Food Bioprocess Technol
DOI 10.1007/s11947-015-1469-0

ORIGINAL PAPER

Inhibitory Effect of the Hybrid Bacteriocin Ent35-MccV on the Growth of *Escherichia coli* and *Listeria monocytogenes* in Model and Food Systems

Leonardo Acuña · Natalia S. Corbalan · Inmaculada C. Fernandez-No · Roberto D. Morero · Jorge Barros-Velazquez · Augusto Bellomio

Received: 6 June 2014 / Accepted: 5 January 2015
© Springer Science+Business Media New York 2015

Abstract Bacteriocins are being used as new food biopreservative agents. In general, bacteriocins produced by Gram-positive bacteria are active against other Gram-positive. Basically, the same principle applies to those produced by Gram-negative bacteria. They have a restricted spectrum of action against related bacteria to those that produce the bacteriocin. Therefore, other hurdles or chemical preservatives are necessary to apply to broaden the spectrum of action of bacteriocins in foods. This is a further and deeper study of the possible application of the hybrid wide-spectrum bacteriocin named Ent35-MccV in food. Its antimicrobial activity was assayed in skim milk and patties as food models against *Listeria monocytogenes* and *Escherichia coli*. The influence of the temperature and digestive proteases on its biological activity and its antimicrobial activity was tested in vitro on a variety of pathogenic and food spoilage bacteria. The results showed that Ent35-MccV could inhibit the growth of both the Gram-positive *L. monocytogenes* and the Gram-negative *E. coli* in model food, and its activity was not affected by heating conditions including autoclaving. *E. coli* strains and *Listeria* spp. are the most affected bacteria, but Ent35-MccV showed antimicrobial activity against some strain of *Salmonella* spp., *Staphylococcus epidermidis*, *Enterobacter aerogenes*, *Morganella morgani*, *Proteus mirabilis*, *Shigella boydii*, *Shigella flexneri*, and *Shigella sonnei*.

Keywords Microcin · Bacteriocin · Biopreservation · Hybrid bacteriocin

L. Acuña · N. S. Corbalan · R. D. Morero · A. Bellomio (✉)
Instituto Superior de Investigaciones Biológicas (INSIBIO), CONICET-UNT and Instituto de Química Biológica "Dr. Bernabé Bloj", Facultad de Bioquímica, Química y Farmacia, UNT, Chacabuco 461, T4000ILI San Miguel de Tucumán, Argentina
e-mail: augustobellomio@fbqf.unt.edu.ar

I. C. Fernandez-No · J. Barros-Velazquez
Department of Analytical Chemistry, Nutrition and Food Science, School of Veterinary Sciences, University of Santiago de Compostela, 27002 Lugo, Spain

Introduction

Foodborne illness results from the consumption of contaminated foods or water. Of the diseases caused by pathogens, those produced by bacteria are of particular concern because they can often be fatal. The pathogenic Gram-positive *Listeria monocytogenes* and the Gram-negative *Salmonella* spp. cause the highest number of deaths (Ray and Bhunia 2007). During the last years, the emergence of alarming outbreaks of Gram-negative enteropathogenic strains of *Escherichia coli* produced a great impact on the public health authorities of countries around the world. Enterohemorrhagic *E. coli* (EHEC) produces bloody diarrhea (Turner 2011). Sometimes, patients develop the dangerous illness named hemolytic-uremic syndrome (WHO 2011).

Foodborne illnesses can be avoided by instrumenting appropriate processing of food as described in the Codex Alimentarius (WHO/FAO) and carrying out Hazard Analysis and the Control of Critical Points (HACCP). In this scenario, the application of hurdle technology to improve the safety of food is gaining grounds for processing (Leistner 1978). In this approach, foods are subjected to

Published online: 24 January 2015

various mild treatments such as light pulses, food irradiation, modified atmosphere packaging, high hydrostatic pressure, and high-intensity pulsed electric fields with the aim that each of them represents a barrier to the survival of pathogenic and spoilage microorganisms that may be present in raw foods (Chen et al. 2004; Jin et al. 2009; Metaxopoulos et al. 2002; Uesugi and Moraru 2009). The goal of this strategy is to increase the shelf life and the microbiological safety of processed foods without affecting the nutritional and organoleptic qualities. Further hurdles are being developed that will allow, if applied intelligently, a reduction of production costs and will improve the quality of industrial products (Leistner and Grahame 2005).

Bacteriocins from lactic acid bacteria are being considered a serious candidate to be employed as biopreservatives for food products. Currently, only two of the over 170 bacteriocins characterized so far are used as food biopreservatives in the food industry (Calo-Mata et al. 2008; Hammami et al. 2010).

The lantibiotic nisin and the linear peptide pediocin PA-1 belonging to class I and class II bacteriocins, respectively, are the only bacteriocins approved to be employed in foods (Cotter et al. 2005). Bacteriocins from lactic acid bacteria (LAB) have been extensively characterized. They are active principally on Gram-positive bacteria phylogenetically related to the producer bacteria, including the opportunistic pathogen *L. monocytogenes*. However, these bacteriocins are generally not active against *E. coli* or other Gram-negative bacteria. Only a few bacteriocins, as the cyclic peptide enterocin AS-48 are active against Gram-negative and Gram-positive bacteria in foods when employed mixed with EDTA or another hurdle (Sánchez-Hidalgo et al. 2011).

Recently, the construction of the chimerical bacteriocin named Ent35-MccV, a hybrid peptide, was reported. The linear class II bacteriocin enterocin CRL35, produced by *Enterococcus mundtii* CRL35, was linked to the N-terminal of the linear microcin V, produced by *E. coli* through a hinge of three glycine residues. The hybrid bacteriocin combines in a single molecule the antilisterial activity of enterocin CRL35 and the anti-*E. coli* activity of microcin V. Such hybrid bacteriocin proved to be active against pathogenic strains of *L. monocytogenes* and *E. coli* O157:H7 (Acuña et al. 2012).

The purpose of this work is to expand the studies on the antimicrobial spectrum of the hybrid bacteriocin Ent35-MccV against foodborne pathogenic and spoilage bacteria, including different species of *Listeria*, Gram-negative pathogens, and histamine-producing strains. Moreover, the effect of high temperature and digestive proteases on the biological activity were tested here and the application as a biopreservative agent on milk and patties as food models.

Material and Methods

Bacteriocin Purification

Ent35-MccV and microcin V were purified as previously described (Acuña et al. 2012; Fath et al. 1994). The strains *E. coli* BL21 [DE3] (pLysS) (pMA24) and *E. coli* MC4100 (pHK11) were used to produce Ent35-MccV and microcin V, respectively. Enterocin CRL35 was chemically synthesized (Bio-Synthesis, Lewisville, TX, USA), based on the amino acid sequence of the mature bacteriocin and was a generous gift from Lucila Saavedra (Saavedra et al. 2004). Bacteriocin concentrations were calculated by serial twofold dilutions spotting 10 μl on plate count agar (PCA) (Oxoid, Hampshire, England) using *E. coli* ATCC 13706 as the indicator organism for both Ent35-MccV and microcin V. *L. monocytogenes* CECT 4032 was used as indicator for enterocin CRL35. Plates were incubated at 37 °C for 24 h. One arbitrary unity (AU) was defined as the reciprocal of the highest dilution that showed a zone of inhibition with at least 5-mm diameter. Protein concentration was determined using Quant-iT™ Protein Assay Kit (Invitrogen, Paisley, UK).

Antimicrobial Spectrum, Bacterial Strains, and Media

The antimicrobial activity was determined against 99 strains by the agar well diffusion assay (see Table 1). Drops of each purified peptide (10 μl, 1000 AU/ml) were spotted in a plate of agar culture medium. Once the droplets were absorbed, the plate was covered with 5 ml of 0.6 % agar previously inoculated with 10^6 cells of the strain to test its sensitivity. The incubation was carried out over night, and the antimicrobial activity was evaluated by measuring the diameter of the inhibition halos. The microorganisms used for evaluation of antimicrobial activities and culture conditions are listed in Table 1. The cultures media used were Plate Count Agar (PCA) (PCA) (Oxoid), de Man, Rogosa and Sharpe (MRS) (Liofilchem, TE, Italy), Brain-Heart Infusion (BHI) (Oxoid), Marine (Cultimed, Barcelona, Spain), and Luria Bertani (LB) (Sigma Chemical Co., St Louis, USA). Fluorocult Violet Red Bile (VRB) (Merck, Darmstadt, Germany) and Palcam Listeria agar base (Merck, Darmstadt, Germany) were used as selective media to *E. coli* and *L. monocytogenes*, respectively. Experiments to test bacteriocin stability and those carried out in complex media, skim milk, and patties were performed with the strains *L. monocytogenes* CECT 4032 or *E. coli* ATCC 13706. They are referred to as *E. coli* or *L. monocytogenes* throughout the manuscript.

