Journal of Microbiology and Antimicrobials Vol. 3(5), pp. 119-125, May 2011
Available online http://www.academicjournals.org/JMA
ISSN 2141-2308 ©2011 Academic Journals

Full Length Research Paper

Purification of a bacteriocin produced by *Enterococcus faecium* and its effectiveness for preservation of fresh-cut lettuce

Bellei B.[1], Miguel M.[2], Mere Del Aguila E. M.[1]*, Silva J. T.[1] and Paschoalin V. M. F.[1]

[1]Universidade Federal do Rio de Janeiro, Instituto de Química, Departamento de Bioquímica, Brazil.
[2]Instituto de Microbiologia Prof. Paulo de Goes, Laboratório de Microbiologia de Alimentos, Rio de Janeiro, RJ, Brazil.

Accepted 30 April, 2011

The bacteriocins produced by *Enterococcus* species (enterocins) show considerable activity against diverse pathogens. One of these antimicrobial peptides was isolated from *Enterococcus faecium* E86 cultured in MRS broth for 24 h at 37°C. The antimicrobial peptide was purified by precipitation in 75% ammonium sulfate, followed by chloroform precipitation and chromatography in Dowex-50, resulting in a specific activity of 52.46 AU/ mg in a final yield of 80% and a 6-fold recovery. The enterocin was purified to homogeneity and had an apparent molecular mass of 3.5 kDa as evaluated by 18% SDS-PAGE. The purified and the partial-purified enterocin were able to inhibit the proliferation of natural microbiota of fresh-cut lettuce as well as that artificially contaminated with *Listeria* spp. After 30 min in the presence of 0.5% acetic acid, the natural microbiota proliferation was reduced by 3 log cycles, while the enterocin in its purified or partially purified form showed an inhibition of the microbiota proliferation by 1 or 2 log cycles, respectively. These results indicate that enterocin could be used as a natural antimicrobial to reduce chemical treatment in minimally processed leafy vegetables.

Key words: Bacterial enzymes, antimicrobial peptide, purification and characterization of enzymes, food safety.

INTRODUCTION

Several studies have investigated the presence of pathogenic microorganisms in salad, in relation to washing treatment and/ or processing steps, which may change bacterial communities in the final products (Shuenzel and Harrison, 2002). Treatment with chlorine-based agents may reduce the bacterial load 100-fold in leafy vegetables (Garcia and Barret, 2002), although with wide variability in decreasing the number of bacteria. With the aim to improve the quality and safety of MPV (minimally processed vegetables) products, and reduce preservatives, the industry is seeking novel and alternative technologies. The use of lactic acid bacteria (LAB) and/ or their natural products for the preservation of foods (biopreservation) appears to be a promising strategy for reducing growth of pathogens, according to the barrier technology strategy (Trias et al., 2008). Bacteriocins are ribosomally-synthesized antibacterial peptides produced by bacteria, and are usually active against genetically closely related species. They have been grouped into four classes, based on their structure and mode of action (Heng et al., 2007).

Several researches have been focused on the production of bacteriocins from members of the LAB group, mainly *Lactobacillus* sp., *Lactococcus* sp., *Leuconostoc* sp., *Enterococcus* sp., and *Pediococcus* sp. (Ghrairi et al., 2008; Millette et al., 2008). The LAB bacteriocins are active against a broad spectrum of food-spoilage and food-borne bacteria. Gram-positive pathogenic bacteria, including *Listeria monocytogenes, Clostridium* sp., and *Bacillus* sp. *L. monocytogenes* can produce a wide variety of human disease ranging from a nonspecific flu-like illness to severe diseases such as sepsis and meningitis (Beuchat, 1996). It is widely

*Corresponding author. E-mail: emda@iq.ufrj.br. Tel: (55 21) 2562-7362. Fax: (55 21) 2562-7266.

distributed in the environment, where it is associated with decaying vegetation, soil, sewage, and animal feces, and has been isolated from several types of vegetables (Marekova et al., 2003).

