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Leuconostoc citreum MB1 as biocontrol agent of *Listeria monocytogenes* in milk

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Cell-free supernatant from *Leuconostoc citreum* MB1 revealed specific antilisterial activity. Preliminary studies demonstrated the proteinaceous, heat-stable, bacteriocin-like trait of the antimicrobial components present in the supernatant. Determination of the genes encoding bacteriocins by PCR and DNA sequencing led to amplification products highly homologous with leucocin A (found in diverse *Leuconostoc* species) and UviB (found in *Leuc. citreum* KM20) sequences. Additionally, antimicrobial activity of cell-free supernatant from *Leuc. citreum* MB1 was revealed by an inhibition halo of the SDS-PAGE gel subjected to a direct detection using *Listeria monocytogenes* as indicator strain. Different assays were carried out to assess the capacity of *Leuc. citreum* MB1 to control *List. monocytogenes* growth: (*i*) inactivation kinetics of the pathogen by antilisterial compounds present in concentrated cell-free supernatant from *Leuc. citreum* MB1, (*ii*) evaluation of optimal *Leuc. citreum* MB1 initial concentration to obtain maximum *List. monocytogenes* ATCC 15313 inhibition, and (*iii*) biocontrol of *List. monocytogenes* ATCC 15313 with *Leuc. citreum* MB1 during growth in milk at refrigeration temperature. According to our results, it is unquestionable that at least one bacteriocin is active in *Leuc. citreum* MB1, since important antilisterial activity was verified either in its cell-free supernatant or in co-culture experiments. Co-culture tests showed that $\sim 10^7$ CFU/ml *Leuc. citreum* MB1 was the optimal initial concentration to obtain maximum pathogen inhibition. Moreover, *Leuc. citreum* MB1 was able to delay *List. monocytogenes* growth at refrigerated temperature.

Keywords: *Leuconostoc*, bacteriocin, antilisterial activity.

Leuconostocs are heterofermentative lactic acid bacteria frequently used in dairy industry. Some species of *Leuconostoc* are very important for fermented dairy products, as they contribute to the formation of openings in blue cheeses and the organoleptic characteristics of butter and cream as well. Additionally, as with the majority of lactic acid bacteria (LAB), *Leuconostoc* may have a role in the prevention of growth and activity of spoilage and pathogenic microorganisms. This feature has been attributed to diverse metabolic end products including organic acids (lactic and citric), bacteriocins, hydrogen peroxide, diacetyl and ethanol (Alakomi et al. 2000; Cleveland et al. 2001; Hemme & Focaud-Scheunemann 2004).

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Bacteriocins are ribosomally synthesised antimicrobial peptides (Cotter et al. 2005) antagonistic against microorganisms that are related or not to the producer bacterium.

Theoretically, purified bacteriocins could be prepared as an ingredient and incorporated afterwards in the food. However, in a practical way, this is difficult to accomplish since it requires regulatory approval. An alternative could be the inclusion of bacteriocin-producing strains to the starter culture, so the antimicrobial compound will be produced in situ while the strain is developing, which is a regulatory-free action and has the advantage of localised and continuous release of the antibacterial compound while the culture is multiplying (Deegan et al. 2006; Trias et al. 2008). Many bacteriocins produced by *Leuconostoc* strains belong to subclass IIa, are pediocin-like, small heat-stable non-modified peptides and they are all active against *Listeria* (Hastings et al. 1991; Stiles, 1994; Papathanasopoulos et al. 1997; Blom et al. 1999; Ennahar et al. 2000).

Listeria monocytogenes is an opportunistic pathogen responsible of listerioris, one of most significant food-borne diseases. Immunodepressed individuals including pregnant women, infants and aged persons, are more susceptible to suffer from infections. *List. monocytogenes* is ubiquitous and has the ability of surviving and growing under adverse environments, especially at refrigeration temperatures, acid conditions and high salt concentrations. These features makes it a potential health threat to consumers of foods that have long, refrigerate shelf lives and are eaten without further cooking prior to consumption, as milk and dairy products (Cataldo et al. 2007; Mellefont et al. 2008).

Most of *Leuconostoc* bacteriocins characterised are produced by *Leuc. mesenteroides* (Daba et al. 1991; Héchard et al. 1992, 1999; Revol-Junelles et al. 1996; Papathanasopoulos et al. 1997; Mataragas et al. 2003; Trias et al. 2008; Xiraphi et al. 2008), followed by those produced by *Leuc. gelidum* (Hastings et al. 1991), *Leuc. carnosum* (Felix et al. 1994) and *Leuc. pseudomesenteroides* (Sawa et al. 2010). According to our knowledge, there is only one reported *Leuc. citreum* strain carrying a bacteriocin gene (Kim et al. 2008). In a previous study carried out by our group (Cardamone et al. 2011), specific antilisterial activity was evidenced in the supernatant of *Leuc. citreum* MB1. Preliminary studies demonstrated the proteinaceous, heat-stable, bacteriocin-like trait of the antimicrobial component. The aim of the present work was to evaluate the ability of *Leuc. citreum* MB1 to control the *List. monocytogenes* growth by the production of antilisterial bacteriocin. In addition, a preliminary identification of the bacteriocin/s produced by this strain was carried out.