Enzyme Sensitivity and Heat Stability

Trypsin, chymotrypsin, proteinase K (pH 7.4), and pepsin (pH 2.4) obtained from Sigma were used to treat Ent35-MccV. All enzymes were employed at a final concentration of 5.0 mg/ml

Table 1 Inhibitory spectrum of Ent35-MccV and parental bacteriocins

Indicator species	Strain	Source	Culture media/temp	Inhibitory activity[a]		
				Ent35-MccV	MccV	Ent35[b]
Gram-positive						
Bacillus cereus	ATCC 14893	CECT	PCA/30 °C	−	−	−
Bacillus licheniformis	ATCC 14580	CECT	PCA/37 °C	−	−	−
Bacillus pumilus	ATCC 7061	CECT	PCA/30 °C	−	−	−
Bacillus subtilis	ATCC 6633	CECT	PCA/30 °C	−	−	−
Bacillus thuringiensis	ATCC 10792	CECT	PCA/30 °C	−	−	−
Carnobacterium divergens	ATCC35677	CECT	PCA/37 °C	−	−	++
Clostridium botulinim	ATCC 19397	CECT	PCA/37 °C	−	−	−
Clostridium perfringens	ATCC 10543	CECT	PCA/37 °C	−	−	−
Lactobacillus sp. 30A*	ATCC 33222	ATCC	MRS/37 °C	+++	−	−
Listeria innocua	4L1Pe	INRA	BHI/37 °C	−	−	−
L. innocua	SR 215	IHT	BHI/37 °C	−	−	−
L innocua	ATCC 33090	CECT	BHI/37 °C	+++	−	+++
Listeria ivanovii	ATCC 19119	CECT	BHI/37 °C	+++	−	+++
Listeria monocytogenes	CECT 4031	CECT	BHI/37 °C	+++	−	+++
L. monocytogenes	CECT 4032	CECT	BHI/37 °C	+++	−	+++
L. monocytogenes	CECT 934	CECT	BHI/37 °C	+++	−	+++
L. monocytogenes	FBUNT2	FBQF	BHI/37 °C	+++	−	+++
L. monocytogenes	Li01	LHICA-FI	BHI/37 °C	+++	−	+++
L. monocytogenes	Li02	LHICA-FI	BHI/37 °C	+++	−	++
L. monocytogenes	Li03	LHICA-FI	BHI/37 °C	+++	−	++
L. monocytogenes	Li04	LHICA-FI	BHI/37 °C	+++	−	++
Listeria seeligeri	ATCC 35967	CECT	BHI/37 °C	+++	−	+++
Listeria welshimeri	ATCC 35897	CECT	BHI/37 °C	+++	−	+++
Staphylococcus epidermidis	FBUNT2	FBQF-CI	LB/37 °C	+++	−	++
*Staphylococcus xylosus**	ATCC 29971	CECT	PCA/37 °C	−	−	−
Gram-negative						
Acinetobacter baumannii	FBUNT1	MALBRAN-CI	PCA/37 °C	−	−	−
Acinetobacter baumannii	ATCC 15308	CECT	PCA/37 °C	−	−	−
*Aeromonas hydrophila**	ATCC 7966	CECT	BHI/30 °C	−	−	−
*Citrobacter freundii**	ATCC 8090	CECT	BHI/37 °C	−	−	−
*Enterobacter aerogenes**	ATCC 13048	CECT	PCA/30 °C	+	−	−
*Enterobacter cloacae**	ATCC 13047	CECT	PCA/30 °C	−	−	−
Enterobacter sakazakii	ATCC29544	CECT	PCA/37 °C	−	−	−
Escherichia coli O157:H7	CECT 4267	CECT	PCA/37 °C	+	−	−
E. coli O157:H7	CECT 4783	CECT	PCA/37 °C	+	−	−
E. coli	ATCC 13706	CECT	PCA/37 °C	+++	++	−
E. coli	D03	LHICA-FI	PCA/37 °C	++	+++	−
E. coli	D05	LHICA-FI	PCA/37 °C	+++	+	−
E. coli	D16	LHICA-FI	PCA/37 °C	++	+	−
E. coli	D41	LHICA-FI	PCA/37 °C	+++	++	−
E. coli	D73	LHICA-FI	PCA/37 °C	+++	++	−
E. coli	D79	LHICA-FI	PCA/37 °C	+++	++	−
E. coli	D83	LHICA-FI	PCA/37 °C	++	−	−
E. coli	E41	LHICA-FI	PCA/37 °C	++	+	−
E. coli	E43	LHICA-FI	PCA/37 °C	−	++	−
E. coli	EC35	LHICA-FI	PCA/37 °C	++	+++	−

Table 1 (continued)

Indicator species	Strain	Source	Culture media/temp	Inhibitory activity[a]		
				Ent35-MccV	MccV	Ent35[b]
E. coli	EC44	LHICA-FI	PCA/37 °C	++	−	−
E. coli	EC114	LHICA-FI	PCA/37 °C	+/−	−	−
E. coli	S39	LHICA-FI	PCA/37 °C	+	++	−
E. coli	S58	LHICA-FI	PCA/37 °C	+	+	−
E. coli	S139	LHICA-FI	PCA/37 °C	+/−	++	−
E. coli	Esc11	IP	PCA/37 °C	++	+++	−
E. coli	NCTC 50365	NCTC	PCA/37 °C	+++	+++	−
E. coli	NCTC 50271	NCTC	PCA/37 °C	++	−	−
*Hafnia alvei**	ATCC 9760	CECT	BHI/30 °C	−	−	−
Klebsiella oxytoca	ATCC 13182	CECT	PCA/37 °C	−	−	−
*Klebsiella pneumoniae**	ATCC 10031	CECT	BHI/37 °C	−	−	−
Morganella morgani	ATCC 8076H	CECT	PCA/30 °C	++	−	−
*M. morgani**	BM56	UAB	PCA/30 °C	−	−	−
Pantoea aglomerans	ATCC 27155	CECT	PCA/30 °C	−	+	−
*Photobacterium damselae**	ATCC 33539	CECT	BHI/30 °C	−	−	
*Proteus mirabilis**	ATCC 14153	CECT	PCA/37 °C	++	−	−
Proteus penneri	ATCC 33519	CECT	PCA/37 °C	−	−	−
*Proteus vulgaris**	ATCC 9484	CECT	BHI/37 °C	−	−	−
Providencia rettgeri	ATCC 29944	CECT	PCA/37 °C	−	−	−
Providencia stuartii	ATCC 29914	CECT	PCA/37 °C	−	−	−
Pseudomonas aeruginosa	FBUNT1	FBQF	PCA/37 °C	−	−	−
Pseudomonas fluorescens	ATCC 13525	CECT	PCA/30 °C	−	−	−
Pseudomonas frágil	ATCC 4873	CECT	PCA/30 °C	−	−	−
Pseudomonas putida	ATCC 12633	CECT	PCA/30 °C	−	−	−
Pseudomonas syringae	ATCC 19310	CECT	PCA/30 °C	−	−	−
*Roultella planticola**	ATCC 33531	CECT	BHI/30 °C	−	−	−
Salmonella enterica	ATCC 4931	CECT	PCA/37 °C	−	−	−
Salmonella enterica arizonae	CECT 4395	CECT	PCA/37 °C	−	−	−
Salmonella enterica Enteritidis	CECT 4300	CECT	PCA/37 °C	−	−	−
Salmonella enterica Enteritidis	CECT 4396	CECT	PCA/37 °C	−	−	−
Salmonella enterica Thypimurium	CECT 4594	CECT	PCA/37 °C	−	−	−
Salmonella enterica houtenae	CECT 5326	CECT	PCA/37 °C	+	++	−
Salmonella enterica salamae	ATCC 43972	CECT	PCA/37 °C	+/−	−	−
Salmonella enterica spp.	Sal1	LHICA-FI	PCA/37 °C	−	−	−
Salmonella enterica spp.	Sal2	LHICA-FI	PCA/37 °C	+/−	−	−
Salmonella enterica spp.	Sal3	LHICA-FI	PCA/37 °C	−	−	−
Salmonella enterica spp.	Sal4	LHICA-FI	PCA/37 °C	−	−	−
Salmonella enterica spp.	Sal5	LHICA-FI	PCA/37 °C	−	−	−
Salmonella enterica Virchou	C2	FI	PCA/37 °C	++	++	−
Serratia liquefaciens	ATCC 12926	CECT	PCA/30 °C	−	−	−
Stenotrophomonas maltophilia	ATCC 13637	CECT	PCA/30 °C	−	−	−
*Stenotrophomonas maltophilia**	25MC6	LHICA-FI	PCA/30 °C	−	−	−
*Stenotrophomonas maltophilia**	15MF	LHICA-FI	PCA/30 °C	−	++	−
Vibrio alginolyticus	ATCC 17749	CECT	Marine/30 °C	−	−	−
Vibrio parahaemolyticus	ATCC 17802	CECT	BHI/37 °C	−	−	−
Vibrio vulnificus	ATCC 27562	CECT	Marine/30 °C	−	−	−
Shewanella algae	ATCC 51192	CECT	BHI/30 °C	−	−	−

