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Potential use of a bacteriocin-like substance in meat and vegetable food biopreservation

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Abstract

Bacillus spp. produces a large number of antimicrobial peptides and includes a variety of species of industrial importance. The aim of the present study was to evaluate the shelf-life extension of fresh beef and lettuce by applying crude extract of a bacteriocin-like substance (CBLS) produced by a native strain of *Bacillus cereus* P9. Food samples were sprayed with the CBLS and stored at 4ºC. Microbiological analyses (count of mesophilic and psychrotrophic aerobic bacteria, total coliforms and molds and yeasts) and pH determination were performed on samples after 0, 3, 6, 9 and 12 days. In addition, the effect of the CBLS on the growth of a pathogenic bacteria added to the food was evaluated. A significant reduction of mesophilic and psychrotrophic aerobic bacteria counts were found in meat treated with crude bacteriocin extract. In adition, lettuce samples inoculated with *B. cereus* and treated with the CBLS, presented a reduction of this microorganism compared to control after 4 h of storage.

Introduction

The proliferation of pathogen and spoilage bacteria should be controlled to guarantee the microbial safety of foods. A combination of factors can be an efficient way to warranty food safety keeping their organoleptic and functional properties (Antolinos *et al*., 2011). During the last few decades, investigation on food preservation has focused on meeting consumer demands for more natural and healthier food (Caminiti *et al*., 2011). This perception, joined with the increasing demand for minimally processed foods with long shelflife and convenience, has stimulated research interest in finding natural and effective preservatives (Chen, 2003). The use of microorganisms and their natural products for the preservation of foods (biopreservation) has been a common practice in the history of mankind (Gálvez, 2007). Biopreservation refers to the extension of the shelf-life and improvement of the safety of foods using microorganisms and/or their metabolites (Settanni and Corsetti, 2008). Bacteriocins may be considered natural preservatives or biopreservatives that fulfill these requirements, since it is assumed that they are degraded by the proteases in gastrointestinal tract (Cleveland *et al*., 2001). Foods can be supplemented with *ex situ* produced bacteriocin preparations, or by inoculation with the bacteriocin-producing strain under conditions that favour production of the bacteriocin in situ (Galvez, 2007). Bacteriocin potential for enhancing food safety and prolonging the shelf-life of final products is routinely investigated and is still under study (Settanni and Corsetti, 2008). In a previous paper (Fangio *et al*., 2010) we reported the isolation of bacteriocinogenic-like strains isolated from Argentinean foods. In this study, the application of a crude bacteriocin sustance as agent of food biopreservation is analysed in the context of foods of animal and vegetable origin.

Materials and Methods

Preparation of crude extracts

The bacteriocin-like producing strain *B. cereus* P9 was grown in brain-heart infusion (BHI) at 37ºC for 24 h. After incubation, the bacterial cells were removed by centrifugation at 10000 g for 15 min. The supernatant was collected and filtered with membrane 0.22 µm (Titan syringe filters; Sri Scientific Resources Inc., USA). This supernatant was designated as crude bacteriocin-like substance (CBLS) (Xie *et al*., 2009)

Antimicrobial activity of the bacteriocin-like substance against food microflora

Food samples treatment

Food samples were prepared from different sources (bovine muscle and lettuce *Lactuca sativa* L.) and sprayed with the CBLS in a final concentration of 3.3 arbitrary units (AU)/g. Each sample was placed in a sterile plastic bag and kept under refrigeration (4 ± 1°C). Each treatment was made in duplicate.

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Microbiological counts and pH determinations of the samples were performed at different time intervals (0, 3, 6, 9 and 12 days).