Materials and methods

Bacterial strains and growth conditions

The bacteriocin-producing strain *Leuc. citreum* MB1 isolated from soft Cremoso Argentino cheese was previously characterised by our group (Cardamone et al. 2011). *List. monocytogenes* ATCC 15313 was used as pathogenic strain. *Leuc. citreum* MB1 was routinely grown in MRS broth (32 °C, 16–18 h), while *List. monocytogenes* ATCC 15313 was grown in BHI broth (37 °C, 16–18 h).

Preliminary identification of the antilisterial compound

PCR amplification and sequencing of the bacteriocin gene. To determine the genes encoding bacteriocins, PCR and DNA sequencing were performed using the primers listed in Table 1. Leucocins A, B, K, Q, N, A-TA33a, mesentericins B, Y, G, H, F, I, C, D, E and UviB were considered. A number of primers previously reported (Xiraphi et al. 2008; Sawa et al. 2010) were applied with some modifications. Based on available sequences of bacteriocins from *Leuconostoc* in GeneBank database, new specific primers were designed. A standard reaction containing PCR buffer 10×, 200 μM dNTPs, 2·25 U/100 μl Taq-polymerase (Life Technologies™, Invitrogen Argentina S.A.) and 0·210 μM of each primer was used. One nanogram of DNA, extracted from *Leuc. citreum* MB1 using a commercial kit (Gen Elute Bacterial Genomic DNA, Sigma) was loaded into the PCR reaction. Amplifications were carried out in a GeneAmp PCR System 2400 (Applied Biosystem) thermalcycler. The PCR conditions included an initial denaturation at 95 °C for 5 min, followed by 35 amplification cycles at 95 °C for 1 min (denaturation), 45 °C for 45 s (annealing) and 72 °C for 1 min and 30 s (extension), and a final extension step at 72 °C for 7 min. Each PCR reaction was analysed by electrophoresis agarose gel (1·8% w/v). Positive PCR products of the expected molecular weight were purified using GenElute™ PCR Clean-Up Kit (Sigma) and sent for sequencing (Macrogen Korea, Seoul, Korea). Sequences were aligned using GeneBank tools for identification purposes.

SDS-PAGE analysis. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on a discontinuos gradient gel was performed according to Laemmli procedure (Laemmli, 1970) modificated by Candioti et al. (2002). A 4% and 20% (w/v) of acrylamide concentration of stacking and separating gels were respectively used. Separation was carried out at alkaline pH (8·3) with a Tris-Glycine carrier buffer, using a Mini Protean II electrophoretic chamber (Bio-Rad Laboratories, California, USA). The working conditions were 150 V and 45 mA. A wide-range (2·5–200 kDa) molecular weight marker was used (Mark12™ Unstained Standard, Novex, Life Technologies™). Cell-free supernatant (CFS) was obtained by centrifugation (12 000 g, 5 min, 5 °C) of overnight *Leuc. citreum* MB1 culture and sterilisation by filtration (0·22-μm pore diameter, Millipore). Two identical gels were prepared, in which 10 and 20 μl CFS from *Leuc. citreum* MB1 were subjected to the electrophoresis. Both gels were afterwards stained/fixed with Coomasie blue and destained with a distilled water/ethanol/acetic acid (1 : 1 : 0·4) solution. Afterwards, one of gels was used for direct detection of antimicrobial activity. Briefly, the gel was boiled (100 °C for 5 min) in distilled water, washed in sterilised bidistilled water and finally revealed by a double-layer method: a BHI agar plate was overlaid with BHI soft agar containing 10^7 CFU/ml *List. monocytogenes* 15313 as indicator strain; the gel was then put onto the soft agar and finally covered by another BHI soft agar layer also containing the pathogenic strain. Incubation was carried out for 24 h at 37 °C, and bacteriocin activity was revealed by a transparent halo of growth inhibition.

Inactivation kinetics of List. monocytogenes *ATCC 15313 with* Leuc. citreum *MB1 concentrated cell-free supernatant*

Bacteriocins can be added as additives in fermented food products, thus avoiding negative interactions between primary starter and bacteriocin-producing strains. The present

Table 1. Primers used for PCR amplification of genes responsible for bacteriocin production in *Leuconostoc citreum* MB1