Table 1 (continued)

Indicator species	Strain	Source	Culture media/temp	Inhibitory activity[a]		
				Ent35-MccV	MccV	Ent35[b]
Shewanella baltica	CECT 323	CECT	BHI/30 °C	−	−	−
Shewanella putrefaciens	ATCC 8071	CECT	BHI/30 °C	−	−	−
Shigella boydii	CECT 583	CECT	PCA/37 °C	++	−	−
Shigella dysenteriae	ATCC 13313	CECT	PCA/37 °C	−	−	−
Shigella flexneri	CECT 585	CECT	PCA/37 °C	+	++	−
Shigella sonnei	ATCC 11060	CECT	PCA/37 °C	++	++	−
Yersinia enterocolitica	CECT 500	CECT	PCA/37 °C	−	−	−

Strains that were sensitive to Ent35-MccV and not its parental bacteriocin or vice versa are underlined. Biogenic amine-producing bacterial species are indicated with an asterisk (*)

CECT Spanish Type Culture Collection, *ATCC* American Type Culture Collection, *INRA* Jouy-en-Josas, France, *IHT* Institute of Hygiene and Toxicology, Karlsruhe, Germany, *FBQF* Facultad de Bioquímica, Química y Farmacia. Universidad Nacional de Tucumán. Argentina, *LHICA*: Laboratorio de Higiene, Inspección y Control de Alimentos, *USC* Spain, *MALBRAN* Administración Nacional de Laboratorios e Institutos de Salud (ANLIS) Dr. Carlos Malbrán, Argentina, *IP*: Institut Pasteur, Paris, France, *NCTC* National Collection of Type Cultures, UK, *UAB* Universidad Autónoma de Barcelona, *FI* food isolate, *CI*: clinical isolate

[a] Symbols represent relative activity by diffusion in agar, evaluated by measuring the average diameter (da) of inhibition zones: −, no inhibition; +, da< 5 mm; ++, 5 mm<da<10 mm; +++, da>10 mm; +/−, 5-mm turbid halo

[b] Ent35: enterocin CRL35

(Wulijideligen et al. 2012). The reaction mixtures for each enzyme were incubated for 2 h at 37 °C, and a control was prepared without enzyme. To test heat stability, Ent35-MccV was exposed to different temperatures (50, 60, 70, 80, 90, and 100 °C for 10 min and sterilization treatment 121 °C, 0.10 MPa for 15 min). To ensure a fast and efficient heat transfer, the assays were performed using 0.2-ml thin-walled PCR tubes (Eppendorf, Hamburg, Germany). Test tubes were dipped in a preheated water bath at the set temperature or autoclaved. Samples were taken from the hot water bath and were immersed in an ice bath, except for when they were autoclaved. The residual antimicrobial activity was evaluated against *L. monocytogenes* and *E. coli* by the agar well diffusion assay.

Determination of Minimal Inhibitory Concentration and Minimum Bactericidal Concentration in Complex Media

The minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Ent35-MccV were tested in BHI broth (Oxoid) and sterile skim milk 10 % *w*/*v* (Central Lechera Asturiana, Oviedo, Spain). *E. coli* ATCC 13706 and *L. monocytogenes* CECT 4032 were used as indicator Gram-negative and Gram-positive strains, respectively. MIC was performed in duplicates with a 96-well microtiter plate. The concentration range tested was from 2 to 500 AU/ml prepared from a stock bacteriocin Ent35-MccV solution titrated against *E. coli* ATCC 13706. The media was inoculated with ~10^4 cells of the sensitive strains. Positive growth controls were included in wells lacking the antimicrobial compound tested. MIC was defined as the lowest dilution of bacteriocin that inhibited growth of the indicator strain after 24 h of incubation at 37 °C. The MBC was calculated by performing viable cell counts after 24 h of incubation at 37 °C for both pathogens. Since *L. monocytogenes* is able to grow at low temperatures, MBC was additionally studied at 4 °C after 7 days of incubation. In order to do this, aliquots of tenfold dilutions of each solution were plated onto selective agar plates after incubation. Plates containing between 30 and 300 colony forming units (CFUs) were considered for counting. The MBC was defined as the minimum concentration of Ent35-MccV that produced a ≥99.9 % reduction of the original inoculum.

Antimicrobial Activity of Ent35-MccV in Contaminated Food Products

Sterile Skim Milk

To determine the antibacterial activity of the hybrid bacteriocin Ent35-MccV against *L. monocytogenes* and *E. coli* in food products, each bacteria was grown overnight in BHI medium (Oxoid) at 37 °C prior to use using the cells for the antibacterial activity assays. Ten milliliters of skim milk powder (Central Lechera Asturiana) reconstituted in distilled water to 10 % (*w*/*v*) and sterilized in autoclave were inoculated with ~10^4 cells of *L. monocytogenes* or *E. coli*. The contaminated samples were treated with 125 or 250 AU of Ent35-MccV per milliliter of milk. Two trials were conducted in parallel, incubating the samples at 37 and 4 °C, respectively. Aliquots were taken at different times and seeded in agar plates with selective

medium to perform the viable counts as detailed in "Microbiological Analysis" section.

The stability of Ent35-MccV in milk was evaluated in a different assay. Milk samples treated with the peptide (250 AU/ml) were kept at 4 °C. They were collected at different times (0, 1, 2, 3, 4, 5, 7, and 10 days), inoculated with 10^4 cells/ml of each strain separately (*L. monocytogenes* or *E. coli*) and incubated 6 h at 37 °C without agitation. Subsequently, different dilutions were plated in their respective media plates, incubated 24 h and the remaining viable cells counted.

Preparation of Ground Beef Patties and Experimental Design

Ground beef patties were prepared from quadriceps femoris muscles obtained from a local supermarket as it was reported previously (Castellano et al. 2011). Approximately 5 mm of the beef surface were aseptically trimmed off to reduce the initial numbers of resident microbiota. The beef was aseptically ground with a sharp knife to form multiple 50–100-mm diameter pieces, placed in sterile bags. Since most processed meat products contain sodium chloride to bind ingredients, stabilize and improve taste and color, 1 % (*w*/*w*) of NaCl was added (Juneja et al. 2013). Finally, the ground meat was inoculated with *L. monocytogenes* or *E. coli* 10^4 CFU/g. Purified Ent35-MccV was reconstituted in peptone water (Merck) and added at a final concentration of 125 AU/g. The bag was manually mixed, and the ground beef patties (25 g) were aseptically weighed and hand-formed. Control patties were prepared using the inoculated mixture treated with peptone water. They were stored at 4 °C in sterile Petri dishes for 10 days, as it was previously reported elsewhere (Chounou et al. 2013). CFU were counted as described in "Microbiological Analysis" section.

The stability of Ent35-MccV in patties was evaluated in a similar way as it was done in milk ("Sterile Skim Milk" section). Ground beef patties were mixed with 125 AU/g and were kept at 4 °C. They were collected at different times, inoculated with 10^4 cells/g of each strain separately (*L. monocytogenes* or *E. coli*) and incubated 6 h at 37 °C without agitation. Subsequently, different dilutions were plated in their respective selective media plates, incubated 24 h, and the viable cells counted.

Microbiological Analysis

Each ground beef patty (25 g) from each experimental case was aseptically transferred to a sterile stomacher bag, diluted with 225 ml of sterile peptone water and homogenized for 2 min using a Mix2 Stomacher (AES Laboratories, Combourg, France). Decimal dilutions were prepared in the same diluents. To count *E. coli* cells, 1 ml of each dilution was poured on Fluorocult® agar prepared as specified by the manufacturer (Merck). After the agar solidified, the plates were overlaid with 3–4 ml of melted Fluorocult® and incubated at 42 °C for 24 h. After incubation, pink to red colonies showing blue florescence after exposure to a 365-nm ultraviolet lamp were counted (Miranda et al. 2008). To count *L. monocytogenes*, 1 ml of each dilution was poured on Palcam Listeria agar base (Merck, Darmstadt, Germany) and incubated 24 h at 37 °C. The plates having between 30 and 300 CFU were selected for counting.

