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Production, stability, gene sequencing and *in situ* anti-*Listeria* activity of mundticin KS expressed by three *Enterococcus mundtii* strains

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ABSTRACT

Three enterococci (WFE3, WFE20 and WFE31) selected as presumptive bacteriocin producers were found to be active against *Listeria monocytogenes*. In this study, due to their potential industrial/food applications, the three bacterial isolates were extensively characterized. Identification was performed by means of a combined 16S rRNA gene sequencing and multiplex PCR approach, and was confirmed with the sequencing of a partial region of a protein-encoding gene, namely *pheS*. The three isolates belonged unequivocally to the species *Enterococcus mundtii*. The randomly amplified polymorphic DNA (RAPD) analysis recognized three distinct strains. The supernatants were mainly active against *Listeria* spp., but some lactic acid bacteria were also inhibited. The proteinaceous nature of the three supernatants was detected after treatment with proteinase K, protease B and trypsin. The bacteriocins were found to be heat resistant, stable in a large pH range and in presence of ethanol. The bacteriocins were not adsorbed onto the surface of the producer cells and their effect was bactericidal. The production of bacteriocins was higher at neutral pHs and temperatures in the range 30–37 °C. The active supernatants did not show cytotoxicity against human erythrocytes and the three strains were susceptible to the action of common antibiotics. The genetic characterization of the bacteriocin genes showed that all three strains produced mundticin KS. They produced it in five food model systems, sterilized by thermal treatment or filtration, prepared from fresh vegetables, cereals, cheeses, meats and fishes. The *in situ* anti-listerial activities of the strains WFE3, WFE20 and WFE31 were quantitatively different.

1. Introduction

Enterococci are considered at the crossroad of food safety (Franz, Holzapfel, & Stiles, 1999); they are reported to be a leading cause of nosocomial infections and to have a significant role in the dissemination and persistence of antimicrobial resistance (Moellering, 1992; Murray, 1990). However, some species within this group are of relevance in food fermentation (Folquié Moreno, Rea, Cogan, & De Vuyst, 2003) and several isolates are commonly employed as probiotics for humans and slaughter animals (Franz, Huch, Abriouel, Holzapfel, & Gálvez, 2011). Enterococci are natural inhabitants of the intestine in warm-blooded animals (Devriese, Collins, & Wirth, 1992), thus, they often occur in foods of animal origin (meat and cheese) (Franz et al., 1999), but they are also commonly found on the above-ground parts of vegetables and cereals (Corsetti et al., 2007; Mundt & Hammer, 1968) and may persist during the fermentation of vegetable products. Furthermore, due to their ability to resist to the technological processes used in the food industries (e.g. pasteurization and addition of acids and salt), *Enterococcus* spp. are usually found in many manufactured food products.

The enterococcal species most frequently identified in fermented foods are *Enterococcus faecium* and *Enterococcus faecalis*, but other species such as *Enterococcus casseliflavus*, *Enterococcus durans* and *Enterococcus mundtii* are common in many raw materials and foods (Corsetti et al., 2007; Franciosi, Settanni, Cavazza, & Poznanski, 2009; Settanni et al., 2012). Besides their contribution to the organoleptic properties of fermented food products, enterococci of food interest are generally investigated for their ability to produce bacteriocins, because these protein antimicrobials produced by bacteria that enjoy a generally recognized as safe (GRAS) status may be considered as "natural" food preservatives (Settanni,

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Valmorri, Suzzi, & Corsetti, 2008). This characteristic is of paramount importance for their application in strategies of biopreservation (Settanni & Corsetti, 2008), that refers to the extension of the shelf-life and improvement of the safety of foods using microorganisms and/or their metabolites (Ross, Morgan, & Hill, 2002).

In comparison to other *Enterococcus* species, the role of bacteriocins produced by *E. mundtii* has been scarcely studied in food systems, although their efficacy has been evaluated in mung bean sprouts (Bennik, van Overbeek, Smid, & Gorris, 1999) and, very recently, in fresh Minas cheese (Vera Pingitore, Todorov, Sesma, & Franco, 2012) and vacuum-packed cold smoked salmon (Bigwood et al., 2012).