Several bacteriocins produced by enterococci, especially by *Enterococcus faecium* strains of different origins (rumen contents, sausages, waste, olives) have been described (Aymerich et al., 1996). To date, the following enterocins from *E. faecium* have been characterized: enterocin A (Casaus et al., 1997), enterocin B (Cintas et al., 1997), enterocin P (Cintas et al., 1998), enterocins L50A and L50B (Floriano et al., 1998), enterocin I (identical to enterocin L50A) (Cintas et al., 2000), enterocin Q (Marekova and Laukova, 2002), and enterocin M (a new variant of enterocin P) (Nes et al., 1996). In general, most enterocins belong to the class IIa bacteriocins, which are thermostable, pediocin-like bacteriocins. The use of bacteriocinogenic enterococci to control contamination in food, feed and in the digestive tract of animals has been reported (Audisio et al., 2000; Cotter et al., 2005; Galvez et al., 2007).

The antimicrobial substance E86 produced by *E. faecium* strain E86 was previously characterized (Miguel et al., 2008). These authors observed that their antimicrobial activity can be abolished by proteinase K, chymotrypsin, pronase, and trypsin treatments, and partially destroyed by pepsin and after exposure to alkaline solutions. The residual activity was 100, 50 and 12.5% after heating at 100°C for 30 min and 1 h, and autoclaving at 121°C for 15 min. The antimicrobial substance could be exposed to pH 2 to 9 without any loss of activity. The antimicrobial substance was active against LAB genera, Clostridium, *Listeria innocua,* and *L. monocytogenes* strains.

The aim of the present study was to purify the peptide protein with antimicrobial activity produced by *E. faecium* E86, and to study the effect of the purified bacteriocin in minimally processed lettuce samples artificially inoculated with *L. innocua*.

MATERIALS AND METHODS

Bacterial strains and culture conditions

BHI, MRS and agar were purchased from BD™, Dowex 50w/ H+ exchanger resin was obtained from Sigma- Aldrich ®. Organic solvents and salts from Merck. Coomassie Blue, acrylamide and bisacrylamide were from ge healthcare life sciences. *E. faecium* E86 was isolated from meat pie, and *L. innocua* strain LI005 was kindly provided by Professor Ernesto Hofer (FiOCRUZ, Rio de Janeiro). The strains were maintained in skim milk at -20°C and propagated in MRS (deManet al., 1969) and BHI broths (Brain Heart Infusion) (Fildes, 1920), respectively at 37°C for 24 h.

Susceptibility to antimicrobial activity

Overnight cultures of *E. faecium E86* were spotted onto modified MRS agar containig 0.2% glucose (wt/vol) and incubated for 24 h at 26 and 37°C. After growth, *E. faecium* were killed by chloroform vapors and the surface of the agar plates were covered with 5.0 ml of soft MRS or BHI agar [with 0.75% agar (wt/vol)] inoculated with 10^7 cells of the indicator microorganisms. The plates were checked for zones of inhibition around the colonies after incubation at the appropriate conditions.

Antimicrobial production

To determine the time course of antimicrobial substance production, 10 ml of an overnight culture of *E. faecium* strain E86 was inoculated in 2 L of MRS broth media 24 h at 37°C. At appropriate intervals, the growth of cells was monitored by measuring the turbidity (OD_{600nm}) for biomass determination and the antibacterial activity detection by the agar well-diffusion method, by using *L. innocua* L1005 as the indicator organism and grown culture was centrifugated at 6000 x *g* for 20 min at 4°C and the cell- free supernatant was adjusted to pH 6.5.

Aliquots (50 µl) were applied in slots on agar plates previously inoculated with *L. innocua* strain L1005. The plates were then incubated for 1 h at room temperature in sterile conditions, followed by incubation at 37°C for 24 h. A clear zone of inhibition was recorded as positive.

The activity of the bacteriocin was defined as the reciprocal of the dilution after the last serial dilution giving an inhibition zone, and was expressed as arbitrary units per milliliter (AU/ ml). The titer of the antimicrobial substance solution, in AU/ml, was calculated as (1000/d) D, where D is the dilution factor and d is the dose, the amount of antimicrobial substance solution added on each spot, (Parente et al., 1996).