The procedure was similar to trials in milk. Decimal dilutions were performed in peptone water, and the same culture media were used for the viable cell count. The difference between the CFU/ml (milk) or CFU/g (meat) in the samples without bacteriocin and the CFU/ml or CFU/g in the samples treated with bacteriocin at initial time (day 0), i.e., the highest inhibitory capacity. Afterward, differences between bacteriocin-treated and non-treated samples were calculated for each time point, and stability assays were reported relative to the initial inhibitory capacity of Ent35-MccV (day 0).

Data Analysis

All experiments in skim milk and patties were performed three times using different overnight bacteria cultures as inoculum for each trial. Every independent assay was carried out in duplicate. Data of CFU from duplicates were averaged first, and then, the three averages of each individual experiment were used to calculate the mean and standard deviation. The colony-forming units and residual activity was plotted versus time with the Origin 6.0 program (MicroCal Software, Northampton, USA), and the error bars presented indicate the standard deviation of the mean (shown only in the positive direction). Data were subjected to analysis of variance (ANOVA) followed by Tukey's test with Statistix 9.0 for Windows (AnalyticalSoftware, Tallahassee, USA). Differences at p value of 0.05 were considered significant.

Results and Discussion

Antimicrobial Spectrum of the Hybrid Bacteriocin

The antimicrobial activity of Ent35-MccV and its component bacteriocins microcin V and enterocin CRL35 were tested over a wide range of bacteria; 54 bacterial species were assayed, including 99 different strains belonging to 27 genera and different serovars. Among these, previously reported foodborne pathogens and food spoilage bacteria were considered and included several clinical isolates and strains of bacteria belonging to different serovars. Bacteria isolated at our laboratory from contaminated food and bacteria reported as histamine producers were also included. The latter are considered of great importance for the meat and, especially for the

fish industry, since they can cause poisoning due to the consumption of meat and, especially, seafood products (Fernández-No et al. 2010).

The results of the inhibition assays are shown in Table 1. Ent35-MccV showed potent antimicrobial activity against various strains of *Listeria* and *E. coli*. Ent35-MccV was inhibitory against 12 of the 14 *Listeria* spp. strains tested. Remarkably, the only two strains that were not sensitive to the hybrid bacteriocin (*Listeria innocua* 4L1Pe and *L. innocua* SR215) were resistant to the parental enterocin CRL35. Among the strains of *Listeria* sensitive to the new bacteriocin, these were isolated from contaminated foods.

On the other hand, many Gram-negative bacteria inhibited by MccV were also inhibited by Ent35-MccV. In particular, the hybrid bacteriocin showed antimicrobial activity against almost all food *E. coli* isolates tested, including the enterohemorrhagic O157:H7 strains.

Another interesting result of these assays was that several strains of Gram-positive and Gram-negative bacteria were affected by Ent35-MccV but were not sensitive to their parental bacteriocins. These were the cases of *E. coli* CECT 4267, CECT 4783, NCTC 50271, and D83; *Shigella boydii*; *Morganella morganii* ATCC 8076H; *Staphylococcus epidermidis*; and the histamine-producing bacteria *Lactobacillus* sp. 30A, *Enterobacter Aerogenes*, and *Proteus mirabilis*. In addition to the abovementioned strains, it is remarkable that Ent35-MccV was active against some *Shigella* and *Salmonella* species. On the other hand, *Salmonella enterica* C2 and Sal2, *Shigella boydii*, *Shigella sonnei*, *Enterobacter aerogenes*, and *Lactobacillus* sp. 30A strains were not completely sensitive to the hybrid bacteriocin since the inhibition zones showed a slight turbidity. Moreover, strains that were inhibited by the parental but not by the hybrid bacteriocin as it were previously noted to *Enterococcus faecalis* FBUNT1 were also observed. In the present paper, it was found that Gram-positive strain *Carnobacterium divergens*, sensitive to enterocin CRL35 and Gram-negative species *Pantoea aglomerans* and *Stenotrophomonas maltophilia* 15MF sensitive to microcin V were insensitive to the hybrid bacteriocin Ent35-MccV. This is not surprising given the alterations in the primary structure of the bacteriocins that make up Ent35-MccV. As a matter of fact, we already characterized the strain *Enterococcus faecalis* FBUNT1 with a similar phenotype in a previous work (Acuña et al. 2012).

In the light of the results obtained on these latter strains, it would be important in the future to test whether different treatments that may be used as hurdles in food processing improve the activity of the hybrid peptide. Moreover, strains that were inhibited by the parental but not by the hybrid bacteriocin as it was previously noted to *Enterococcus faecalis* FBUNT1 (Acuña et al. 2012) were also observed. This is not surprising given the alterations in the primary structure of the bacteriocins that make up Ent35-MccV. Gram-positive strain *C. divergens* was sensitive to enterocin CRL35 and Gram-negative species *Pantoea aglomerans* and *Stenotrophomonas maltophilia* 15MF were sensitive to microcin V. By contrast, the hybrid bacteriocin Ent35-MccV showed no antimicrobial activity against these strains. In this regard, new hurdle combination using antimicrobial peptides were reported. Nisin Z in combination with natural preservatives as the surfactant lauric arginate increased its antimicrobial activity against *E. coli*, *L. monocytogenes*, and *Brochothrix thermosphacta*. Also, nisin with white mustard essential oil had synergistic effects against *L. monocytogenes* (Techathuvanan et al. 2014). Furthermore, combination using nisin or several chemical preservatives as methyl paraben, propyl paraben, and sodium nitrite increased inhibitory effects of sakacin C2 against *Staphylococcus aureus* and *E. coli* (Li et al. 2014). Physical and chemical combinations with antimicrobial peptides were also assayed. Huq et al. (2015) showed greater antilisterial activities when they used essential oils and nisin in combination with -irradiation (Huq et al. 2015). Regarding physical barriers, in recent years, synergistic effects have been reported among antimicrobial peptides like nisin or pediocin AcH in combination with physical agents such as pulsed electric fields, high-pressure processing, and intense light pulses that were successfully applied in food products (Smigic and Rajkovic 2014). In addition, several studies reported effective results using nisin in foods together with chemical or physical hurdles that alter the permeability of the outer wall of Gram-negative bacteria (Chalón et al. 2012). The antilisterial activity is a common characteristic among enterococcal bacteriocins (Cintas et al. 1997; Cotter et al. 2005; Giraffa et al. 1994). The structure and composition of the outer membrane of Gram-negative bacteria is a formidable barrier that does not allow bacteriocins of Gram-positive bacteria to reach their target: the inner membrane (Acuña et al. 2011; Chalón et al. 2012). However, some bacteriocins produced by LAB with activity against Gram-negative bacteria have been described (Gao et al. 2013; Sánchez-Hidalgo et al. 2011). With respect to microcins, they have a spectrum of bactericidal activity against *Enterobacteriaceae* (Duquesne et al. 2007). To our knowledge Ent35-MccV is the first well-characterized linear bacteriocin with such a wide inhibitory spectrum against a large number of foodborne pathogens and spoilage Gram-negative and Gram-positive bacteria.

Susceptibility of Ent35-MccV to High Temperature and Digestive Proteases

From an industrial point of view, it is very important to know the thermal stability of Ent35-MccV antimicrobial activity since many foods are subjected to heat treatments during cooking and processing to decrease or eliminate the microbial load. Ent35-MccV was subjected to different heat treatments in order to evaluate its thermal stability. As it can be seen in

Table 2, the hybrid bacteriocin kept intact its antimicrobial activity against *E. coli* and *L. monocytogenes*, after suffering 10 min of heat treatment at different temperatures. Only a small decrease in activity against both strains was observed after a sterilization treatment.

On the other hand, the application of a bacteriocin to a food system requires keeping the necessary antimicrobial activity to inhibit the bacterial contaminants in the food, but after ingestion, such antimicrobial activity must not persist in the human gut because it could cause an imbalance of the normal microbiota. Accordingly, it would be desirable to render Ent35-MccV susceptible to at least one digestive protease. When Ent35-MccV was treated with different proteolytic enzymes in order to assess their potential use in food preservation strategies, there was a loss of the antimicrobial activity after incubation with pepsin (stomachal enzyme), with the intestinal proteases trypsin and chymotrypsin, as well as with the broad-spectrum serine protease proteinase K. After treatment with pepsin, only a small cloudy inhibition halo was observed (less than 5 mm in diameter), as compared with the clear halos with a diameter greater than 10 mm of the control untreated extracts. Treatment with any of the other enzymes tested voided the antimicrobial activity of the peptide. These results indicate that the hybrid bacteriocin may be inactivated in vivo, not expecting to affect the intestinal flora of the hosts in the event of incorporation in food as biopreservative.