Primer†	Sequence (5′–3′)	Target gene	Accession number	Reference/origin
Leucocins				
lcnAF	ATGATGAACATGAAACCTAC	*lcnA-UAL187*	M64371	Xiraphi et al. (2008)
lcnAR	TTACCAGAAACCATTTCCAC			Xiraphi et al. (2008)
lcnAGF	AAACTGTCTTCTCCATTATTC	*lcnA (A-UAL187, A-QU15) and lcnB*	M64371, AB499610	This work
lcnAGR	TTCTGACCAATTACACCAAG			Sawa et al. (2010)
lcnAPAF‡§	TAAGTATTATGGTAACGGAG			This work
lcnAQU15F‡¶	ATGAATAACATGAAATCTGC	*lccA-QU15*	AB499610	This work
lcnATAF	TACTACTTGTACTTTGGATG	*lcnA-TA33a*	AF036713	Xiraphi et al. (2008)
lcnATAR	TGGTCTTTGGTAAAGGTG			Xiraphi et al. (2008)
lcnBF	ATGAATAACATGAAATCTGC	*lcnB*	S72922	Xiraphi et al. (2008)
lcnBR¶	TTACCAGAAACCATTTCCAC			Xiraphi et al. (2008)
lcnQF	ATGAAAAATCAGTTAATGTCTTTC	*lccQ*	AB499611	Sawa et al. (2010)
lcnQR	TTAGTGCCAACGTTTGTAATC			Sawa et al. (2010)
lcnNF	ATGAATAAAGAATATAATAGC	*lccN*	AB499611	This work
lcnNR	TCAATGCTTATGCTTATAG			This work
lcnKF	ATGAAAAAATTCAAAGAACTAAAAG	*lcnK*	AF420260	Xiraphi et al. (2008)
lcnKR	TTAATTGTTAATGGTTGAAGAG			Xiraphi et al. (2008)
Mesentericins				
mesBF	ATGCAAGATAAAACAAATTTG	*mesB*	AF143443	Xiraphi et al. (2008)
mesBR	TTATTTGTGGTTCTTGATC			Xiraphi et al. (2008)
mesYF	ATGACGAATATGAAGTCTGTGG	*mesY*	X81803	Xiraphi et al. (2008)
mesYR	TTACCAAAATCCATTTCCACC			Xiraphi et al. (2008)
mesGF	ATGTTCGTCAAAATAAATTTG	*mesG*	AF143443	This work
mesGR	TTATCTTGTTCCCACAATC			This work
mesHF	ATGGCTAAGTATATTGTCTC	*mesH*	AF143443	This work
mesHR	TTAATCAAAAATTAGATATTTAG			This work
mesFF	ATGATAGAATTTTCTAAATTTACC	*mesF*	AF143443	This work
mesFR	TTATATTGATAATATGTCTTTAATG			This work
mesIF	AAAAAGTATCGGTATTTAGAAG	*mesI*	X81803	This work
mesIR	TTACTGATCGAAAACGCTGTAAAAC			This work
mesCF	ATGCCTGATTTAAACATAAATG	*mesC*	X81803	This work
mesCR	ACATTTGTCGTTAATTGCTG			This work
mesDF	ATGGTTAAAACTCCAATGTTTC	*mesD*	X81803	This work
mesDR	TTAAATAGTGACGCATAGAAGC			This work
mesEF	ATGTTTGATCCAAAATACTTAG	*mesE*	X81803	This work
mesER	TTAGTGTTTCCTAGCAGAATG			This work
Other				
uviBF	ATGCAAAAAGATGAATTATGG	*uviB*	6063826	This work
uviBR	CTATTGAAATAAATTGCCC			This work

†F: forward primer; R: reverse primer
‡For these two forward primers, lcnBR was used as reverse primer
§Primer targeted to active peptide
¶ Primer targeted to leader peptide

test was aimed to evaluate the antilisterial ability of *Leuc. citreum* MB1 concentrated supernatant which contains antilisterial bacteriocins. Following this purpose, concentrated cell-free supernatant (CCFS) from a fresh *Leuc. citreum* MB1 culture (16–18 h, 32 °C) was obtained after centrifugation (12 000 g, 5 min, 5 °C), concentration (5×, in a rotary evaporator) and filter-sterilisation (0·22 μM pore diameter, Millipore). Aliquots of a fresh *List. monocytogenes* ATCC 15313 culture (16–18 h, 37 °C) were inoculated in 10 ml BHI broth, at a low ($7{\cdot}3\times10^3$ CFU/ml) and a high initial cell load ($8{\cdot}7\times10^7$ CFU/ml). Then, the CCFS was added in volumes of 50 or 500 μl, and incubated for 48 h at 37 °C. Additionally, other aliquots of the pathogenic strain inoculated at the same low and high initial concentrations, were pre-grown at 37 °C until $OD_{560} \sim 0{\cdot}15$ (for low initial cell load) or $OD_{560} \sim 0{\cdot}5$ (for high initial cell load), subsequently added of 50 or 500 μl CCFS and incubated at 37 °C for 48 h. The latter condition emulates a post-processing contamination. Tubes of BHI broth inoculated only with *List. monocytogenes* ATCC 15313 were used as controls. Growth of the pathogenic strain was followed by measuring OD_{560} at certain intervals of time.

Co-cultures experiments

Optimal Leuc. citreum *MB1 initial concentration for* List. monocytogenes *ATCC 15313 biocontrol*. Diverse initial cell loads of *Leuc. citreum* MB1 and *List. monocytogenes* ATCC 15313 were used to find maximum pathogen inhibition. With this aim, overnight cultures (16–18 h, 32 °C) of *Leuc. citreum* MB1 and the pathogenic strain (16–18 h, 37 °C) were co-inoculated in 10 ml reconstituted sterilised skim milk at: (*i*) 10^6 and 10^4 CFU/ml; (*ii*) 10^4 and 10^4 CFU/ml or (*iii*) 10^6 and 10^2 CFU/ml of *Leuconostoc* and *Listeria* initial concentrations, respectively. Tubes of skim milk inoculated only with *Leuc. citreum* MB1 or *List. monocytogenes* ATCC 15313 at each initial concentration were used as growth controls. Co-culture and control tubes were incubated at 34 °C for 48 h in a water bath. This temperature was selected to allow growth of both pathogenic and bacteriocinogenic strains. At 0, 24 and 48 h, pH values were measured and viable cell counts in BHI agar (*Listeria*) or MRS agar (*Leuconostoc*) of both controls and co-cultures were performed.