The *in situ* antimicrobial efficacy of bacteriocin may be limited by their binding to food components (fat or protein particles) and food additives (e.g. triglyceride oils), inactivation by proteases or other inhibitors, changes in solubility and charge, changes in the cell envelope of the target bacteria (Aasen et al., 2003; Settanni et al., 2008). Furthermore, bacteriocin production can be influenced by the culture conditions (Settanni et al., 2008). It is reported that bacteriocin activities do not always correlate with cell concentration or growth rate of the producer (Kim, Hall, & Dunn, 1997), as well as that higher levels of bacteriocin production may be obtained in sub-optimal conditions (Aasen, Moretro, Katla, Axelsson, & Storro, 2000; Todorov & Dicks, 2004). On the contrary, Settanni et al. (2008) found that stressing conditions and lack or low concentrations of nutritional factors determined a reduction in bacteriocin production by several *E. mundtii* strains.

This work was performed to evaluate the inhibitory activities of three *E. mundtii* strains, to study their production under several growth conditions and after different enzymatic, thermal and chemical treatments, to genetically investigate their structure, to determine their production in different food model systems, and to monitor their anti-*Listeria* potential *in situ* during fermentation.

2. Materials and methods

2.1. Strains and growth conditions

Enterococci (isolates WFE3, WFE20, WFE31) scored positive for antimicrobial compound production by well diffusion assay (WDA, see Section 2.3), during a general screening aimed at characterizing LAB from wheat flours (work in preparation), and *E. mundtii* PON10063 of flour origin were cultured in MRS (Oxoid, Basingstoke, England) for 24 h at 30 °C. The bacterial strains used as indicators (sensitive to the inhibitory activity) are listed in Table 1. *Listeria innocua* 4202 (obtained from the culture collection of National Food Biotechnology Centre, Cork, Ireland) and all *Listeria monocytogenes* DHPS strains (belonging to the culture collection of the Department of Sciences for Health Promotion and Mother-Child Care "G. D'Alessandro" – University of Palermo, Italy) were propagated in Brain Heart Infusion (BHI) (Oxoid) at 37 °C for 24 h, *Lactobacillus sakei* LMG 2313 (obtained from the Laboratory of Microbial Gene Technology, Ås, Norway) in modified-MRS (mMRS) (maltose and fresh yeast extract were added at final concentrations of 1% and 10%, respectively, and the final pH was adjusted to 5.6) at 30 °C for 24 h, *Citrobacter freundii* PSS60, *Enterobacter* spp. PSS11, *Escherichia coli* PSS2, *Klebsiella oxytoca* PSS82, *Serratia grimesii* PSS72 and *Stenotrophomonas maltophilia* PSS52 (belonging to the culture collection of the Agricultural Microbiology laboratory – Department of Agricultural and Forestry Science – University of Palermo, Italy) were propagated in Nutrient Broth (NB) (Difco Laboratories, Detroit, MI) at 37 °C for 24 h, *Pseudomonas putida* PSS21 (of the same collection) was cultivated in NB at 20 °C for 24 h, while all other strains were propagated as indicated by the respective culture collection.

2.2. Identification of enterococci at species level and strain differentiation

The DNA from LAB cultures was extracted by the Instagene Matrix kit (Bio-Rad, Hercules, CA) as described by the manufacturer. Crude cell extracts were used as template DNA for PCR.

Genotypic identification of LAB was first carried out by 16S rRNA gene sequencing. PCRs were performed as described by Weisburg, Barns, Pelletier, and Lane (1991). DNA fragments were visualized and the amplicons of about 1600 bp were purified by the QIA-quick purification kit (Qiagen S.p.a., Milan, Italy) and sequenced using the same primers employed for PCR amplification. DNA sequencing reactions were performed by PRIMM (Milan, Italy). The sequences were compared by a BLAST search in GenBank/EMBL/DDBJ database and on EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012). The multiplex PCR assay based on *sodA* gene reported by Jackson, Fedorka-Cray, and Barrett (2004) was applied to confirm species identity. Finally, *pheS* partial sequence was obtained for strain WFE31 as previously reported (Naser et al., 2005) and compared in GenBank/EMBL/DDBJ database. DNA amplifications were performed by means of T1 Thermocycler (Biometra, Göttingen, Germany).