Ent35-MccV in Complex Media

The MICs and MBCs were determined in complex media as a first approach to the study of the action of the hybrid bacteriocin on food products. The culture media selected for this purpose were BHI (due to its meat derived composition) and sterile skim milk. The stock solution of Ent35-MccV used to perform these tests was titrated against *E. coli* MC4100 as previously detailed in "Bacteriocin Purification" section.

Table 2 Effect of enzymes and heating on antimicrobial activity of Ent35-MccV

Treatment	Inhibitory activity	
Heat	*L. monocytogenes*	*E. coli*
50 °C, 10 min	+++	+++
60 °C, 10 min	+++	+++
70 °C, 10 min	+++	+++
80 °C, 10 min	+++	+++
90 °C, 10 min	+++	+++
100 °C, 10 min	+++	+++
121 °C, 15 min	++	++
Enzyme		
Pepsin (pH 2.4, 2 h)	+/−	+/−
Trypsin (pH 7.4, 2 h)	−	−
Chymotrypsin (pH 7.4, 2 h)	−	−
Proteinase K (pH 7.4, 2 h)	−	−

Symbols represent inhibition zones after treatment: −, no inhibition; +, da <5 mm; ++, 5 mm<da<10 mm; +++, da>10 mm; +/−, 5-mm turbid halo

The results obtained showed that the MICs and MBCs varied depending on the temperature and the culture media in which the assays were carried out. No difference was observed between MIC and MBC against *E. coli*. The bacteriocin concentration required to inhibit the growth or kill *E. coli* at 37 °C in skim milk (64 AU/ml) was slightly lower than in BHI (170 AU/ml). On the other hand, the action of Ent35-MccV against *L. monocytogenes* at 37 °C exhibited a different pattern. Thus, it was found that the MIC against *L. monocytogenes* was 16 AU/ml in BHI and 4 AU/ml in milk. This means that the growth of *L. monocytogenes* was inhibited at a concentration of bacteriocin at least four times lower than that required to inhibit the growth of *E. coli* in each medium. Unlike what was observed for *E. coli*, the MBCs against *L. monocytogenes* were 250 and 500 AU/ml in BHI and milk, respectively, these resulting to be considerably higher than the MICs regardless of the culture medium. In other words, hybrid bacteriocin inhibits the growth of *L. monocytogenes* much better than *E. coli*, although it is more efficient to kill *E. coli* than *Listeria* spp. This result is consistent with previously reported results using other Gram-positive and Gram-negative (Acuña et al. 2012).

In turn, the MBC against *L. monocytogenes* did not show great difference with the MBC against *E. coli* in BHI, but differences were observed between the values of MBCs against *E. coli* and *L. monocytogenes* in skim milk, since almost three times more concentration of Ent35-MccV was required to achieve the MBC against *L. monocytogenes*. The probable cause of the antilisterial activity of the hybrid bacteriocin was affected by some component present in the milk.

Finally, considering that *L. monocytogenes* is a pathogen able to grow at refrigeration temperatures, MBCs was also determined at 4 °C. MBC values in both media were slightly lower (125 AU/ml in BHI and 250 AU/ml in milk) than the counterpart values obtained at 37 °C. This observation is consistent with experiments of inhibitory concentrations at different temperatures reported previously for enterocin CRL35 (Minahk et al. 2005).

Antimicrobial Activity of Ent35-MccV in Food Products

The effectiveness of bacteriocins in food systems is often low due to several factors such as adsorption of the peptide to food components like particles of fat, poor solubility, the generation of oxidation processes that destabilize its biological activity, inactivation or degradation by enzymes, presence of additives, effect of pH, or uneven distribution in the food matrix (Cleveland et al. 2001). The antimicrobial efficiency of the hybrid bacteriocin was evaluated in food products using Gram-

positive and Gram-negative bacterial indicator strains. To perform these studies, two different models of food were employed. Sterile skim milk was used as dairy model, and beef burgers were used as a meat model. Samples of these foods were treated without or with two different concentrations of bacteriocin 125 and 250 AU/ml and were inoculated with *E. coli* or *L. monocytogenes* as described above. The evolution of viable cells along time was determined after the treatment.

Effect of Ent35-MccV in Sterile Skim Milk

As shown in Fig. 1, 250 AU/ml of Ent35-MccV applied to sterile skim milk reduced the initial bacterial load of 10^4 CFU/ml of *L. monocytogenes* in almost two log units after 24 h at 37 °C. Moreover, Ent35-MccV was able to inhibit the growth of this pathogen by a factor of four magnitude orders as compared to the sample control without bacteriocin, after 48 h at 37 °C. However, slight increases in the bacterial counts at 48 h were observed, these observations being in agreement with Vignolo et al. (2000) who observed that the population of *Listeria* treated with enterocin CRL35 decreased initially but increased upon further incubation at 30 °C. *L. monocytogenes* had a similar behavior when treated with other bacteriocins as lactocin 705, nisin, or a bacteriocinogenic LAB in milk and other food systems (Chi-Zhang et al. 2004; Schillinger et al. 1998; Schmidt et al. 2009).

On the other hand, when the assay was performed at refrigeration temperatures, the bacterial load of the samples treated with bacteriocin decreased along time. There was a 2 and 3 log reduction of CFU/ml in the samples treated with 125 and 250 AU/ml of bacteriocin, respectively, with respect to the control assays, after 10 days of storage. Moreover, no growth of *L. monocytogenes* in milk samples treated with Ent35-MccV was observed at any time of the assay. However, Pucci et al. (1988) reported inhibition of *L. monocytogenes* by the class IIa bacteriocin pediocin PA-1 in several dairy systems but with a resurgence of the pathogen after 7 days at 4 °C (Pucci et al. 1988). The results obtained in our study are more in agreement with the previously reported results by Mitra et al. (2011) who noted that non-viable *L. monocytogenes* cells were detected at 13 h under refrigeration in milk after the addition of the class I bacteriocin nisin Z (Mitra et al. 2011). In addition, although it was mentioned that nisin and pediocin PA-1 are the only bacteriocins permitted in the food, the literature offers many examples of bacteriocins used in food preservation at laboratory scale. Between them, enterocin CRL35 (one of the parental bacteriocins of Ent35-MccV) was tested as biopreservative in the manufacture of artisan cheeses proving to be very effective controlling the growth of *L. monocytogenes* (Farias et al. 1994). Also, the two-component bacteriocin lacticin 3147 exhibited a great potential for the preservation of dairy products (Morgan et al. 1999; Ryan et al. 1996; Sobrino-López and Martín-Belloso 2008). It has also been reported that lacticin 3147 inhibited

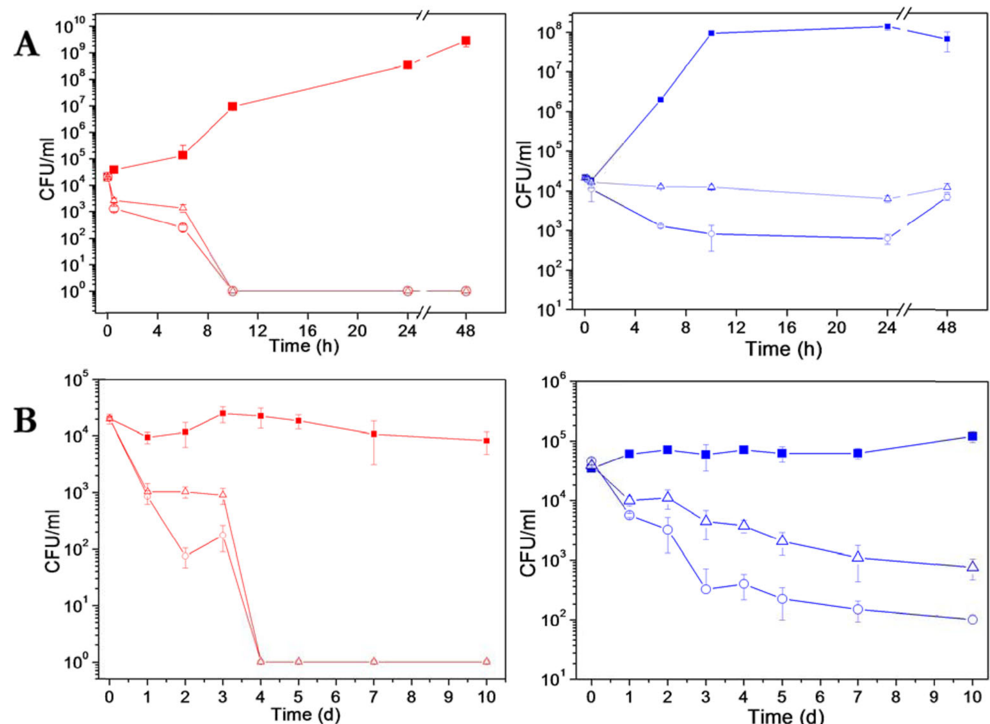

Fig. 1 Antimicrobial activity of Ent35-MccV in skim milk. Assays were carried out at 37 °C (**a**) and 4 °C (**b**) against *E. coli* (*left*) and *L. monocytogenes* (*right*). Cell viability is expressed as CFU/ml at several times. The results represent the average of three independent experiments. All the graphs show cells without treatment (*squares*), cells treated with 250 AU/ml of Ent35-MccV (*circles*), and cells treated with 125 AU/ml of Ent35-MccV (*triangles*)