Biocontrol of List. monocytogenes *by* Leuc. citreum *MB1 in milk at refrigeration temperature*. The ability of *Leuc. citreum* MB1 to control *List. monocytogenes* ATCC 15313 growth was studied in a fermented-milk model. Tubes of skim milk were co-inoculated with *Leuc. citreum* MB1 and *List. monocytogenes* ATCC 15313 at 10^7 and 10^2 CFU/ml, respectively. Tubes of skim milk inoculated only with *Leuconostoc* or *Listeria* were used as controls. One set of inoculated tubes was immediately incubated at 8 °C, while the other was pre-incubated at 30 °C for 5 h and then kept at 8 °C during 28 d. Pre-incubation step was intended to accelerate the growth of the strains before incubation at 8 °C. Viable cell counts in BHI (*Listeria*) or MRS (*Leuconostoc*) agar were carried out at the beginning and throughout the experience.

Results and discussion

Preliminary identification of the antilisterial compound

PCR amplification and sequencing of the bacteriocin gene. From all bacteriocin-specific primers tested, those for leucocin A (lcnAPAF and lcnBR) and UviB (uviBF and uviBR) showed amplification products with the expected size (~130 and ~300 bp, respectively). Amplification products of expected size were sequenced and compared with nucleotide sequences of the corresponding bacteriocins deposited in GenBank (Fig. 1). Amplification product of ~130 bp (named *a-mb1*, putative leucocin A-MB1) exhibited high homology (>98%) with genes codifying for active peptide from leucocin A: A-QU 15 (accession no. AB499610), A-UAL 187 (accession no. M64371) and B-Ta11a (accession no. S72922). No amplification was obtained with primers designed on the basis of the leader peptide from leucocins described above. In comparison with previous findings (Sawa et al. 2010), putative leucocin A-MB1 found in *Leuc. citreum* MB1 would share the same nucleotide sequence as that codifying for active peptide. However, structural genes (including immunity) and nucleotide sequence codifying for leader peptide could be different. Similarly leucocin A is produced by diverse *Leuconostoc* species, such as *Leuc. pseudomesenteroides* QU 15 (leucocin A-QU 15, Sawa et al. 2010), *Leuc. gelidum* UAL 187 (leucocin A-UAL 187, Hastings et al. 1991), *Leuc. carnosum* Ta11a (leucocin B-Ta11a, Felix et al. 1994). Even though their structure is highly homologous, they present some differences principally in their leader peptides or structural genes (Sawa et al. 2010).

PCR product of ~300 bp (named *u-mb1*, putative bacteriocin U-MB1) showed complete (100%) homology with bacteriocin UviB sequence (accession no. YP_001728928). This is the first report of a gene with high homology with bacteriocin UviB, which is also codified in the genome of a *Leuc. citreum* strain (Kim et al. 2008).

SDS-PAGE analysis

SDS-PAGE was used to estimate the molecular mass of bacteriocins present in CFS obtained from *Leuc. citreum* MB1. However, the antimicrobial compound did not give definite bands in the Coomasie Blue-stained gel, possibly due to the low mass of these peptides and thus the complexity of a proper resolution of the bands. On the other hand, the gel subjected to direct detection of the antimicrobial activity by using *List. monocytogenes* ATCC 15313 as indicator strain, revealed a clear zone of inhibition (Fig. 2). The molecular mass was estimated to be much lower than 14·4 kDa, which is the last band resolved in the gel. Molecular masses reported for leucocins produced by *Leuconostoc* strains studied ranged between 2500 and 4000 kDa (Hastings et al. 1991; Papathanasopoulos et al. 1997; Xiraphi et al. 2008; Sawa et al. 2010). In the present work, estimation of molecular masses by SDS-PAGE was not certain. However, their assessment from products obtained by PCR amplification lead to molecular masses in the range of previously reported bacteriocins.

Inactivation kinetics of List. monocytogenes ATCC 15313 with Leuc. citreum *MB1 concentrated cell-free supernatant*

Results are shown in Fig. 3. *Leuc. citreum* MB1 concentrated cell-free supernatant (CCFS) containing antilisterial bacteriocins was generally able to delay the growth of *List. monocytogenes* ATCC 15313 in BHI broth for approximately 24 h at 37 °C. The effectiveness of the bacteriocin appeared to be dependent on both bacteriocin concentration and initial number of pathogen bacteria. In Fig. 3a, growth kinetics of *List. monocytogenes* at $7{\cdot}3 \times 10^3$ CFU/ml initial cell load, added with 50 or 500 µl CCFS are shown. In general, the addition of CCFS at the beginning of the experience, inhibit the growth of *List. monocytogenes* ATCC