Microbiological evaluation

On days 0, 3, 6, 9 and 12, samples were homogenized in phosphate buffer pH 7, at a 1:10 ratio. Furthermore decimal dilutions were made using the same diluent. An aliquot of each dilution was plated on Plate Count Agar (Britania, Buenos Aires) for determining the growth of mesophilic aerobic bacteria (35 ± 0.5°C, 2 days) and psychrotrophic aerobic bacteria (20 ± 2°C, 3-5 days), Violet Red Bile Agar (Britania, Buenos Aires) for the determination of total coliforms and HyL media (Britania, Buenos Aires) to determine the growth of molds and yeasts (25°C, 7 days).

Determination of pH

On days 0, 3, 6, 9 and 12, all samples were subjected to determinations of pH. Five g of sample was homogenized with 50 mL of boiled 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 pHmeter HI 9321 (HANNA, Buenos Aires).

Antimicrobial activity of bacteriocin-like substance against pathogenic bacteria inoculated to food

Indicator bacteria suspension

To prepare the bacterial suspension, indicator strain B. cereus was seeded in nutrient agar and incubated 24 h at 35 ± 0.5°C. Colonies of *B. cereus* were added under aseptic conditions in physiological solution contained in test tubes. The suspension was homogenized with a vortex Fbr (Decalab SRL, Argentina) until reach the turbidity corresponding to tube 0.5 of McFarland and diluted to a concentration of 10^5 CFU/mL.

Inoculation of the samples

Food samples from different sources (bovine muscle and lettuce *Lactuca sativa* L.) were prepared and sprayed with the CBLS in a final concentration of 3.3 AU/g. The suspension of the indicator strain was then added in the same way to achieve an initial count of 10^2 - 10^3 CFU/g. Each sample was placed in a sterile plastic bag and kept under refrigeration (4 ± 1°C).

Microbiological evaluation

Samples were taken for determination of *B. cereus* count, at different time (0, 3, 6, 9 and 12 days for meat and 0, 2, 4, 6, 8, 24 and 48 h for lettuce). Ten g of sample was homogenized for 3-5 min in a Stomacher 400 Circulator Homogenizer with phosphate buffer pH 7, at a 1:10 ratio. Furthermore decimal dilutions were made using the same diluent. An aliquot of each dilution was plated on agar-mannitol egg yolk-polymyxin (MYP) for determining the growth of *B. cereus.*

Statistical analysis

The antimicrobial activity of strains in the different treatments were compared using analysis of variance (ANOVA) with Tukey's test using SPSS 15.0 (SPSS Inc., Chicago, Illinois, USA) for Windows.

Result and Discussion

Antimicrobial activity on the natural microflora of chilled meat

In this paper, the microbiological analyses of meat samples without treatment showed a mesophilic aerobic bacteria count within the limits set by the Argentine Food Code for minced meat and ICMFS (1986) for billet meat, both imposes a maximum of 10^7 CFU/g. Mesophilic aerobic bacteria count was also below values reported by Rai *et al.* (2010) (10^5-10^9 CFU/g) and Luning *et al.* (2011) (10^6 to 6.3 x 10^6 CFU/g) in fresh billet meat, indicative of an acceptable quality meat (Soldatou *et al*., 2009).

To assess the consequences of the incorporation of bioactive substances to improve life in fresh beef, the mesophilic aerobic bacteria counts were measured in samples untreated and treated with CBLS that were stored 12 days under refrigeration (Figure 1). These values show that the initial microbiological quality of meat was acceptable, although at the end of storage time, spoilage sings were showed. In control samples, the population of mesophilic aerobic bacteria exhibited only a small increase in the total count of bacteria after the first 3 days of incubation, although after 12 days, the rate of growth of the total bacteria count had increased in 7.9 x 10^2 CFU/g. In addition, in samples treated with the CBLS P9, mesophilic aerobic bacteria count after 6 days of incubation was lower than in the control, indicating an inhibitory action by this antimicrobial extract at that time. However, treated and untreated samples showed no significant ($P > 0.05$) differences after 12 days of storage. Other researchers have also observed a similar reduction in the total populations of mesophilic bacteria, when meat samples are packed with materials containing bacteriocins (Dawson *et al.*, 2005). Guerra *et al.* (2005) studied the effect of nisin adsorbed on cellophane used as packaging of meat, determining that the final level of total bacterial count after 12 days of incubation (approximately