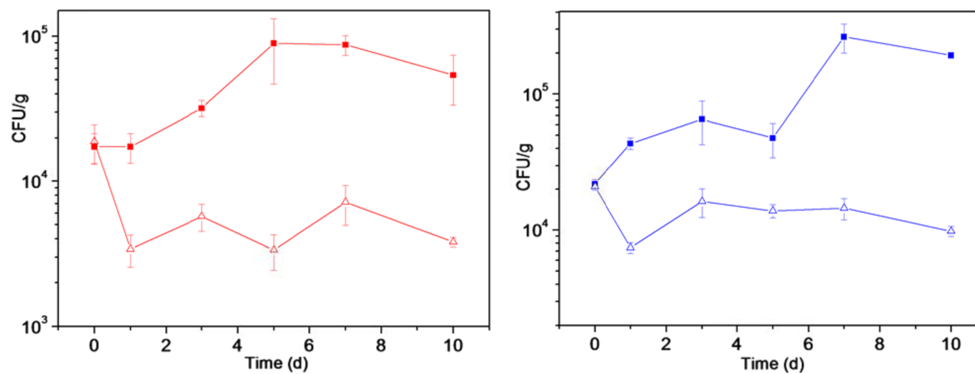

Fig. 2 Antimicrobial activity of Ent35-MccV in ground beef patties stored at 4 °C for 10 days. Cell viability is expressed as CFU/g at different times. *Left*: *E. coli* cells without bacteriocin (*squares*) and Ent35-MccV 125 AU/g (*triangles*). *Right*: *L. monocytogenes* control without bacteriocin (*squares*) and Ent35-MccV 125 AU/g (*triangles*). The results represent the average of three independent experiments

the growth and reduced the number of *L. monocytogenes* and *Staphylococcus aureus* in infant milk (Morgan et al. 1999).

When Ent35-MccV 125 or 250 AU/ml were assayed in milk samples at 37 °C against *E. coli* ATCC 13706, a complete elimination of viable cells was observed after 10 h of incubation. In like manner, when the experiment was performed at 4 °C, after the fourth day, no viable cells were detected. Either in the trial conducted at 37 °C or at refrigeration temperature, both concentrations of the bacteriocin tested caused total disappearance of CFUs.

These results indicate that Ent35-MccV could be effective for the biopreservation of refrigerated dairy foods and may prevent a possible contamination caused by *Listeria* or *E. coli* strains.

Effect of Ent35-MccV in Beef Burger Patties

It was previously reported that enterocin CRL35 was effective in controlling *L. monocytogenes* in a model of beef and shrimp (Concha et al. 1999; Vignolo et al. 2000), and the class IIa bacteriocin pediocin PA-1 was able to decrease the population of *L. monocytogenes* in a piece of beef (Nielsen et al. 1990). Patties inoculated with *L. monocytogenes* were treated with 125 AU/g of the hybrid bacteriocin. No significant decrease of the initial bacterial load was observed. However, the growth of *Listeria* was inhibited throughout the treatment. The final difference of CFU/g between bacteriocin-treated patties and untreated control after 10 days at refrigeration temperature was greater than one order of magnitude (Fig. 2). On the other hand, Ent35-MccV, at a final concentration of 125 AU/g, was able to reduce the initial bacterial load of *E. coli* almost one order of magnitude. Figure 2 shows a marked decrease in the CFUs from the first day and along the treatment in treated samples, compared to the control without bacteriocin which continued to grow slowly. Finally, after 10 days, the difference between untreated and Ent35-MccV-treated patties was a ~1.2 log CFU/g decrease.

Gram-negative food pathogens are causing great concern due to the emergence of outbreaks due mainly to enterohemorrhagic *E. coli* producing hemolytic-uremic syndrome and species of *Salmonella* and *Shigella*. The main problem of the bacteriocins produced by LAB is their inability to cross the outer membrane of Gram-negative bacteria, since

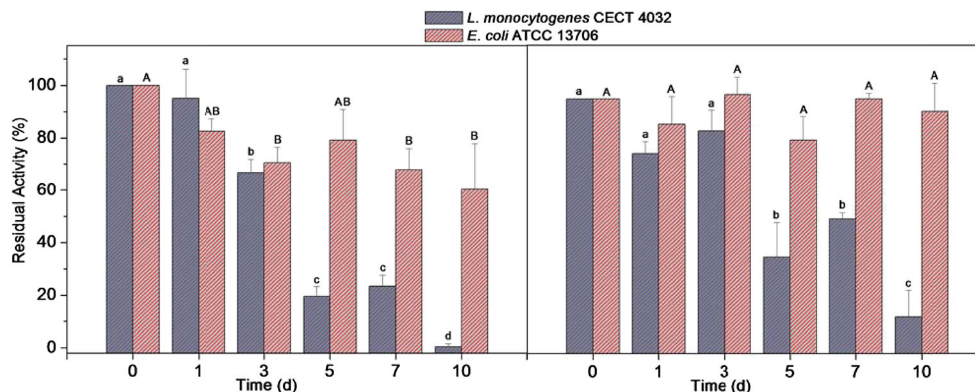

Fig. 3 Effect of storage at 4 °C on the stability of Ent35-MccV in ground beef patties (*left*) and milk (*right*). The residual activity is represented as percentage relative to the maximum measured value taken at initial time (day 0) against *E. coli* and *L. monocytogenes*. *Columns with the same letters* (*uppercase letters* for *E. coli* and *lowercase letters* for *L. monocytogenes*) are not significantly different according to Tukey's honestly significant difference (P=0.05)

these do not have a protein receptor for bacteriocins. In this aspect, nisin was effective against *E. coli* when added in combination with lactoferrin in different liquid cultures (Murdock et al. 2007). Also, the cyclic bacteriocin enterocin AS-48 was able to control the growth of this pathogen in crops and fruit juices when used in combination with heating or chelating agents. Regarding microcins (bacteriocins produced by Gram-negative bacteria), the only one which was tested as a food biopreservative was a mutant of microcin J25 (MccJ25) (Salomón and Farías 1992). MccJ25 is a class I bacteriocin produced by *E. coli* not susceptible to degradation by the intestinal proteases. Using genetic engineering techniques, a single amino acid substitution at residue 12 introducing a chymotrypsin cleavage site was built. The mutant microcin is then degraded in the intestine, proving to be active against Gram-negative pathogens in food models without altering the intestinal flora in rats (Pomares et al. 2009).

Ent35-MccV Stability in Model Foods

The biopreservation models tested until here involved food contamination in a first stage of its preparation or processing. However, the contamination may also occur in later stages of handling or storage, since *L. monocytogenes* is a ubiquitous bacterium (Saavedra et al. 2012). Accordingly, its presence is a common fact in food processing plants, transport vehicles and storage areas for raw materials. Moreover, operators could contaminate food when handling because it is normal to find *Listeria* in feces of healthy individuals (Rørvik 2000). Because of this, it was interesting to study whether Ent35-MccV could remain stable in the food for a considerable period of time, and in consequence, elucidate if it would be able to prevent a possible bacterial food contamination should this happen at a later stage of storage after the addition of the bacteriocin. Therefore, in order to test Ent35-MccV stability in biological products, its inhibitory activity was followed up to 10 days determined at different days after being added to the different food models. The experiments were carried out in the two food models tested: sterile skim milk and ground beef patties. Samples of these foods were treated with Ent35-MccV and kept in individual bags (ground beef patties) or in individual falcon tubes (skim milk) at refrigeration temperature. The residual antimicrobial activity was quantified periodically for 10 days.