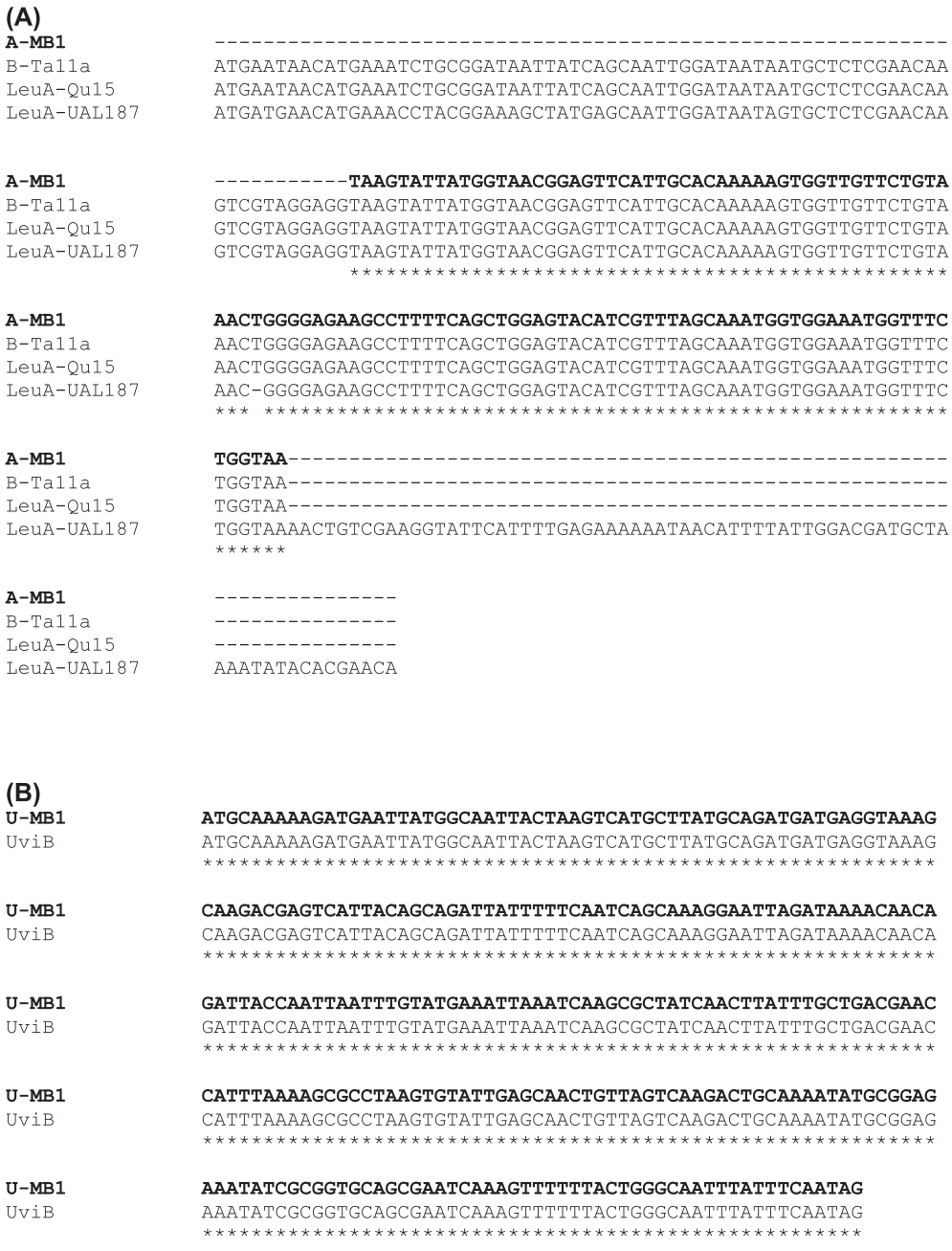

Fig. 1. Alignment of nucleotide sequences of the products obtained by PCR amplification using specific primers, with sequences of bacteriocins deposited in GenBank. (A) A-MB1, ~ 130 bp-product, highly homologous with sequences from "leucocin A" group, (B) U-MB1, ~ 300 bp-product, highly homologous with bacteriocin UviB sequence.

15313 during 30 h. After this period, the pathogen started to grow exponentially, although much slower than the control. On the other side, the addition of 50 or 500 µl of CCFS after pre-incubation of the pathogen until DO_{560} ~ 0·15, held up the development of *List. monocytogenes* for the subsequent 12 h of incubation. For the pathogen inoculated with the highest volume of CCFS (500 µl), the inhibitory effect was even longer. In all cases, the addition of CCFS controlled and even inhibited the growth of *List. monocytogenes* for at least 24 h. Figure 3b shows growth kinetics of *List. monocytogenes* at a higher initial cell load ($8{\cdot}7 \times 10^7$ CFU/ml). In this case, the addition of 50 or 500 µl at the beginning of the experience, led to a 12 h-delay in *List. monocytogenes* growth. Moreover, inoculation with 500 µl CCFS led to a very slow growth of the pathogen until 24 h of incubation. Pre-incubation of the pathogen until DO_{560} ~ 0·5 before the addition of 50 or 500 µl of CCFS, allowed a rapid decrease in pathogen numbers during approximately 24 h, followed by a reduced development of *List. monocytogenes* during the next 32 h of