Strain differentiation was performed by random amplification of polymorphic DNA (RAPD)-PCR analysis in a 25-μL reaction mix using single primers M13, AB111, and AB106 as reported by Settanni et al. (2012). PCR products were separated by electrophoresis on 2% (*w*/*v*) agarose gel (Gibco BRL, Cergy Pontoise, France) and visualized by UV transillumination after staining with SYBR® safe DNA gel stain (Molecular probes, Eugene, OR, USA). GeneRuler 100 bp Plus DNA ladder (M·Medical Srl, Milan, Italy) was used as a molecular size marker. RAPD patterns were analyzed using the Gelcompare II software version 6.5 (Applied-Maths, Sin Marten Latem, Belgium).

2.3. Assays for antibacterial activity

After propagation, the three strains WFE3, WFE20 and WFE31 were centrifuged at 10,000×*g* for 5 min, washed in Ringer's solution (Sigma–Aldrich, Milan, Italy) and re-suspended in the same solution to achieve an optical density (OD) of ca. 1.00, measured by 6400 Spectrophotometer (Jenway Ltd., Felsted Dunmow, UK) at 600 nm wavelength, which approximately corresponds to a concentration of 10^9 CFU/mL, to standardize bacterial inocula. Cell suspensions were inoculated in MRS at a final concentration of approximately 10^6 CFU/mL and incubated at 30 °C for 24 h. The antimicrobial activity of the active supernatants (20 μL) was tested by WDA (Schillinger & Lücke, 1989) following the modifications of Corsetti, Settanni, and Van Sinderen (2004). *L. sakei* LMG 2313, *L. innocua* 4202 and *L. monocytogenes* ATCC 19114 were used as indicator strains. Inhibition was scored positive in presence of a detectable clear area around the well of the producer strain. The antibacterial activity of the supernatants was measured by the critical dilution assay of Barefoot and Klaenhammer (1983). The activity was defined as the reciprocal of the highest dilution showing definite inhibition of the indicator strain and was expressed as activity units per milliliter (AU/mL). The inhibitory substances were then characterized for their inhibitory spectra against other food related bacteria (Table 1). Tests were carried out in triplicate.

2.4. Characterization of the active supernatants

In order to evaluate the proteinaceous nature of the inhibitory compounds, the active supernatants, obtained after separation of the bacterial cells (10,000×*g* for 5 min) in the stationary phase of growth, were characterized for their sensitivity to proteolytic enzymes using proteinase K (12.5 U/mg), protease B (45 U/mg) and

Table 1
Inhibitory spectra of *E. mundtii* WFE3, WFE20, and WFE31 against food-associated bacteria.