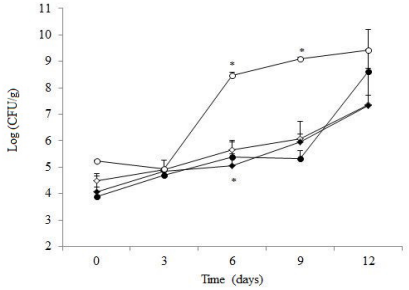

Figure 1. Mesophilic (diamond) and psychrotrophic (circle) aerobic microorganism counts (Log CFU/g) of control (open) and treated (fill) samples of bovine muscle stored at 4ºC. An asterisk indicates statistically significant difference ($p < 0.05$) between counts of control and treated samples at the same time of storage.

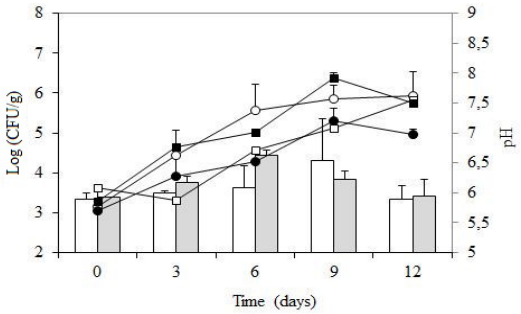

Figure 2. Total coliforms (circle) and yeast and mold (squares) counts (Log CFU/g) and pH (column) of control (open) and treated (fill) samples of bovine muscle stored at 4ºC.

7.9 x 10^3 CFU/g) was significantly lower ($p < 0.05$) that the total count of bacteria in the initial level, revealing that the bioactive substances provided good protection against bacterial growth. Ercolini *et al.* (2010) also studied the effect of nisin adsorbed on meat packages, reporting that total bacteria count was not affected by the use of antimicrobial film in the first 5 days, although after 22 days and until the end of time storage, they remained 2 log units lower than control values.

Furthermore, the results of psychrotrophic microorganisms showed that meat treated with the CBLS, reached level of spoilage between 9 and 12 days, while for the control these values were obtained between 3 and 6 days of storage (Figure 1). García *et al.* (1995) reported that levels of 10^7 CFU/g indicate the presence of bad odor and values of 10^8 CFU/g reveal the development of sliminess on the surface of meat.

In adittion, the count of these microorganisms in the treated meat was significant lower ($p < 0.05$) than those measured in the control at sixth and ninth day of storage. Fiorentini *et al.* (2001) and Vázquez *et al.* (2009) reported a reduction on mesophilic and psychrotrophic flora from treated meat with crude bacteriocin substances during 12 days of storage.

In this research we determined an initial count of 1.2 x 10^3 CFU/g of total coliforms (Figure 2), considered as hygiene indicators (Zeitoun *et al.*, 1994). Castellano *et al.* (2011) reported values close to 10^3 CFU/g. Monitoring the growth of these microorganisms in the control showed a significant increase of 5 x 10^2 CFU/g at 9 days of storage, higher that the increase of about 10^1 CFU/g reported by Castellano *et al.* (2011) for meat stored for 9 days.