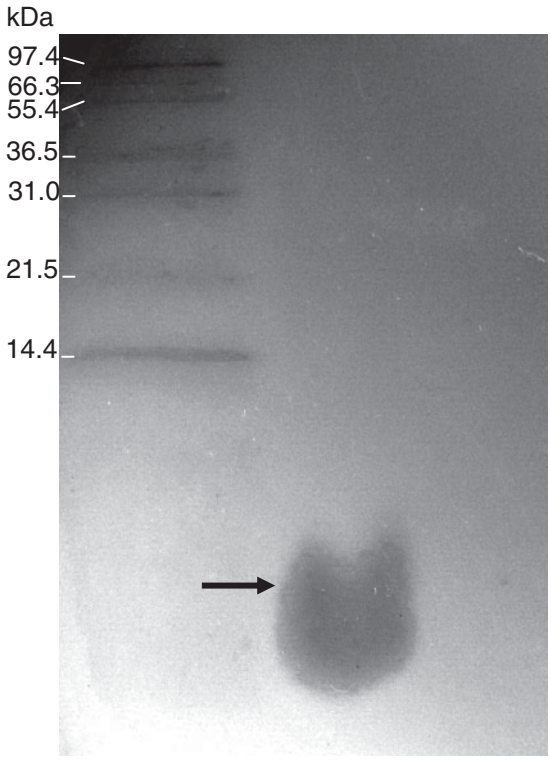

Fig. 2. SDS-PAGE gel subjected to direct detection of antimicrobial activity present in *Leuc. citreum* MB1 cell-free supernatant containing bacteriocin. *List. monocytogenes* ATCC 15313 was used as indicator strain. Inhibition zone is indicated with a black arrow. A wide-range (2·5–200 kDa) weight molecular marker was used.

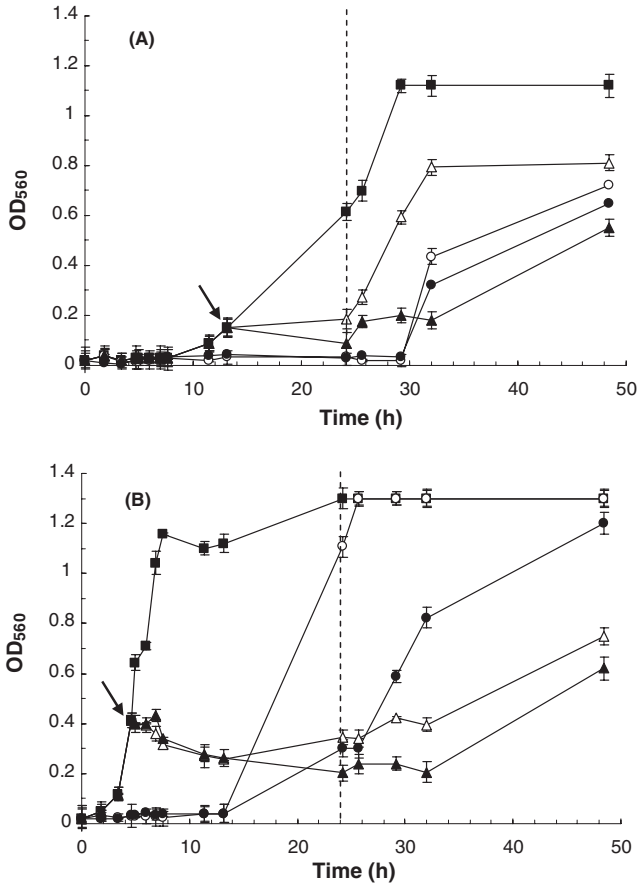

Fig. 3. Inactivation kinetics at 37 °C in BHI broth of *List. monocytogenes* ATCC 15313 by *Leuc. citreum* MB1 concentrated cell-free supernatant (CCFS). *List. monocytogenes* ATCC 15313 initial cell loads of $7{\cdot}3 \times 10^3$ CFU/ml (A) and $8{\cdot}7 \times 10^7$ CFU/ml (B); added of 50 µl (○) or 500 µl (●) CCFS at the beginning, and 50 µl (△) or 500 µl (▲) CCFS after reaching $OD_{560} \sim 0{\cdot}15$ (10^4 CFU/ml initial cell load) or $OD_{560} \sim 0{\cdot}4$ (10^7 CFU/ml initial cell load). *List. monocytogenes* ATCC 15313 without addition of CCFS (control) (■). Dotted vertical lines indicate 24 h incubation; black arrows indicate the addition of CCFS. Values are the mean of three determinations ± standard determination.

incubation. The latter results showed that efficiency of CCFS in *List. monocytogenes* inhibition is more remarkable when the supernatant was added on the pre-incubated pathogen. Recently, it has been shown that a set of bacteriocins Class IIa produced by Gram-positive species can employ the membrane components of the mannose phosphotransferase system (Man-PTS) on sensitive cells as receptor molecules (Kjos et al. 2011). In our work, inhibitory effect of the CCFS was more pronounced when added to the pre-incubated pathogen cells. This effect could be attributable to an increased expression of those receptors linked to a high metabolism of the pathogenic strain.

Our results showed that the inhibitory effect of the CCFS against *List. monocytogenes* ATCC 15313 was greater at the lowest initial cell load tested ($\sim 10^4$ CFU/ml) of the pathogenic strain. Vignolo and co-workers (1996) arrived at similar conclusions studying the effect of lactocin 705 on the growth of *List. monocytogenes* in meat at 20 °C. They observed a partial reduction in pathogen cell loads only by 40% at 24 h when the initial concentration of the pathogenic bacteria was 1×10^7, and a reduction by 44% when the initial cell load of pathogen bacteria was 1×10^4. Pucci et al. (1988) evaluated the lysis of *List. monocytogenes* by PA-1 bacteriocin during 7 h. They observed a greater reduction in number of pathogen bacteria when the maximal bacteriocin concentration was used. The addition of higher concentrations of bacteriocin should confer a protective effect against *Listeria* over longer periods (Pucci et al. 1988; Motlagh et al. 1992; Trias et al. 2008). The extent of inhibitory effect will depend on many factors, principally of the initial number of pathogen bacteria and the bacteriocin concentration.