Figure 3 shows that Ent35-MccV retained more than 60 % of its antimicrobial activity at least 3 days in both food models. In sterile skim milk, 95 % of its activity against *E. coli* and 20 % against *L. monocytogenes* remained after 10 days in preparations of sterile skim milk (Fig. 3, right panel). On the other hand, on ground beef patties, Ent35-MccV completely lost its antilisterial activity after 10 days on ground beef patties, while it retained 20 % of its initial activity after 1 week at 4 °C. Moreover, Ent35-MccV maintained the 60 % of its initial activity against *E. coli* after 10 days (Fig. 3, left panel). The shelf life and display life reported for ground meat and derivatives in overwrapped air-permeable packaging systems is 2–3 days (Delmore 2009). During this period, Ent35-MccV antimicrobial activity against both pathogens remained above 60 %. These results suggest that Ent35-MccV could retain preservative effects during a significant time for highly perishable food products. However, antilisterial activity decreased along time. Thus, after 1 week, only 20 % of initial activity remained, and there was a total loss after 10 days.

Conclusions

In summary, the hybrid bacteriocin Ent35-MccV exhibited broad inhibition spectrum against Gram-positive and Gram-negative emergent foodborne pathogens and spoilage strains. The peptide inhibited the growth of *L. monocytogenes*, *E. coli*, and *Shigella*.

Apart from its antimicrobial activity, the other properties that make it a promising food preservative are the sensitivity to protease, thermal stability, and the maintenance of antimicrobial activity in food models after storage at refrigeration temperature. Then, from the perspective of the processing, the thermal stability of the peptide allows its use from the beginning of food production. Thus, the hybrid bacteriocin may be added in a first moment to the fresh food to prevent contamination with microorganisms sensitive and extend the shelf life after cooking or other heat treatment. As far as it is known, the results of this study indicate that Ent35-MccV is the first example of a lineal genetically engineered bacteriocin used alone as an effective approach to control Gram-negative and Gram-positive foodborne and spoilage bacteria in food products. For bacteriocins to be massively employed on food industries, it is necessary to adjust scientific, legal, and administrative aspects which will achieve their approval as biopreservatives (Benmechernene et al. 2013). The results demonstrate that hybrid bacteriocins can be a solution to develop additives suitable to the needs of the food industries.

Acknowledgments Financial support was provided by Grants PIP 0779 from CONICET, PICT 2998 from the Agencia Nacional de Promoción Científica y Tecnológica ANPCyT, and PIUNT D548/1 from UNT. This work was also funded by the project 10PXIB261045PR from Xunta de Galicia and by the project AGL2010-19646 from the Spanish Ministry of Science and Technology. The work of L. Acuña is supported by CONICET and USC-Santander fellowships. N. Corbalan is recipient of a CONICET fellowship. The work of I.C. Fernandez was supported by a "Lucas Labrada" research contract from Xunta de Galicia. The authors thank Carlos Franco and José M. Miranda for providing generously the *E. coli*, *Salmonella* spp., and *L. monocytogenes* food isolates.

References

Acuña, L., Morero, R., & Bellomio, A. (2011). Development of wide-spectrum hybrid bacteriocins for food biopreservation. *Food and Bioprocess Technology, 4*(6), 1029–1049. doi:10.1007/s11947-010-0465-7.

Acuña, L., Picariello, G., Sesma, F., Morero, R. D., & Bellomio, A. (2012). A new hybrid bacteriocin, Ent35–MccV, displays antimicrobial activity against pathogenic Gram-positive and Gram-negative bacteria. *FEBS Open Bio, 2*, 12–19. doi:10.1016/j.fob.2012.01.002.

Benmechernene, Z., Fernandez-No, I., Kihal, M., Böhme, K., Calo-Mata, P., & Barros-Velazquez, J. (2013). Recent patents on bacteriocins: food and biomedical applications. *Recent patents on DNA & gene sequences, 7*(1), 66–73.

Calo-Mata, P., Arlindo, S., Boehme, K., de Miguel, T., Pascoal, A., & Barros-Velazquez, J. (2008). Current applications and future trends of lactic acid bacteria and their bacteriocins for the biopreservation of aquatic food products. *Food and Bioprocess Technology, 1*(1), 43–63. doi:10.1007/s11947-007-0021-2.

Castellano, P., Belfiore, C., & Vignolo, G. (2011). Combination of bioprotective cultures with EDTA to reduce Escherichia coli O157:H7 in frozen ground-beef patties. *Food Control, 22*(8), 1461–1465. doi:10.1016/j.foodcont.2011.02.018.

Chalón, M. C., Acuña, L., Morero, R. D., Minahk, C. J., & Bellomio, A. (2012). Membrane-active bacteriocins to control Salmonella in foods: are they the definite hurdle? *Food Research International, 45*(2), 735–744. doi:10.1016/j.foodres.2011.08.024.

Chen, C. M., Sebranek, J. G., Dickson, J. S., & Mendonca, A. F. (2004). Combining pediocin with postpackaging irradiation for control of Listeria monocytogenes on frankfurters. *Journal of Food Protection, 67*(9), 1866–1875.

Chi-Zhang, Y., Yam, K. L., & Chikindas, M. L. (2004). Effective control of *Listeria monocytogenes* by combination of nisin formulated and slowly released into a broth system. *International Journal of Food Microbiology, 90*(1), 15–22.

Chounou, N., Chouliara, E., Mexis, S. F., Stavros, K., Georgantelis, D., & Kontominas, M. G. (2013). Shelf life extension of ground meat stored at 4 °C using chitosan and an oxygen absorber. *International Journal of Food Science and Technology, 48*(1), 89–95.

Cintas, L. M., Casaus, P., Håvarstein, L. S., Hernández, P. E., & Nes, I. F. (1997). Biochemical and genetic characterization of enterocin P, a novel sec-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. *Applied and Environmental Microbiology, 63*(11), 4321–4330. Accessed 22 June 2010.

Cleveland, J., Montville, T. J., Nes, I. F., & Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology, 71*(1), 1–20. Accessed 25 January 2011.

Concha, R., Farías, M. E., Kümmerlin, R., & Sesma, F. (1999). Enterocin-35, a bacteriocin with activity against *Listeria monocytogenes*. Possible use in the food industry. *Revista Latinoamericana de Microbiología, 41*(3), 133–138.

Cotter, P. D., Hill, C., & Ross, R. P. (2005). Bacteriocins: developing innate immunity for food. *Nature reviews. Microbiology, 3*(10), 777–788. doi:10.1038/nrmicro1273.

Delmore, J.R. (2009). Beef Shelf-life. http://www.beefresearch.org/cmdocs/beefresearch/pe/beef_shelf_life.pdf. Accessed 15 Dec 2014.

Duquesne, S., Destoumieux-Garzón, D., Peduzzi, J., & Rebuffat, S. (2007). Microcins, gene-encoded antibacterial peptides from enterobacteria. *Natural Product Reports, 24*(4), 708–734. doi:10.1039/b516237h.

Farias, M. E., De Ruiz Holgado, A. A. P., & Sesma, F. (1994). Bacteriocin production by lactic acid bacteria isolated from regional cheeses: inhibition of foodborne pathogens. *Journal of Food Protection, 57*(11), 1013–1015.

Fath, M. J., Zhang, L. H., Rush, J., & Kolter, R. (1994). Purification and characterization of colicin V from *Escherichia coli* culture supernatants. *Biochemistry, 33*(22), 6911–6917.

Fernández-No, I. C., Böhme, K., Gallardo, J. M., Barros-Velázquez, J., Cañas, B., & Calo-Mata, P. (2010). Differential characterization of biogenic amine-producing bacteria involved in food poisoning using MALDI-TOF mass fingerprinting. *Electrophoresis, 31*(6), 1116–1127. doi:10.1002/elps.200900591.

Gao, Y., Li, D., & Liu, X. (2013). Evaluation of the factors affecting the activity of sakacin C2 against *E. coli* in milk. *Food Control, 30*(2), 453–458. doi:10.1016/j.foodcont.2012.07.013.

Giraffa, G., Neviani, E., & Tarelli, G. T. (1994). Antilisterial activity by enterococci in a model predicting the temperature evolution of Taleggio, an Italian soft cheese. *Journal of Dairy Science, 77*(5), 1176–1182. doi:10.3168/jds.S0022-0302(94)77055-7.

Hammami, R., Zouhir, A., Le Lay, C., Ben Hamida, J., & Fliss, I. (2010). BACTIBASE second release: a database and tool platform for bacteriocin characterization. *BMC Microbiology, 10*, 22. doi:10.1186/1471-2180-10-22.

Huq, T., Vu, K. D., Riedl, B., Bouchard, J., & Lacroix, M. (2015). Synergistic effect of gamma (γ)-irradiation and microencapsulated antimicrobials against *Listeria monocytogenes* on ready-to-eat (RTE) meat. *Food Microbiology, 46*, 507–514. doi:10.1016/j.fm.2014.09.013.

