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POTENTIAL OF BACTERIOCIN-LIKE SUBSTANCES PRODUCED BY *Lactobacillus plantarum* UTNCys5-4 TO INHIBIT FOOD PATHOGENS IN RAW MEAT

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Abstract

In Ecuador the mild climate, the inappropriate storage condition and food manipulation are suitable environments for spoilage/pathogens growth, therefore, to satisfy with the consumer demands on food quality, searching for novel natural preservative is of interest. Lactic acid bacteria are very attractive to be exploited from the biotechnological point of view. In this research, the effectiveness of bacteriocin-like substances produced by Lactobacillus plantarum UTNCys5-4 (Genbank No KY041686.1) isolated from native wild-type tropical fruits to control the spoilage bacterial growth in raw meat was evaluated. The microbiological analysis indicated that the beef meat filets purchased from local market exhibit contamination. A 2.3-fold reduction of total coliforms viability was registered when treated the raw meat with crude-extract containing Cys5-4 bacteriocin up to day 12 of storage with refrigeration. A significant change of meat pH was observed in the non-bacteriocin treated samples along with the increase in the concentration of released ammonia indicating the degradation of protein. A visible change in color of meat was observed only in the samples treated with nitrite. The study revealed the high potential of bacteriocin Cys5-4 to inhibit the growth of spoilage bacteria, indicating its promising approach to be use in preservation.

Key words*: bacteriocin, spoilage bacteria, preservation.*

INTRODUCTION

To guaranty the food safety and security of the products, the proliferation of the spoilage and bacterial strains should be controlled. During the last two decades the research have been centered on identification of new natural antimicrobial compounds to replace the food chemicals for the purpose of conservation (Deegan et al., 2006; Arena et al., 2016). Biopreservation is a robust and natural tool to extend shelf life and to enhance the safety of foods by application of naturally occurring microorganisms or their antimicrobial compounds (i.e. bacteriocins). For many years, lactic acid bacteria (LAB) attracted significant attention for food industry due to their GRAS status (Generally Considered as Safe) (Biscola et al., 2013). Bacteriocins, known as antimicrobial compounds produced at ribosomal level, are not considered antibiotics and does not produce illicit allergic reaction in humans or animals, therefore are investigated for their potential as natural preservatives in food. Despite of many LAB bacteriocin producers, nisin remains the only component commercialized as food additive (Delves-Broughton, 2005; Gálvez et al., 2007; Hartmann et al., 2011; Nath et al., 2014). The potential *ex vitro* of some bacteriocin was reported (Fiorentini et al., 2001; Banerjee et al., 2013; Fangio and Fritz, 2014; Zamfir et al., 2014). Ecuador is known for its biodiversity in either plants, birds and animals with regions across the mountains from land to the ocean where the climate is very changeable, and the temperature varies with the altitude. Among undeveloped natural areas, Amazon was included in the governmental policy as important biological resources to be investigated. Globally, the metropolitan life grew and eating outside is becoming habitual.

In this context, Ecuador is not an exception since a substantial proportion of the ready-to-eat food is sold on the streets. Despite the growth of alimentary sector and low regulation for food consumer, there is no effective improvement on the food manipulation or

hygienic control. Most of artisanal minimally processed foods, typical dishes (i.e. mote), natural fruit or cereals fermented drinks (i.e. chicha morada), meat and fish, appears to contain a significant number of spoilage bacteria. This must be related with the inappropriate manipulation and storage, inadequate cooking, contaminated equipment or poor personal hygiene, therefore the risk of developing diseases is elevated. In spite of, considerable human illness related to food contaminants have been reported by the Ministry of Public Health (2014). Consequently, an attention was assumed to increase the consumer protection by preventing contamination, improving communication about safety by facilitating relevant research on food preservation. In this context our research proposed the exploration of the native wild-type microbiota of tropical fruits to identify newly antimicrobial substances (Benavidez et al., 2016; Tenea and Yepez, 2016). It is believed that the microorganisms from this region might provide a newly source of functional compounds to be examined. Thus, a large-scale study selection of native bacteriocinogenic LAB strains of native fruits was early reported (Garzon et al., 2017). Among several strains showing highly antimicrobial potential towards food pathogens founded often in artisanal products the *Lactobacillus plantarum* UTNCys5-4 demonstrated elevated antimicrobial potential *in vitro* against several pathogenic bacteria. Recently, we showed that this bacteriocin exerted activity *ex vitro* in artisanal drinks controlling the pathogen growth overtime in combination with refrigeration (Tenea and Barrigas, accepted manuscript). In this study, the effect of the crude-extract containing bacteriocin Cys5-4 was evaluated in beef raw meat to monitor and control the pathogen growth as overall good manufacturing practice to ensure an adequate safety and quality of meat-based food products.