Co-cultures experiments

Optimal Leuc. citreum *MB1 initial concentration for* List. monocytogenes *ATCC 15313 biocontrol.* To determine the range of pathogen concentrations that the bacteriocinogenic strain is able to control, diverse combinations of bacteriocinogenic and pathogenic strains were tested. Initial *List.*

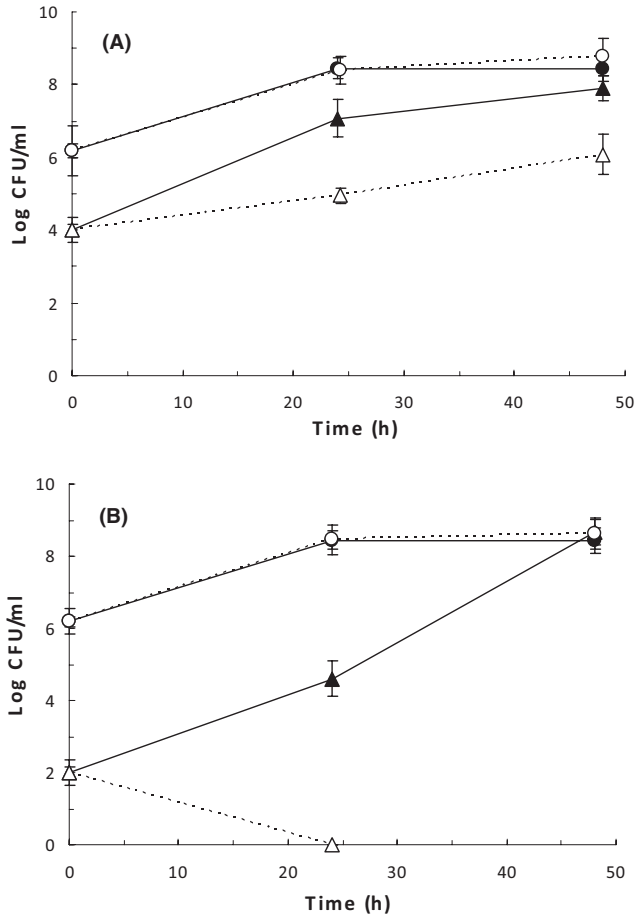

Fig. 4. Effect of *Leuc. citreum* MB1 initial cell loads on *List. monocytogenes* 15313 growth, in co-culture (skim milk, 34 °C, 48 h). Relative *Leuconostoc* and *Listeria* initial inocula: (A) 10^6 and 10^4 CFU/ml; (B) 10^6 and 10^2 CFU/ml. Symbols correspond to *Leuc. citreum* MB1 control (●), *Leuc. citreum* MB1 in co-culture (○), *List. monocytogenes* 15313 control (▲) and *List. monocytogenes* 15313 in co-culture (△).Values are the mean of three determinations ± standard determination.

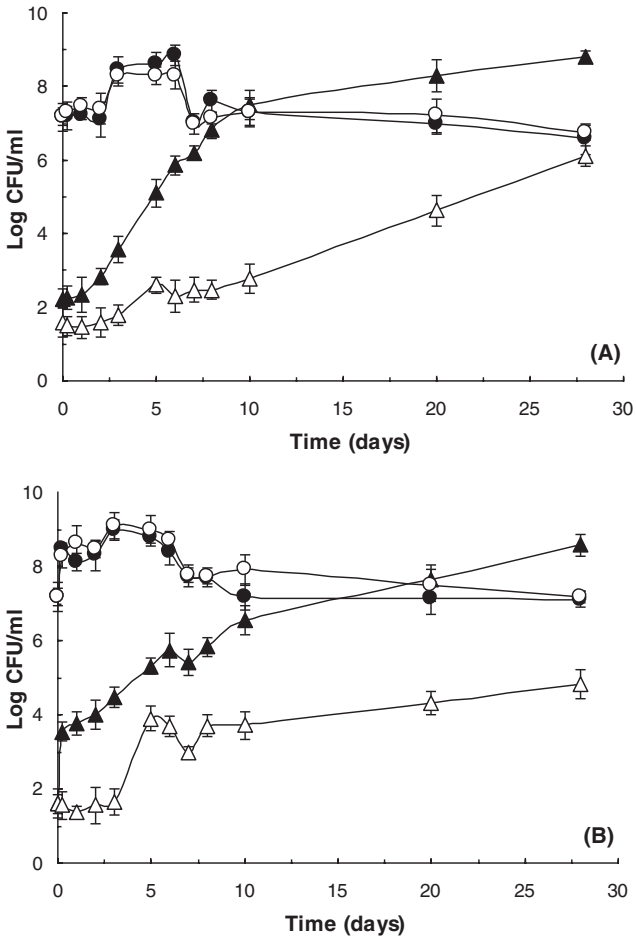

Fig. 5. Co-culture growth of *Leuc. citreum* MB1 and *List. monocytogenes* ATCC 15313 in skim milk, at 10^6 CFU/ml *Leuconostoc* and 10^2 CFU/ml *Listeria* initial cell loads. (A) incubation at 8 °C for 28 d; (B) pre-incubated at 30 °C for 5 h, followed by incubation at 8 °C for 28 d. Symbols correspond to *Leuc. citreum* MB1 control (●), *Leuc. citreum* MB1 in co-culture (○), *List. monocytogenes* 15313 control (▲) and *List. monocytogenes* 15313 in co-culture (△). Values are the mean of three determinations ± standard determination.