Purification of antimicrobial substance

E. faecium E86 was grown in 4 L of MRS broth until reaching OD 600 nm of 0.1 (log phase). The cells were harvested by centrifugation at 10,000 x *g* for 20 min and the supernatant was precipitated by ammonium sulfate at 75% saturation and stirred for 1 h. The precipitate was collected by centrifugation at 10,000 x *g* for 20 min, and the resulting pellet was resuspended in distilled water. The resuspended precipitate was dialyzed against 10 volumes of distilled H_2O for 24 h. Chloroform (1:2) was added to the dialyzed precipitate, stirred vigorously, and centrifuged at 9,000 x *g* for 25 min (Burianik and Yousef, 2000).

The pellet was resuspended in 0.1 M Tris-HCl buffer pH 7.0 and centrifuged under the same conditions. The pellet was dried overnight and resuspended in 2 ml of 0.1 M Tris-HCl buffer pH 7.0, with agitation, and held overnight at 7°C to allow particles to hydrate and dissolve. The resuspended pellet was applied onto a Dowex 50W-H^+ exchanger column, equilibrated with 0.02 M Na_2HPO_4 pH 2.0, with a flow rate of 4 ml/ min. Polypeptide chains were eluted in a stepwise mode using: 0.02 M Na_2HPO_4 pH 2.0 (acid fraction), 0.02 M Na_2HPO_4 pH 7.0 (neutral fraction), and 1 N NH_2OH (basic fraction), at the same flow rate (Porfirio et al., 1997). Unless otherwise specified, all purification steps were performed at 4°C. The antimicrobial activity of the cell-free supernatant and the fractions from each purification step was assayed by the agar well-diffusion method, as described earlier. Protein concentration was determined according to Lowry (1951) using bovine serum albumin as standard.

Molecular size approximation

Fractions (2 ml) containing antimicrobial activity were pooled and precipitated with 60% TCA (Jiang et al., 2004), centrifuged at

10,000 x *g* at 4°C for 20 min, and the pellet was washed with 90% acetone and incubated at room temperature for 20 min. The acetone was removed by centrifugation (10,000 x *g* for 15 min), and the resulting pellet was analyzed by 18% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing and reducing conditions according to Laemmli (1970) in a Hoeffer MiniVE electrophoresis unit (Amersham Biotech). The molecular weight of the bacteriocin was estimated by comparing molecular mass markers. The gel was divided into two vertical parts; one part was stained with Coomassie blue R-250, and the other part was assayed for the detection of antimicrobial activity.

Detection of bacteriocin activity in polyacrylamide gel

To determine bacteriocin activity, the electrophoresis gel was extensively washed with sterile water for 3 h for SDS removal; drained off on sterile filter paper for 2 to 3 s, and fixed with acetic acid, isopropanol, and water in a proportion of 1: 2: 7. The slice was placed on a Petri dish of convenient diameter; the gel was flooded with precooled BHI medium and contaminated with *L. innocua* (10^5 CFU/ml) in 0.7% soft agar medium and incubated at optimal growth conditions for the indicator organism (Park et al., 2003).

Determination of the effectiveness of enterocin decontamination on lettuce

The effectiveness of enterocin in inhibiting the proliferation of natural microbiota or artificially contaminated lettuce was evaluated by using fresh-cut lettuce or lettuce seed with *L. innocua* L1005 (10^9 CFU/ ml). One gram of lettuce (not treated) was cut into pieces and dipped for 5 min in 5 ml of saline (natural microbiota), or in 5 ml of 0.5% acetic acid or in 5 ml of *L. innocua* strain L1005 (10^9 CFU/ ml) suspension, at room temperature during 30 min. Next, the lettuce pieces were placed on sterile filter paper to drain excess water and dipped in an adequate concentration of purified (32 AU) or partially purified (128 AU) (ammonium sulfate precipitate fraction) enterocin or sterile water for 5 min at room temperature. At several time intervals, the liquid was drained, and the lettuce pieces were washed in 1.0 ml of sterile, serially diluted in saline solution and spread on plates of Oxford and BHI agar. Plates were incubated at 37°C for 24 h, and the number of colonies showing features typical of Listeria was enumerated, in order to calculate viable cell counts. Experiments were run in triplicate.

