective system even under an ambient temperature of 30°C. The application of a biopreservation system to blood could reduce the microbiological load, extending its shelf life, and thus avoiding immediate processing after blood collection.

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Table 1. Effect of Biopreservation System (BS) on the growth of *Enterobacteriaceae*, coliforms, *Pseudomonas spp.*, *Staphylococcus aureus* and lactic acid bacteria in avian blood stored at 30°C for 48 h.

Bacteria group	Treatment	Mean (SD) bacteria count (log ufc/ml)			Significance ¹ , $P <$
		Time 0 h	Time 24 h	Time 48 h	
Enterobacteriaceae	No BS	5.62 (0.03) ^a	8.36 (0.19) ^a	8.34 (0.04) ^a	0.001
	With BS	5.45 (0.59) ^a	5.70 (0.51) ^b	8.91 (0.59) ^a	
Coliforms	No BS	5.52 (0.39) ^a	8.16 (0.34) ^a	8.23 (0.27) ^a	0.001
	With BS	5.06 (0.38) ^b	5.66 (1.04) ^b	8.78 (0.55) ^a	
<i>Pseudomonas spp.</i>	No BS	2.48 (0.36) ^a	6.65 (0.63) ^a	6.28 (0.61) ^a	0.001
	With BS	2.61 (0.41) ^a	2.24 (0.35) ^b	5.83 (0.18) ^b	
<i>Staphylococcus aureus</i>	No BS	4.92 (1.04) ^a	6.41 (0.53) ^a	5.64 (0.77) ^a	0.001
	Eith BS	4.23 (0.15) ^a	1.96 (0.19) ^b	6.52 (1.77) ^a	
Lactic acid bacteria	No BS	4.99 (0.06) ^a	7.93 (0.22) ^a	7.76 (0.09) ^a	0.001
	With BS	8.98 (0.07) ^b	8.79 (0.44) ^b	8.78 (0.40) ^b	

^{a, b} Means within groups with no common superscript letter are significantly ($P < 0.05$).

¹ Significance value of repeated measures (ANOVA).

Table 2. Mean (SD) effect of Biopreservation System (BS) on the pH in avian blood stored at 30°C for 48 h

Treatments	Time 0 h	Time 24 h	Time 48 h	Significance ¹ , $P < ^1$
No BS	7.58 (0.04) ^a	6.88 (0.33) ^a	7.07 (0.13) ^a	0.001
With BS	6.02 (0.18) ^b	4.58 (0.26) ^b	6.67 (0.15) ^b	

^{a, b} Means within groups with no common superscript letter are significantly ($P < 0.05$).

¹ Significance value of repeated measures (ANOVA)

Table 3. Mean (SD) effect of Biopreservation System (BS) on haemolysis in avian blood stored at 30°C for 48 h

Treatments	Haemolysis (Abs 414nm)			Significance ¹ , $P <$
	Time 0 h	Time 24 h	Time 48 h	
No BS	0.23 (0.04) ^a	0.60 (0.27) ^a	0.66 (0.33) ^a	0.001
With BS	0.21 (0.02) ^a	0.26 (0.21) ^b	0.23 (0.19) ^b	

^{a, b} Means within groups with no common superscript letter are significantly ($P < 0.05$).

¹ Significance value of repeated measures (ANOVA)

SD: standard deviation.