The initial count of molds and yeasts was 3.9 x 10^3 CFU/g (Figure 2), which was down below the values reported by Luning *et al.* (2011) who indicated counts of mold and yeast of 6.3 x 10^6 CFU/g, while Rai *et al.* (2010) established ranges of yeast counting of 10^4 to 10^6 CFU/g and filamentous molds of 10^{13} CFU/g. However, there was not a significant reduction ($P > 0.05$) in growth of total coliforms or molds and yeasts in meat treated with the CBLS. There have been numerous studies on bacteriocin-like substances that have been effective for the control of Gram-negative bacteria added to food (Todorov and Dicks, 2005). In our study "*in vitro*" (Fangio *et al.*, 2010) CBLS did not present activity against the tested Gram-negative bacteria. These results preliminarily indicated that this substance would not inhibit efficiently the growth of Gram-negative bacteria "*in situ*". However, although the addition of the extract do not exert their antagonist action directly on these microbial populations, their introduction into a complex matrix such as food, both in terms of physiochemical and microbiological, may produce an imbalance in the natural flora resulting in an inhibition. Since foods are complex ecosystems in which the different microbial populations interact (through cooperation, competition for nutrients, ammensalism or antagonism, etc.), treatment with antimicrobials such as bacteriocins affect not only the bacteria target, but also other members of the microbial community. Ercolini *et al.* (2010) studied the growth of Enterobacteriaceae in meat packaged in containers with the bacteriocin nisin bioactive, determining that this microbial populations counts for the samples packaged in packaging bioactive held from 1 to about 3 log cycles lower than counts control samples, with a final load of about 10^4 CFU/g after 32 days.

pH variation of chilled meat

Both pH and a_w are the most important physicochemical parameter of food quality. The pH of the meat may affect its color, tenderness and eating quality (Jelenikova *et al.*, 2008). Acceptable meat quality shows a pH below 6 after 24 h of slaughter (Mach *et al.*, 2008). In this research initial pH value was 5.83 (Figure 2). This initial pH is important because the addition of CBLS could

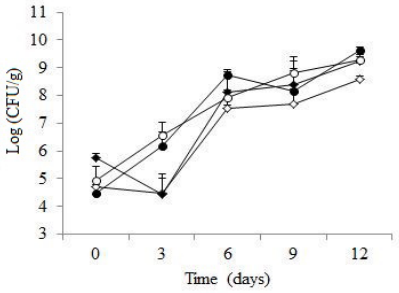

Figure 3. Mesophilic (diamond) and psychrotrophic (circle) aerobic microorganisms counts (Log CFU/g) of control (open) and treated (fill) samples of lettuce stored at 4ºC.

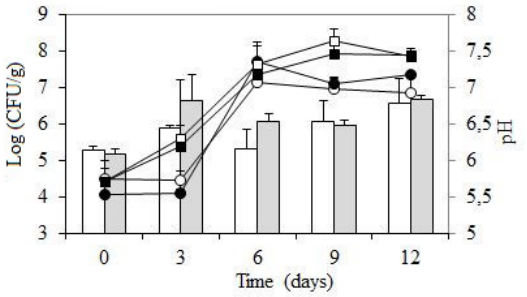

Figure 4. Total coliforms (circle) and yeast and mould (squares) counts (Log CFU/g) and pH (column) of control (open) and treated (fill) samples of lettuce stored at 4ºC.

significantly affect the pH of meat samples from the beginning of the experiment, resulting in a more prolific condition for colonization of microflora. Fiorentini *et al.* (2001) obtained initial pH of meat samples of 6.07, which decreased to 5.81 after adding the cell-free bacteriocinogenic supernatants and remained at 6.08 for the control. In addition, Vázquez *et al.* (2009) obtained an average initial value of 5.74, which after treatment with cell-free bacteriocinogenic supernatants remained at 5.69. After 6 days of storage, it was showed an increase in the pH values, keeping this value until 9 days of storage. Such increase in pH reflects the degree of deterioration of meat through the degradation of proteins with free amino acid production, leading to the formation of alkaline compounds as NH_3 and amines (Vázquez *et al*., 2009). The pH of treated meat did not show significant differences ($P > 0.05$) with control samples.