RESULTS AND DISCUSSION

Antimicrobial production

The search for methods of food conservation that minimize the use of artificial substances has increased the number of studies of bacteriocins for this purpose (Molinos et al., 2005). *E. faecium* E86 was grown in MRS broth for 24 h, and the production of the antimicrobial substance was detected in the beginning of the log phase (after 7 h, OD_{660nm} = 0.06), reaching the peak after 9 to 11 h (0.1≤ D.O.≤ 0.145). The titer of activity was 2,560 AU/ ml. The production of the antimicrobial substance decreased after 19 h of growth (Figure 1). The decrease in production of bacteriocin when the bacteria are in the stationary phase is frequently observed for other bacteriocins (Miguel et al., 2008). This decreased activity was ascribed to the adsorption of the antimicrobial substance molecules on the surface of producer cells (Folquie-Moreno et al., 2003). Another possibility is that the bacteriocin molecules are degraded by proteases produced during their growth.

The antimicrobial substance produced by *E. faecium* E86 has a broad spectrum of inhibition. The bacteriocin was tested against *Listeria* sp. (25 isolates) and *Enterococcu*s sp. (82 isolates) (Table 1). All strains of *L. innocua* (10) and *L. monocytogenes* (15) isolated from food were susceptible to *E. faecium* E86, and 69 isolates of *Enterococcus* (*faecalis, durans, casseliflavus, faecium, munditi,* and *raffinosus*) from food or clinical samples were also susceptible to the bacteriocin. As a result of the risks and severity of infections caused by *L. monocytogenes* in human beings, and based on the susceptibility of *L. innocua* to the *E. faecium* E86 bacteriocin, this specie replaced *L. monocytogenes* as the indicator organism in all subsequent experiments.

Purification of antimicrobial substance

Cells were harvested by centrifugation, and the supernatant was submitted to ammonium sulfate and chloroform precipitation. The resultant sample was applied to a Dowex-50 exchanger column, and the proteins left the column in the first two fractions eluated. This purification system resulted in a yield of 80% and a 6-fold increase in the specific activity (Table 2). As with other antimicrobial substances, a marked increase in specific activity occurred after some purification steps. This finding could reflect the presence in culture supernatants of inhibitory compounds that are removed during the purification and/ or the dissociation of high-molecular-weight aggregates into their smaller, more active forms (Floriano et al., 1998). A review of antimicrobial substance purification methods (Carolissen-Mackay et al., 1997) highlighted the disadvantages of the complex and time-consuming purification protocols existing at that time, which usually resulted in low protein yields.

Zymographic analysis of bactericidal activity

The apparent molecular mass of the peptide as judged by 8% SDS-PAGE was approximately 3.5 kDa, similar to other enterocins that have been characterized (Aymerich et al., 1996; Folquie-Moreno et al., 2003). SDS gel was already cut into two parts: one part of the gel was stained with Coomassie blue R-250 (Figure 2 A), and the other part was used for zymographic analysis of bacteriocin activity (Figure 2 B). The zone of growth inhibition of *L. innocua* corresponded to the same band present in the gel stained with Coomassie blue, although the band was more diffuse, indicating that the purified protein showed

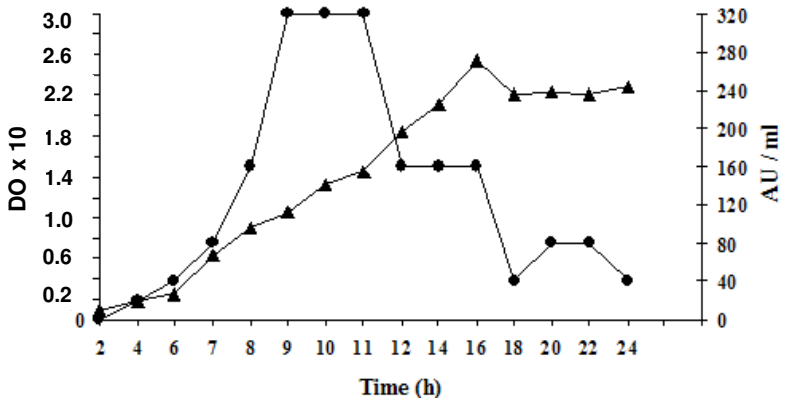

Figure 1. Growth of *Enterococcus faecium* in MRS broth (▲) and the production of antimicrobial substance E86 (•).