monocytogenes cell loads added (10^2 and 10^4 CFU/ml) were selected on the basis of most probable pathogen levels in postprocessing contamination (Pucci et al. 1988). Our results showed that equal *Leuconostoc* and *Listeria* initial cell loads (10^4 CFU/ml), allowed normal growth of the pathogenic strain (data not shown). However, when *Leuc. citreum* MB1 initial concentration was higher than that of *List. monocytogenes* ATCC 15313, partial or total growth inhibition of the pathogenic strain was observed. Specifically, at 10^6 CFU/ml *Leuconostoc* and 10^4 CFU/ml *Listeria* initial cell loads, the growth of the pathogenic strain in co-culture was maintained two log orders lower than the control (Fig. 4a) throughout the experiment. Moreover, at 10^6 CFU/ml *Leuconostoc* and 10^2 CFU/ml *Listeria* initial concentrations, viable cell counts of the pathogenic strain were undetectable after 48 h of incubation at 34 °C (Fig. 4b). Efficacy of the bioprotective effect depends on initial relative concentrations of both *Leuc. citreum* MB1 and *List. monocytogenes* ATCC 15313, being higher when the bacteriocinogenic strain is in a predominant number from the start. Similar behaviour was obtained by Trias et al. (2008) when inoculating wounds of apples and lettuce leaf cuts with different doses of pathogenic and bacteriocinogenic *Leuc. mesenteroides* strains: the highest *List. monocytogenes* inhibition was revealed in co-culture with any of the three *Leuc. mesenteroides* strains tested at the highest initial concentration tested (10^9 CFU per wound or per gram).

Comparison of our results are difficult since other reported in vitro co-culture experiments were carried out using different bacteriocinogenic LAB strains, for example, *Lactococcus lactis* (Liu et al. 2008) and *Lactobacillus casei* CRL 705 (Vignolo et al. 1996). In any case, the consensus

result is that bacteriocinogenic strains reached their maximum effectiveness when inoculated at higher initial numbers than that of the pathogenic strain, which indicates the importance of maintaining a high standard manufacturing process together with strict sanitation.

Biocontrol of List. monocytogenes *ATCC 15313 by* Leuc. citreum *MB1 in milk at refrigeration temperature*. Results are shown in Fig. 5. In general, growth of *List. monocytogenes* ATCC 15313 was clearly delayed when it was co-inoculated in skim milk with *Leuc. citreum* MB1, as viable cell counts of the pathogen were below 10^2 CFU/ml during the first 3 d for both directly incubated at 8 °C and pre-incubated at 30 °C co-cultures. Afterwards and for 10 d, direct incubation at 8 °C allowed a very low growth rate in co-cultured *List. monocytogenes* 15313. After 28 d of refrigerated storage, viable cell counts of the pathogenic strain were raised more pronouncedly, although they were maintained at least two log orders lower than the control (Fig. 5a).

Pre-growth at 30 °C for 5 h and further incubation at 8 °C of *List. monocytogenes* 15313 together with *Leuc. citreum* MB1, allowed considerable delay of the pathogen growth and even after 28 d, the number of pathogenic cells was extremely inferior to that of the control ($6{\cdot}7\times10^4$ and $3{\cdot}8\times10^8$ CFU/ml, respectively) (Fig. 5b). *Leuconostoc* growth in skim milk was not affected by the presence of the pathogenic strain as shown by similar counts in co-culture and control tubes.

Leuc. citreum MB1 ability to control *List. monocytogenes* 15313 growth at 8 °C was greater in the pre-incubated (30 °C, 5 h) culture. This previous treatment at higher temperature certainly allowed rapid development of *Leuc. citreum* MB1. Superiority in cell number and the production of high amounts of antimicrobial compounds at an earlier stage facilitated *Leuc. citreum* MB1 to control the growth of the pathogenic strain. Similar behaviour was obtained by Vignolo et al. (1996) when studying the effect of in situ-produced bacteriocin by *Lb. casei* CRL 705 in meat. They observed no changes in the number of inoculated *Listeria* cells during 24 h, indicating a partial inhibition and bacteriostatic effect of the produced bacteriocin.

Since bacteriocins may not be considered as inhibitors by themselves, for optimal effectiveness they should be used together with other preservation methods, as part of a general multihurdle food preservation system, in which the combined effects of pH, temperature and oxygen availability are studied together with the added bacteriocinogenic LAB (Ennahar et al. 2000; Castellano et al. 2008).

Conclusions

Leuc. citreum MB1 belongs to a group of adventitious *Leuconostoc* strains previously isolated from dairy environment. This strain has previously been shown to grow well in milk and produce antibacterial bacteriocin-like compounds specific against *Listeria*.

Our results showed that *Leuc. citreum* MB1 harbours two different genes (*a-mb1* and *u-mb1*) compatible with the active fraction of two known bacteriocins: leucocin A and bacteriocin UviB. It is unquestionable that at least one bacteriocin is active in *Leuc. citreum* MB1, since important antilisterial activity was verified in its cell-free supernatant. We verified that the addition of *Leuc. citreum* MB1 concentrated cell-free supernatant conferred a protective effect against the pathogenic strain during 24 h. Moreover, high initial concentrations of *Leuc. citreum* MB1 are able to control *List. monocytogenes* growth in milk during refrigerated storage. This feature, together with its ability to grow at low temperatures, may be very advantageous to control the growth of *Listeria*.

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