Growth inhibition of exogenous bacteria added to chilled meat

As discussed above, the effectiveness of bacteriocins *in vitro* systems are not always reflected in food systems, due to the complex factors that influence the growth of bacteria and production of metabolites. In this work we studied the activity of CBLS against a *B. cereus* strain added to meat samples. The results indicated no significant differences ($P > 0.05$) between control and treated samples in the growth of *B. cereus.* This result contrast with the studies "*in vitro*" (Fangio *et al.*, 2010), which showed an antimicrobial activity on *B. cereus* from this CBLS. Although previously it was described an antimicrobial action against some endogenous bacteria of the flesh, the low concentration of the antimicrobial substance in the CBLS, together with a high concentration of the added testing bacteria and the interaction with the matrix substances in food as fat and proteolitic enzymes may cause an increased difficulty to inhibit a particular pathogenic strain.

For example, Vignolo *et al.* (1996) analyzed the effect of Lb. curvatus CRL705 and its bacteriocin (AL705 lactocina) on the growth of a strain of *L. monocytogenes* added to meat samples, concluding that the efficacy of antimicrobial activity depended on both the concentration of the pathogenic bacteria and the concentration of the bacteriocins or producing strain. In this case, inhibition of *L. monocytogenes* on beef was higher when levels of lactocina AL705 were high and initial quantity of cells from the pathogenic strain was low.

Antimicrobial activity on the natural microflora of chilled lettuce

The lettuce is a food that may have a large number of microorganisms, including coliforms (Oliveira *et al.*, 2011), total aerobic bacteria, molds and yeasts, etc. (Abadias *et al.*, 2008; Caponigro *et al.*, 2010), enteric pathogens bacteria such as *Escherichia coli* O157: H7 (Oliveira *et al.*, 2011; Sagong *et al*., 2011) and *Salmonella*. In this study, the initial count of mesophilic aerobic bacteria in the control was 5×10^4 CFU/g (Figure 3) agreed with the results of Caponigro *et al.* (2010), who determined count values of these microorganisms in a range of 10^4 to 5×10^8 CFU/g, and Nguz *et al.* (2005) who reported values of 1.2×10^2 to 5×10^9 CFU/g in mixtures of vegetables. After 12 days of storage, values indicate an increase of 6.3×10^3 CFU/g in control samples. Corbo *et al*. (2006) obtained similar values for monitoring the growth of total mesophilic bacteria on lettuce samples with an initial count of 10^4 – 3.1×10^6 CFU/g and registering values of 10^7 - 10^8 CFU/g after 2 weeks of storage. However, no significant differences ($P > 0.05$) between control and treated samples count were showed.

Similarly, the growth of psychrotrophic aerobic bacteria shows an initial value for the control of 7.9×10^4 CFU/g which increased to almost double after 12 days (Figure 3). Abadias *et al.* (2008) determined less than 10^5 CFU/g of psychrophilic microorganisms (6.5ºC) for 3.4% of the samples. It is known that the low temperature regime is a weak barrier against psychrotrophic pathogens such as *Listeria*

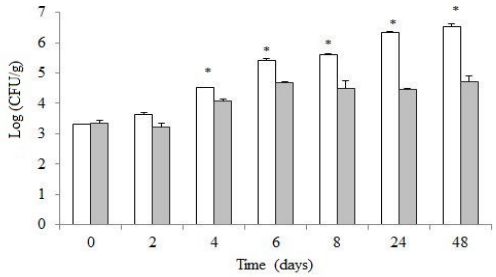

Figure 5. Effect of CBLS P9 on the growth of *B. cereus*. Untreated (white bar) and treated (grey bar) lettuce samples. An asterisk indicates statistically significant difference ($p < 0.05$) between counts of control and treated samples at the same time of storage.

monocytogenes (Sagong *et al*., 2011).

The initial values of coliforms in control samples (2.5×10^4 CFU/g) (Figure 4) agrees with the values of 10^2 to 1.5×10^7 CFU/g and 3.9×10^1 to 7.9×10^6 CFU/g, reported by Caponigro *et al.* (2010) and Nguz *et al.* (2005) respectively. Furthermore Nguz *et al.* (2005) detected values of *E. coli* in the range of 3.9 to 10^3 CFU/g in mixed vegetables. Wießner *et al.* (2009) found values from 7.9×10^4 to 6×10^5 for organic lettuce.