Strains used as indicators for the inhibition tests[a]	Source	*Enterococcus* strains tested as bacteriocin producers			
		PON10063	WFE3	WFE20	WFE31
Enterococcus hirae DSM 20160^T	Unknown	–	10,666 ± 3695A	12,800 ± 0A	10,666 ± 3695A
Listeria innocua 4202	Unknown	–	42,666 ± 14,780A	51,200 ± 0A	68,266 ± 29,560A
Listeria monocytogenes ATCC 19114	Animal tissue	–	273,066 ± 118,241A	51,200 ± 0B	819,200 ± 0C
L. monocytogenes DHPS129	Human stool	–	51,200 ± 0A	34,133 ± 14,780A	85,333 ± 29,560A
L. monocytogenes DHPS131	Human stool	–	17,067 ± 7390A	12,800 ± 0A	34,133 ± 14,780A
L. monocytogenes DHPS133	Human stool	–	204,800 ± 0A	170,667 ± 5920A	204,800 ± 0A
L. monocytogenes DHPS179	Salmon	–	34,133 ± 14,780A	17,067 ± 7390AB	68,266 ± 29,560AC
L. monocytogenes DHPS180	Ricotta cheese	–	17,067 ± 7390A	6,400 ± 0A	17,067 ± 7390A
L. monocytogenes DHPS182	Ricotta cheese	–	25,600 ± 0A	68,266 ± 29,560A	34,133 ± 14,780A
L. monocytogenes DHPS184	Rice salad	–	85,333 ± 29,560A	34,133 ± 14,780AB	136,533 ± 59,120AC
L. monocytogenes DHPS185	Beef	–	136,533 ± 59,120A	136,533 ± 59,120A	136,533 ± 59,120A
L. monocytogenes DHPS186	Mozzarella salad	–	85,333 ± 29,560A	25,600 ± 0BC	68,266 ± 29,560AC
L. monocytogenes DHPS187	Roasted chicken	–	170,667 ± 5920A	546,133 ± 236,483B	409,600 ± 0B
L. monocytogenes DHPS188	Green salad	–	204,800 ± 0A	68,266 ± 29,560B	68,266 ± 29,560B
L. monocytogenes DHPS1BO	Chopped meat	–	68,266 ± 29,560A	17,067 ± 7390BC	21,333 ± 7390AC
L. monocytogenes DHPS2BO	Fresh salami	–	68,266 ± 29,560A	12,800 ± 0B	17,067 ± 7390B
L. monocytogenes DHPS3BO	Fresh salami	–	6400 ± 0A	17,067 ± 7390AC	25,600 ± 0BC
L. monocytogenes DHPS4BO	Ripened salami	–	68,266 ± 29,560A	5333 ± 1847B	17,067 ± 7390B
L. monocytogenes DHPS5BO	Ripened salami	–	17,067 ± 7390A	17,067 ± 7390A	25,600 ± 0A
L. monocytogenes DHPS6BO	Ripened salami	–	17,067 ± 7390A	17,067 ± 7390A	17,067 ± 7390A
L. monocytogenes DHPS7BO	Ripened salami	–	17,067 ± 7390A	6,400 ± 0A	10,667 ± 3695A
L. monocytogenes DHPS11BO	Meat factory	–	12,800 ± 0A	12,800 ± 0A	12,800 ± 0A
L. monocytogenes DHPS12BO	Ripened salami	–	12,800 ± 0A	6,400 ± 0AB	34,133 ± 14,780AC
L. monocytogenes DHPS13BO	Gorgonzola cheese	–	12,800 ± 0A	12,800 ± 0A	17,067 ± 7390A
L. monocytogenes DHPS20BO	Gorgonzola cheese	–	204,800 ± 0A	17,067 ± 7390B	25,600 ± 0B
L. monocytogenes DHPS22BO	Taleggio cheese	–	68,266 ± 29,560A	34,133 ± 14,780A	51,200 ± 0A
L. monocytogenes DHPS24BO	Taleggio cheese	–	25,600 ± 0A	17,067 ± 7,390AB	68,266 ± 29,560AC
Lactobacillus farciminis DSM 20180	Sausage	–	1600 ± 0A	1066 ± 462B	800 ± 0C
Lactobacillus curvatus ssp. *curvatus* ATCC 25601^T	Milk	–	1066 ± 462A	200 ± 0B	167 ± 58B
Lactobacillus delbrueckii ssp. *bulgaricus* ATCC 11842^T	Yogurt	–	667 ± 231A	200 ± 0B	400 ± 0C
Lactobacillus fermentum DSM 20391	Unknown	–	1066 ± 462A	400 ± 0B	667 ± 231C
Lactobacillus paralimentarius DSM 13238^T	Sourdough	–	3200 ± 0A	800 ± 0B	2,133 ± 924A
Lactobacillus paraplantarum DSM 10667^T	Beer	–	333 ± 115A	200 ± 0A	133 ± 58A
Lactobacillus pentosus ATCC 8041^T	Unknown	–	800 ± 0A	167 ± 58B	533 ± 231A
Lactobacillus pentosus DSM 20199	Unknown	–	8533 ± 3695A	1333 ± 462B	2667 ± 924B
Leuconostoc mesenteroides DSM 20343^T	Fermented olives	–	25,600 ± 0A	6400 ± 0B	8533 ± 3695B
Pediococcus acidilactici LMG 11384^T	Barley	–	2667 ± 924A	667 ± 231AB	1333 ± 462AC
Statistical significance:					
Strains		ns	***	***	***