Table 1. Antimicrobial spectra of the bacteriocin produced by *Enterococcus faecium* E86 against different *Listeria* and *Enterococcus* species.

Indicator strains	Origin	No. of susceptible/tested strains
Listeria monocytogenes	Food	15/15
L. innocua	Food	10/10
Enterococcus faecalis	Clinical and food	39/47
E. durans	Clinical	2/2
E. casseliflavus	Clinical	1/1
E. faecium	Clinical and food	23/28
E. galinarum	Clinical	2/2
E. munditii	Clinical	1/1
E. raffinosus	Clinical	1/1
Total		94/107

10^7 strains of *Lysteria* and *Enterococcus* species tested on agar plate method. Susceptibility was determined by the presence of a clear zone around the colonies tested.

Table 2. Purification of the antimicrobial substance produced by *Enterecoccus faecium* E86.

Purification step	Total protein (mg)	Total activity (UA)	Specific activity (UA/mg)	Yield (%)	Purification
75% ammonium sulfate precipitation	2952.5	25600	8.67	100	1
Chloroform precipitation	400.0	20480	51.2	80	5.9
Dowex 50W chromatography	390.4	20480	52.46	80	6.05

Arbitrary unit (AU) was defined as the amount of the antimicrobial peptide required to produce a clear zone of inhibition using 10^5 CFU/ml of *L. innocua* L1005 (indicator organism), as described in Materials and methods.

the expected molecular mass and activity of an antimicrobial substance. Since the inhibition growth was indicated by a clear area around the polypeptide band.

Enterocin effectiveness in decontaminating artificially contaminated lettuce

The purified and the partially purified (ammonium sulfate precipitate fraction) enterocin were able to inhibit the proliferation of *L. innocua* strain L1005 on artificially contaminated lettuce. 32 AU/ml of antimicrobial substance was used to treat lettuce artificially contaminated by *L. innocua.* Purified and partially purified (ammonium sulfate precipitate fraction) enterocin were able to reduce the proliferation of *L. innocua* by 4 log cycles (10^9 to 10^5 CFU/ml), and by 2 log cycles (10^9 to 10^7 CFU/ml), respectively, after 15 min exposure. The

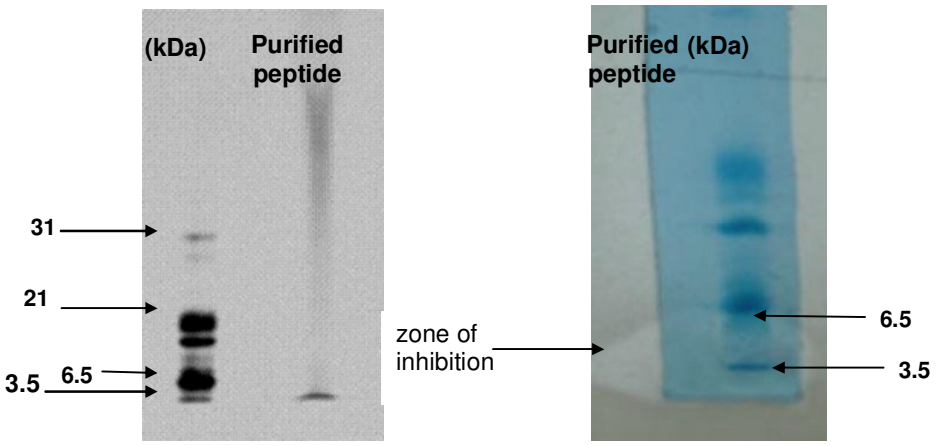

Figure 2. Analysis and identification of the purified antimicrobial substance from *Enterococcus faecium* E86. The purified antimicrobial substance was analyzed by 18% SDS-PAGE: (A) Gel stained with Coomassie blue R-250: line 1, peptide ladder of Amresco® with molecular mass ranging from 3.5 to 31 kDa, and line 2, the purified protein; (B) Detection of antimicrobial activity against *Listeria innocua* (10^5 CFU/ml): line 1, the purified protein, and line 2, peptide ladder of Amresco®.