MATERIALS AND METHODS

Bacterial strains and crude-extract preparation

The *L. plantarum* UTNCys5-4 (Genbank No. KY041686.1) isolated from tropical wild-type fruits of *Malus* sp. (Sucumbíos Provence) was used. To prepare the crude-extract (CE), the bacteria was grown in broth MRS (Merck) at 32°C for 24 hours and the supernatant was collected by centrifugation at 13,000 x *g* for 20 minutes, 4°C, following by the filtration using 0.22µm porosity syringe filter and storage with refrigeration before use. As standard CE from *Lactobacillus plantarum* ATCC8014 (LP) was used.

Meat microbiological evaluation

The meat samples consisting of bovine muscle were purchased from an ambulatory local vender and microbiologically analyzed in concordance with the Ecuadorian Normative, NTE INEN 1529-15 and 1529-8. Briefly, 5g meat were inoculated in peptone (1%), homogenized and incubated for 24 hours at 37°C. Moreover, decimal dilutions made on sterile water were plated on Plate Count Agar (Difco) to determine the growth of mesophilic aerobic and psychrotrophic aerobic bacteria (35 ± 0.5°C, 48 hours); moreover, aliquots were plated on SS (*Shigella-Salmonella*, Difco), incubated for 48 hours at 37-40°C to determine the presence of *Salmonella* and *Shigella*; moreover aliquots were placed on chromocult agar (Merck) to determine the total coliforms and eosin methylene blue (Difco), to determine the presence of *E. coli*; furthermore DRBC agar plates (Difco), for the enumeration of yeasts and molds (incubation at 25°C for 7 days) were used.

Effect of Cys5-4 crude-extract on raw meat

The meat filets (75g / each treatment) were divided for different assays: a) treatment with CE of Cys5-4 at the final concentration of 18 AU/g; b) treatment with the same concentration of CE of LP. c) treatment with nitrite at 200 ppm/kg according with the INEN Normative for meat preservation (1338. 2012); d) meat control (untreated). Briefly, the samples were immersed for 10 min in individual sterile trays containing the crude-extract of each bacteria, as described above and nitrite and maintained for 10 min to dry under the laminar bench to avoid any cross contamination. The experiments were performed in triplicate starting with different batch of meat and kept in refrigeration for 12 days in polystyrene food delivery boxes wrapped with sterile plastic bag (Ziploc).

pH determination

Determination of pH at different intervals of 0, 1, 3, 6, 9 and 12 days was performed. Five g of meat sample was homogenized with 50 mL distilled water cooled at 25°C.

The mixture was stirred for 30 minutes and decanted. The pH value was measured in the supernatant, using a pH meter (RoSH, Balance Instrument Co., Ltd).

Microbiological evaluation

The meat filets treated with crude-extract of Cys5-4, LP, nitrite and control (untreated) were microbiologically analysed at different intervals of time (0, 1, 3, 6, 9 and 12 days) using agar plate assay method to determine the number of total viable cell counts (Pratush et al., 2012).

Determination of ammonia

Determination of ammonia in filtrate samples of meat was performed with the addition of Nessler reagent (0.09 mol/L solution of potassium tetraiodomercurate (II) in 2.5 mol/L potassium hydroxide and EDTA (20 mM).

The addition of the Nessler reagent will produce a yellow to brown color dependent on the concentration of ammonia found in the sample.

By monitoring the color change the concentration of released ammonia can be determined by spectrophotometry at the wavelength of 400-450 nm.

The EDTA solution was used to avoid precipitation. Briefly, 5g from each meat sample treated with crude-extract, nitrite and control, was placed in the beakers, treated with solution of NaOH 6N for 15 minutes, aliquots of 300 µl were transferred in the 10 ml balloons and 100µl of EDTA was added.

Moreover, the samples were treated with 100µl of the Nessler reagent for 10 minutes followed by calibration with sterile distilled water, and immediately determine the absorbance at 450 nm using the spectrophotometer (Nova60, Millipore, Merck) with previously determined ammonia standard curve (90-200 ppm).

A value greater than 120 ppm being associated with spoilage contamination (Hijaz et al., 2007)**.** To determine the minimum value an uncontaminated meat muscle was used.