The initial values of molds and yeasts in samples of lettuce were 2.5×10^4 CFU/g (Figure 4). These results coincide with the values detected by Abadias *et al.* (2008) in 75.9% samples of fresh (less than 10^5 CFU/g). These initial values increased in 2.5×10^3 CFU/g at the end of the refrigeration cycle. Neither total coliform or molds and yeast showed a reduction due to the treatment with CBLS. Similar results were obtained by Randazzo *et al.* (2009) who determinated no differences in the growth of mesophilic bacteria, coliforms, *Pseudomonas* spp., lactic acid bacteria (LAB) and molds and yeasts of samples of lettuce treated with a bacteriocinogenic extract an the control. The results reflect the complex influence of food factors on the overall impact of the bacteriocins in food. For example, previous studies have shown that bacteria can be protected from disinfectants applied in washing by the irregularities of the surface of leaves and plant tissue damages (Caponigro *et al*., 2010). This phenomenon coupled with the cooling, could partially explain the lack of efficacy of treatment with antimicrobial substances.

Variation of pH on refrigerated lettuce

The vegetable salad has a large microbial population, particularly bacteria, which can contribute to the natural breakdown of individual vegetative organs of the plant, mainly due to their high surface/weight ratio and a relatively high pH (Caponigro *et al*., 2010). In the present investigation, initial pH value of lettuce was 6.13 (Figure 4), similar to the initial pH of 6.08 reported by Weissinger *et al.* (2000) in samples of lettuce. Such as the results obtain for meat samples, there was not a variation in the pH of the control and treated samples. In addition, lettuce samples showed an increase in pH as storage time passed, coinciding with the increase of the microbial population.

Inhibition of growth of pathogens in chilled lettuce

The effect of antimicrobial peptides on pathogenic bacteria added to samples of lettuce has been reported (Allende *et al*., 2007; Molinos *et al*., 2008; Randazzo *et al*., 2009; Hartmann *et al*., 2011). Unlike what happen with meat, lettuce samples inoculated with B. cereus and treated with the CBLS, presented a reduction of microorganism compared to control after 4 h of storage (Figure 5).

Molinos *et al.* (2008) reported a reduction of viable cell counts $10^1 – 3.1 \times 10^1$ CFU/g of *B. cereus* after washing lettuce samples with solutions of enterocin AS-48. In addition, Molinos *et al.* (2008) determined that the combination of enterocin AS-48 with several other antimicrobials and disinfectants improved considerably the bactericidal effect. Allende *et al.* (2007) obtained similar results when they studied the effect of a mixture of bacteriocins (nisin, plantaricin, lacticin, coagulin and pediocin PA-1) produced by LAB on *L. monocytogenes* added to lettuce, finding a reduction of 1.5×10 to 3.9×10^1 CFU/g of the microbial population. Randazzo *et al.* (2009) studied the effect of commercial nisin and bacteriocinogenic extract obtained from lactic bacteria, determining a reduction of 7.9×10^1 CFU/g of *L. monocytogenes* compared with the control after 7 days of refrigeration. Also Cai *et al.* (1997) obtained a similar reduction (2.5×10^1 CFU/g) of *Listeria* spp. in ready to eat salads treated with nisin.

Conclusion

A crude bacteriocin-like substance was applied to meat and lettuce samples to determinate its preservation action on endogenous microflora and on added *B. cereus.* Treatments results in a reduction on mesophilic and psychrotrophic aerobic bacteria counts of meat samples and a reduction on *B. cereus* counts of lettuce sample. However, the application of biopreservation technology to these products should be integrated as part of an overall good manufacturing practice program. According to the results, CBLS in combination with refrigeration is a promising approach for maintaining product safety.

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