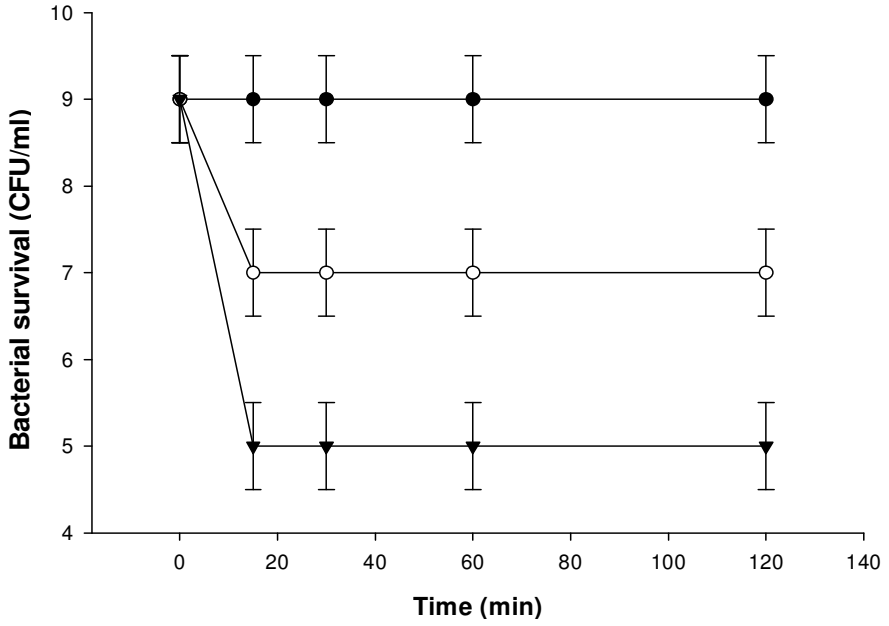

Figure 3. Decontamination effect of the purified peptide from *Enterococcus faecium E86* on lettuce artificially contaminated by *Listeria innocua*. 32 AU/ml of purified (▲) or partially purified enterocin 128AU/ml (O - ammonium sulfate precipitate) was used to treat 1 g of lettuce artificially contaminated by *L. innocua* (10^9 CFU/ml) for 30 min. Data represent means ± SD of three independent experiments.

purified enterocin inhibited 99.99% of *L. innocua* proliferation, whereas the partially purified fraction inhibited nearly 90% of cell proliferation (Figure 3).

These results indicate that enterocin in its purified or even partially purified form can be very effective against *Listeria* sp. Since the assays were performed by using the same quantity of bactericidal activity present in the purified or partially purified fraction, it could be concluded that this increase in activity result from the removal of proteins, peptides, and other cellular components from the producer or even from the culture medium, which could prevent the bonding of enterocin to the target cell.

This adverse effect may have been reduced when the purified peptide was used.

Additionally, the purified fraction has the advantage of not contributing to the enrichment of the lettuce, since it does not contain culture media components that could enhance the growth of the raw vegetable microbiota and increase the number of contaminants. The effectiveness of a natural bactericidal substance has been described previously; another study (Bennick et al., 1999) found that the application to mungbean sprouts of an antimicrobial substance produced by *E. mundtii* was able to reduce the proliferation of *L. monocytogenes* by two log cycles.

Combined effect of enterocin and acetic acid on natural microbiota of fresh-cut lettuce

Acetic acid has also been widely used as a food preservative for killing food-borne pathogenic bacteria on leafy vegetables (Samelis et al., 2001). In this study, after 30 min in the presence of 0.5% acetic acid, the natural microbiota proliferation was reduced by 3 log cycles, while the enterocin (128 AU/ml) in its purified or partially purified form showed a lower but not less significant inhibition of the microbiota cell proliferation, by 1 or 2 log cycles, respectively (Table 3). As observed for samples artificially contaminated, the purified enterocin is more effective in reducing natural microbiota than is the partially purified enterocin.

When both were combined with acetic acid, a synergistic effect was observed, reducing proliferation by 4 or 6 log cycles compared to the control. Bacteriocin activity may be a function of pH, it is well known that organic acid and their salts can potentiate the bacteriocin effect (Stiles, 1996), cationic nature seems to be crucial for bacteriocidal activity. Although the purified enterocin was more effective in decontaminating leafy vegetables, the partial purified fraction can be further exploited on the large-scale of enterocin production, since the preparation of the partial-purified enterocin is an effortless and low-cost procedure, and the product can be stored at 4°C until its use.