RESULTS AND DISCUSSIONS

The contamination of ready-to-eat and fresh food products with pathogenic/ spoilage microorganisms and its persistence, growth, multiplication and/ or toxin production has emerged as an important public health concern. Food-borne illness is a major international problem and the worthy cause of the reduced economic growth. Thus, several synthetic additives to preserve food or enhance their organoleptic characteristics are used (Castellano et al., 2017). To assure the quality and safety of the food several natural methods were proposed including the use of bacteriocin secreted by lactic acid bacteria (Arena et al., 2016). In this study, the effect of crude-bacteriocin produced by the native strain *L. plantarum* Cys5-4 was evaluated in raw beef meat. Although the meat muscle is a sterile microenvironment (Mach et al., 2008) the microbiological analysis indicated that the purchased meat does not comply with the quality standards as *Salmonella*/ *Shigella* and *E. coli* were identified (Table 1). The thee meat batches purchased at two weeks intervals showed the presence of a larger number of coliforms (9.57-9.82 log CFU/g) which, according with the legislation are considered no acceptable for consuming. Nonetheless, the meat is sold out without any restriction.

Table 1. Presence of contamination in meat

Meat Samples	Coliforms (log CFU/g)	*Salmonella/ Shigella*	*E. coli*	Yeasts/ molds	pH
Batch # 1	9.57	+/+	+	-/ -	5.83
Batch # 2	9.80	+/+	+	+/+	6.10
Batch # 3	9.82	-/+	+	+/-	6.15

We suggested that a possible contamination pathway might be after the animal slaughtering. Is possible that due to the inappropriate manipulation or storage the surface of meat might exposed at the spoilage bacteria. The molds were presented in batch #2, while yeasts were presented in batch #2 and #3. The presence of spoilage/ pathogenic bacteria in meat juices for example, is a question of cross contamination of other food products. Bacteria also can be founded on equipment, hands, and the air. The Imbabura Province as other regions of the country has the established inspection

programs applicable for meat produced and sold in the market, but seems they are susceptible to application for the small local venders. However, the state inspection program must enforce requirements for all producers. For the safety concerns and concordance with the normative, no *Salmonella* / *Shigella* are accepted in food products. The analysis of the three meat batches showed the contrary (Table 1). Food may act as a vector for the transfer of antimicrobial resistant bacteria and antimicrobial resistance genes to humans. When examined the antibiotic resistance of the pathogens found in meat using diffusion agar assay with antibiotic dicks, a tetracycline, ampicillin and cefuroxime resistance was detected for *E. coli* and *Shigella* (data not shown). Moreover, to evaluate the effect of the incorporation of bioactive substances to control the pathogens growth in fresh meat, independent samples immersed in solution containing CE-Cys5-4, CE-LP, nitrite stored for 12 days under refrigeration were microbiologically analyzed at different intervals of incubation time. In control samples, the total population of mesophilic aerobic bacteria were slightly reduced overtime due to the refrigeration. The meat samples treated with CE-Cys5-4 showed about 2.3 log CFU/g decrease in each individual batch of treated meat, indicating an inhibitory action associated to the presence of antimicrobial substances contained in the CE (Table 2).

Table 2. Viability of spoilage bacteria in meat

Incubation time (day)	Viability (log CFU/g)			
	Control	Cys5-4	LP	Nitrite
0	9.57	9.57	9.57	9.57
1	9.60	9.57	9.54	9.58
3	8.53	6.46	6.40	6.34
6	7.25	5.84	5.46	5.74
9	6.76	4.47	5.90	5.39
12	6.99	5.90	6.14	6.13

Control: untreated meat; Cys5-4: meat + crude-extract Cys5-4; meat + crude-extract LP; meat+ nitrite

The overall reduction of the cell counts was observed in the samples treated with LP up to day 6 (5.46 log CFU/ml), while at the day 9 a slightly increase was registered, indicating that the crude extract of LP was no longer active at that point. A reduction in cell counts was observed in the samples treated with nitrite, but the meat showed a browned color at day 6 compared with the meat samples treated with CE-Cys5-4 or CE-LP maintaining the same color as purchasing. Figure 1 displayed the cell growth in plates after 9 days of refrigeration with visible decrease in the treatments. Previous research showed the potential of cell free supernatant containing a bacteriocin produced by *Bacillus cereus* P9 in beef meat (Fangio and Fritz, 2014). A similar reduction in the total populations of mesophilic bacteria, when meat samples are packed with materials containing bacteriocins was observed (Dawson et al., 2005). At Day 12 the total cell counts increases indicating that activity of the active component might be inactivated.