Jin, T., Liu, L., Sommers, C. H., Boyd, G., & Zhang, H. (2009). Radiation sensitization and post irradiation proliferation of Listeria monocytogenes on ready-to-eat deli meat in the presence of pectin-nisin films. *Journal of Food Protection, 72*(3), 644–649.

Juneja, V. K., Altuntaş, E. G., Ayhan, K., Hwang, C.-A., Sheen, S., & Friedman, M. (2013). Predictive model for the reduction of heat resistance of *Listeria monocytogenes* in ground beef by the combined effect of sodium chloride and apple polyphenols. *International Journal of Food Microbiology, 164*(1), 54–59. doi:10.1016/j.ijfoodmicro.2013.03.008.

Leistner, L. (1978). Hurdle effect and energy saving. In W. K. Downey (Ed.), *Food quality and nutrition* (pp. 553–557). London: Applied Science Publishers.

Leistner, L., & Grahame, W. (2005). Update on hurdle technology approaches to food preservation. In P. M. Davidson, J. N. Sofos, & A. L. Branen (Eds.), *Antimicrobials in Food, Third Edition* (3rd ed., pp. 621–641). CRC Press.

Li, D., Liu, X., & Gao, Y. (2014). Synergistic Effects of sakacin C2 in combination with food preservatives. In T.-C. Zhang, P. Ouyang, S. Kaplan, & B. Skarnes (Eds.), *Proceedings of the 2012 International Conference on Applied Biotechnology (ICAB 2012)* (pp. 455–464). Springer Berlin Heidelberg. http://link.springer.com/chapter/10.1007/978-3-642-37916-1_47.

Metaxopoulos, J., Mataragas, M., & Drosinos, E. H. (2002). Microbial interaction in cooked cured meat products under vacuum or modified atmosphere at 4 degrees C. *Journal of Applied Microbiology, 93*(3), 363–373.

Minahk, C. J., Saavedra, L., Sesma, F., & Morero, R. (2005). Membrane viscosity is a major modulating factor of the enterocin CRL35 activity. *Biochimie, 87*(2), 181–186. doi:10.1016/j.biochi.2004.10.013.

Miranda, J. M., Vázquez, B. I., Fente, C. A., Calo-Mata, P., Cepeda, A., & Franco, C. M. (2008). Comparison of antimicrobial resistance in *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes* strains isolated from organic and conventional poultry meat. *Journal of Food Protection, 71*(12), 2537–2542.

Mitra, S., Mukhopadhyay, B. C., & Biswas, S. R. (2011). Potential application of the nisin Z preparation of *Lactococcus lactis* W8 in preservation of milk. *Letters in Applied Microbiology, 53*(1), 98–105. doi:10.1111/j.1472-765X.2011.03075.x.

Morgan, S. M., Galvin, M., Kelly, J., Ross, R. P., & Hill, C. (1999). Development of a lacticin 3147-enriched whey powder with inhibitory activity against foodborne pathogens. *Journal of Food Protection, 62*(9), 1011–1016.

Murdock, C. A., Cleveland, J., Matthews, K. R., & Chikindas, M. L. (2007). The synergistic effect of nisin and lactoferrin on the inhibition of *Listeria monocytogenes* and *Escherichia coli* O157:H7. *Letters in Applied Microbiology, 44*(3), 255–261. doi:10.1111/j.1472-765X.2006.02076.x.

Nielsen, J. W., Dickson, J. S., & Crouse, J. D. (1990). Use of a bacteriocin produced by *Pediococcus acidilactici* to inhibit *Listeria monocytogenes* associated with fresh meat. *Applied and Environmental Microbiology, 56*(7), 2142–2145. Accessed 2 June 2014.

Pomares, M. F., Salomón, R. A., Pavlova, O., Severinov, K., Farías, R., & Vincent, P. A. (2009). Potential applicability of chymotrypsin-susceptible microcin J25 derivatives to food preservation. *Applied and Environmental Microbiology, 75*(17), 5734–5738. doi:10.1128/AEM. 01070-09.

Pucci, M. J., Vedamuthu, E. R., Kunka, B. S., & Vandenbergh, P. A. (1988). Inhibition of *Listeria monocytogenes* by using bacteriocin PA-1 produced by *Pediococcus acidilactici* PAC 1.0. *Applied and Environmental Microbiology, 54*(10), 2349–2353.

Ray, B., & Bhunia, A. (2007). *Fundamental Food Microbiology, Fourth Edition* (4th ed.). CRC Press.

Rørvik, L. M. (2000). *Listeria monocytogenes* in the smoked salmon industry. *International Journal of Food Microbiology, 62*(3), 183–190.

Ryan, M. P., Rea, M. C., Hill, C., & Ross, R. P. (1996). An application in cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. *Applied and Environmental Microbiology, 62*(2), 612–619.

Saavedra, L., Minahk, C., de Ruiz Holgado, A. P., & Sesma, F. (2004). Enhancement of the enterocin CRL35 activity by a synthetic peptide derived from the NH2-terminal sequence. *Antimicrobial Agents and Chemotherapy, 48*(7), 2778–2781. doi:10.1128/AAC. 48.7.2778-2781.2004.

Saavedra, L., Bellomio, A., Hebert, E., Minahk, C. J., Suarez, N., & Sesma, F. (2012). *Listeria: epidemiology, pathogenesis and novel potential treatments. In applications of natural products in food*. Nueva York: Nova Science Publishers.

Salomón, R. A., & Farías, R. N. (1992). Microcin 25, a novel antimicrobial peptide produced by *Escherichia coli*. *Journal of Bacteriology, 174*(22), 7428–7435.

Sánchez-Hidalgo, M., Montalbán-López, M., Cebrián, R., Valdivia, E., Martínez-Bueno, M., & Maqueda, M. (2011). AS-48 bacteriocin: close to perfection. *Cellular and Molecular Life Sciences: CMLS, 68*(17), 2845–2857. doi:10.1007/s00018-011-0724-4.

Schillinger, U., Chung, H. S., Keppler, K., & Holzapfel, W. H. (1998). Use of bacteriocinogenic lactic acid bacteria to inhibit spontaneous nisin-resistant mutants of *Listeria monocytogenes* Scott A. *Journal of Applied Microbiology, 85*(4), 657–663.

Schmidt, S. E., Holub, G., Sturino, J. M., & Taylor, T. M. (2009). Suppression of *Listeria monocytogenes* Scott A in fluid milk by free and liposome-entrapped nisin. *Probiotics and Antimicrobial Proteins, 1*(2), 152–158. doi:10.1007/s12602-009-9022-y.

Smigic, N., & Rajkovic, A. (2014). Hurdle technology. In V. R. Rai & J. A. Bai (Eds.), *Microbial food safety and preservation techniques* (1st ed.). Boca Raton: CRC Press.

Sobrino-López, A., & Martín-Belloso, O. (2008). Use of nisin and other bacteriocins for preservation of dairy products. *International Dairy Journal, 18*(4), 329–343. doi:10.1016/j.idairyj.2007.11.009.

Techathuvanan, C., Reyes, F., David, J. R. D., & Davidson, P. M. (2014). Efficacy of commercial natural antimicrobials alone and in combinations against pathogenic and spoilage microorganisms. *Journal of Food Protection, 77*(2), 269–275. doi:10.4315/0362-028X.JFP-13-288.

Turner, M. (2011). Microbe outbreak panics Europe. *Nature News, 474*(7350), 137–137. doi:10.1038/474137a.

Uesugi, A. R., & Moraru, C. I. (2009). Reduction of Listeria on ready-to-eat sausages after exposure to a combination of pulsed light and nisin. *Journal of Food Protection, 72*(2), 347–353.

Vignolo, G., Palacios, J., Farias, M. E., Sesma, F., Schillinger, U., Holzapfel, W., & Oliver, G. (2000). Combined effect of bacteriocins on the survival of various *Listeria* species in broth and meat system. *Current Microbiology, 41*(6), 410–416.

WHO. (2011). A Public Health Review of the enterohaemorrhagic *Escherichia coli* outbreak in Germany. http://www.euro.who.int/en/health-topics/disease-prevention/food-safety/publications/2011/a-public-health-review-of-the-enterohaemorrhagic-escherichia-coli-outbreak-in-germany. Accessed 25 Feb 2014.

WHO/FAO. (n.d.). CODEX Alimentarius: Standards. http://www.codexalimentarius.org/standards/en/. Accessed 4 Apr 2014.

Wulijideligen, N., Asahina, T., Hara, K., Arakawa, K., Nakano, H., & Miyamoto, T. (2012). Production of bacteriocin by *Leuconostoc mesenteroides* 406 isolated from Mongolian fermented mare's milk, airag. *Animal Science Journal, 83*(10), 704–711. doi:10.1111/j.1740-0929.2012.01010.x.