Conclusion

This study represents the first report on the purification and partial characterization of an antimicrobial substance produced by *E. faecium* E86. This is also the first indication that bacteriocins could provide a novel, safe alternative and effective barrier, which, combined with other control measures such as pH and temperature, can maximize protection from food borne pathogens on MPV (minimally processed vegetables) products. The development of appropriate barriers in terms of the number required, the intensity of each, and the sequence of applications to achieve a specified outcome is expected to have significant potential for future use with minimally processed leafy vegetables.

ACKNOWLEDGEMENTS

This research was sponsored by CAPES (Conselho de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Cientifico e Tecnológico), and FUJB (Fundação Universitária José Bonifácio).

REFERENCES

Audisio CM, Oliver G, Apella MC (2000). Protective effect of *Enterococcus faecium* J96, a potential probiotic strain, on chicks infected with *Salmonella pullorum*. J. Food Prot., 10: 1333-1337.

Aymerich T, Holo H, Havartein LS, Hugas M, Garriga M, Nes IF (1996). Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. Appl. Environ. Microbiol., 62: 1676-1682.

Bennick MHJ, Van Overbeek W, Smid EJ, Gorris LGM (1999). Biopreservation in modified atmosphere stored mung bean sprouts: the use of vegetable-associated bacteriocinogenic lactic acid bacteria to control the growth of *Listeria monocytogenes*. Lett. Appl. Microbiol., 28: 226-232.

Beuchat LR (1996). Pathogenic microorganisms associated with fresh produce. J. Food Prot., 59: 204-216.

Burianik LL, Yousef AE (2000). Solvent extraction of bacteriocins from liquid cultures. Lett. Appl. Microbiol., 31: 193-197.

Carolissen-Mackay V, Arendse G, Hastings JW (1997). Purification of bacteriocins of lactic acid bacteria problems and pointers. Int. J. Food Microbiol., 34: 1-16.

Casaus P, Nielsen T, Cintas LM, Nes IF, Hernandez PE, Holo H (1997). Enterocin B, a new bacteriocin from *Enterococcus faecium* T136 which can act synergistically with enterocin A. Microbiology, 143: 2287-2294.

Cintas LM, Casaus P, Havarstein LS, Hernandez PE, Nes IF (1997). Biochemical and genetic characterization of enterocin P, a novel sec-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. Appl. Environ. Microbiol., 63: 4321-4330.

Cintas LM, Casaus P, Herranz LS, Havarstein LS, Holo H, Hernandez PE, Nes IF (2000). Biochemical and genetic evidence that *Enterococcus faecium* L50 produces enterocins L50A and L50B, the sec-dependent enterocin P, and a novel bacteriocin secreted without an N-terminal extension termed enterocin Q. J. Bacteriol., 182: 6806-6814.

Cintas LM, Casaus P, Holo H, Hernandez PE, Nes IF, Havarstein LS (1998). Enterocins L50A and L50B, two novel bacteriocins from *Enterococcus faecium* L50 are related to staphylococcal haemolysins. J. Bacteriol., 180: 1988-1994.

Cotter PD, Hill C, Ross RP (2005). Bacteriocins: developing innate immunity for food. Nature Rev. Microbiol., 3: 777-788.

Fildes P (1920). The nature of the effect of blood-pigment upon the growth of *B. influenzae*. Br. J. Exp. Pathol., 1: 129-130.

Floriano B, Ruiz-Barba JL, Jimenez-Diaz R (1998). Purification and genetic characterization of enterocin I from *Enterococcus faecium* 6T1a, a novel antilisterial plasmid encoded bacteriocin which does not belong to the pediocin family of bacteriocins. Appl. Envion. Microbiol., 64: 4883-4890.

Folquie-Moreno MR, Callewaert R, Devreese J, Beeumen JV, De Vuyst L (2003). Isolation and biochemical characterization of enterocins produced by enterococci from different sources. J. Appl. Microbiol., 94: 214-229.