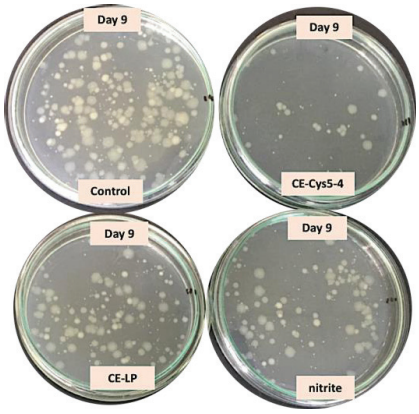

Figure 1. The presence of viable cells in meat treated or no with bacteriocins at day 9 of storage. CE: crude-extract of Cys5-4 and LP; nitrite: meat with nitrite

Other research using nisin adsorbed on the cellophane for packaging the meat, indicated that the final level of total bacterial count was considerably reduced after 12 days of incubation that the total count of bacteria in the initial level (Ercolini et al., 2010). The pH may affect the color, tenderness and quality (Jelenikova et al., 2008; Luning et al., 2011). In this research, the initial pH values for each batch seems to be acceptable, but an increase was recorded with the incubation time in the non-treated samples. If at Day 0 the values the pH varied from 5.83 to 6.15, at the Day 12 increased up to 6.44 log CFU/g indicated that the presence of contaminant induced protein degradation which possible determine the release of amino acids and alkaline compounds as ammonia. Table 3 showed the overall variation of pH in meat with and without treatment.

Table 3. Changes of pH in beef meat batch #1 during refrigeration with and without bacteriocin

pH	Day 1	Day 3	Day 6	Day 9	Day 12
Control	5.83	5.83	5.95	6.02	6.44
LP	5.72	5.77	5.80	5.92	6.00
Cys5-4	5.69	5.77	5.80	5.81	5.93
Nitrite	5.79	5.8	5.89	6.10	6.24

Control: untreated meat; Cys5-4: meat + crude-extract Cys5-4; meat + crude-extract LP; meat+ nitrite

In the samples treated with crude-extract of Cys5-4 the pH was maintained from 5.69 to 5.93 indicating that the addition of bacteriocin might protect for the proliferation of spoilage bacteria. This data correlated well with the reduction of the total coliforms in meat batch inoculated with bacteriocin. The ammonia concentration varied overtime indicating that a degradation of meat occurred due to the contamination (Figure 2). In the sample treated with bacteriocin was lower that its control counterpart. For example, in batch #3 the control overhead 170 ppm. Previous study indicated that the ammonia background levels varied little between the different meat muscles as well as varied with the aging and the increase of contamination (Pivarnik et al., 2001).

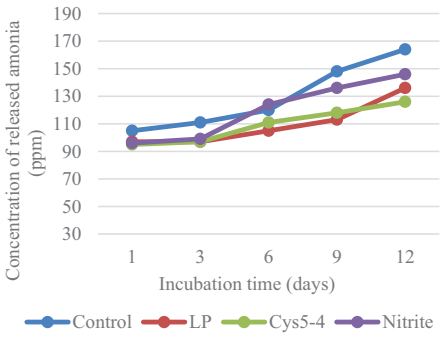

Figure 2. Variation of ammonia released in contaminated meat overtime. Control: meat untreated; LP, Cys5-4: meat + Cys5-4; LP meat with CE-LP, Nitrite: meat treated with 200 ppm/kg nitrite

These backgrounds levels of ammonia are important as FDA recommends analysis of similar non-ammonia-exposed foods to determine the normal background levels. The product can be released if the ammonia content does not exceed the normal value corresponding to the meat type used or origin (Hijaz et al., 2007). In case of beef filets the minimum value are about 90 ppm. In the present study the minimum ammonia value determined in the meat sample free of contamination varied from 86 to 90 ppm. Contrary with other studies where the effect of certain bacteriocins was monitored in food artificial added contaminants (Amin, 2012; Fango and Fritz, 2014) in the present investigation all batches of meat purchased from the local vender were founded contaminated, however the registered reduction of cell counts was of already existing pathogens being significant when bacteriocin added compared with nitrite.

CONCLUSIONS

Taken together, the results indicated the potential of crude-extract containing bacteriocin Cys5-4 to control the growth of pathogens in contaminated beef meat. The reduction of pathogenic microorganisms on meat filets treated with bacteriocin Cys5-4 in combination with refrigeration is a promising approach for maintaining the product safety. Thus, its application as natural preservative in meat as part of overall good manufacturing practice program might protect the meat for further contamination during manipulation, transportation, storage and released product for the consumer.

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