Galvez A, Abriouel H, Lopez RL Omar NB (2007). Bacteriocin-based strategies for food biopreservation. I. J. Food Microbiol., 120: 51-70.

Garcia E, Barrett DM (2002). Preservative treatment for fresh-cut fruits and vegetables. In O. Lamikanra (Ed.), Fresh-cut Fruits and Vegetables: Science, Technology and Market, Washington: CRC Press, pp. 267-303.

Ghrairi T, Frere J, Berjaud JM, Manai M (2008). Purification and characterization of bacteriocins produced by *Enterococcus faecium* from Tunisian rigouta cheese. Food Control, 19: 162-169.

Heng NCK, Wescombre PA, Burton JP, Jack RW, Tagg JR (2007). In: Riley, MA, Chavan MA (Eds.), The Diversity of Bacteriocins in Gram-positive Bacteria Bacteriocins: Ecology and Evolution. Springer-Verlag, Berlin Heidelberg.

Jiang L, He L, Fountoulakis M (2004). Comparison of proteins precipitation methods for sample preparation prior to proteomic analysis. J. Chromatogr., 1023: 317-320.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 277: 680-685.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-276.

Marekova M, Lauková A (2002). Enterocin M- a new bacteriocin produced by *Enterococcus faecium* AL41 and its potential use. Proceedings of "XX. Days of Animal Physiology", 20.10.–23.10 2002, Trest, Czech Republic, P. O. 38 [in Slovak].

Mareková M, Lauková A, DeVuyst L, Skaugen M, Nes IF (2003). Partial characterization of bacteriocins produced by environmental strain *Enterococcus faecium* EK13. J. Appl. Microbiol., 94: 523-530.

Miguel MAL, Castro A, Selma L (2008). Inhibition of vancomycin and high level aminoglycoside resistant enterococci strains and *Listeria monocytogenes* by bacteriocin-like substance producing *Enterococcus faecium* E86. Current Curr. Microbiol., 57: 429-436.

Millette M, Dupont C, Shareck F, Ruiz MT, Archambault D, Lacroix M (2008). Purification and identification of the pediocin produced by *Pediococcus acidilactici* MM33, a new human intestinal strain. J. Appl. Microbiol., 104: 269-275.

Molinos AC, Abriouel H, Omar NB, Valdivia E, Lopez RL, Maqueda M, Canãmero MM, Galvez A (2005). Effect of immersion solutions containing enterocin AS-48 on *Listeria monocytogenes* in vegetable foods. Appl. Envion. Microbiol., 71: 7781-7787.

Nes IF, Diep DB, Havarstein LS, Brurberg MB, Eijsink V, Holo H (1996). Biosynthesis of bacteriocins in lactic acid bacteria. Anton von Leeuwenhook Int., 70: 113-128.

Parente E, Brienza C, Moles M, Ricciardi A (1994). A comparison of methods for the measurement of bacteriocin activity. J. Microbiol. Meth., 22: 95-108.

Park SH, Itoh K, Fujisawa T (2003). Characteristics and identification of enterocins produced by *Enterococcus faecium* JCM 5804. J. Appl. Microbiol., 95: 294-300.

Porfirio Z, Prado SM, Vancetto MDC, Fratelli F, Alves EW, Raw I, Fernandes BL, Camargo ACM, Lebrum I (1997). Specific peptides of casein pancreatic digestion enhance the production of tetanus toxin. J. Appl. Microbiol., 83: 678-684.

Samelis J, Sofos JN, Kendall PA, Smith GC (2001). Influence of the natural microbial flora on the acid tolerance response of *Listeria monocytogenes* in a model system of fresh meat decontamination fluids. Appl. Environ. Microbiol., 67: 2410-2420).

Shuenzel KM, Harrison MA (2002). Microbial antagonist of foodborne pathogens on fresh, minimally processed vegetables. J. Food Prot., 65: 1909-1915.

Stiles ME (1996). Biopreservation by lactic acid bacteria. Ant van Leeuw, 70: 331-345.

Trias R, Bañeras L, Badosa E, Montesinos E (2008). Bioprotection of golden delicious apples and iceberg lettuce against food-borne bacterial pathogens by lactic acid bacteria. Int. J. Food Microbiol., 123: 50-60.