The results are expressed in activity units (AU)/mL and indicate mean value ± SD of three replicates. The activity was measured in MRS supernatants.
–, no inhibition.
P value: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns = not significant.
Uppercase letters indicate different statistical significances (overall $P < 0.05$, Tukey's correction). Means within a given column with the same letter are not statistically different from each other.

[a] The following strains were not inhibited by any active supernatant: *Citrobacter freundii* PSS60, *Enterococcus durans* DSM 20633^T, *Enterococcus faecium* DSM 20477^T, *Enterobacter* spp. PSS11, *Escherichia coli* PSS2, *Klebsiella oxytoca* PSS82, *Kocuria varians* DSM 20033^T, *Lactobacillus alimentarius* DSM 20181, *Lactobacillus amylovorus* DSM 20531^T, *Lactobacillus amylolyticus* DSM 11664^T, *Lactobacillus brevis* ATCC 14869^T, *Lactobacillus buchneri* LMG 6852^T, *Lactobacillus casei* DSM 20011^T, *Lactobacillus casei* LMG 6904^T, *Lactobacillus delbrueckii* ssp. *delbrueckii* DSM 20074^T, *Lactobacillus delbrueckii* ssp. *lactis* ATCC 12315^T, *Lactobacillus farciminis* DSM 20184^T, *Lactobacillus fermentum* ATCC 14931^T, *Lactobacillus fructivorans* DSM 20203^T, *Lactobacillus hilgardii* DSM 20051, *Lactobacillus hilgardii* DSM 20176^T, *Lactobacillus paracasei* ssp. *paracasei* NCFB 151^T, *Lactobacillus paracasei* ssp. *tolerans* LMG 9191^T, *Lactobacillus plantarum* ATCC 14917^T, *Lactobacillus reuteri* DSM 20056^T, *Lactobacillus rhamnosus* LMG 6400^T, *Lactobacillus sakei* LMG 2313, *Lactococcus lactis* ssp. *cremoris* DSM 20069^T, *Lactococcus lactis* ssp. *lactis* DSM 20481^T, *Pseudomonas putida* PSS21, *Serratia grimesii* PSS72, *Stenotrophomonas maltophilia* PSS52, *Streptococcus thermophilus* DSM 20617^T.

trypsin (10.6 U/mg) at a final concentration of 1 mg/mL in phosphate buffer (pH 7.0). All enzymes were purchased from Sigma–Aldrich (Milan, Italy). The supernatants were incubated for 2 h at 37 °C and the remaining activity was determined by well diffusion assay (WDA) (Settanni, Massitti, Van Sinderen, & Corsetti, 2005). The effect of α-amylase and lipase, heat treatment, pH and organic solvent on the antimicrobial activity was evaluated as described by Corsetti, Settanni, Braga, Lopes, and Suzzi (2008). Tests were carried out in triplicate.

2.5. Adsorption studies and effect of bacteriocins

The effect of the pH on the adsorption of the active proteins onto producer cells was evaluated as reported by Todorov et al. (1999) and Yang, Johnson, and Ray (1992).

The effect of the antimicrobial compounds on the sensitive cells was evaluated as follows: the supernatants (4 mL) were adjusted to pH 6.5, treated with catalase as reported by Corsetti et al. (2008) and concentrated under vacuum (Hetovac VR-1, Heto Lab Equipment, Birkerod, Denmark); the dried supernatants were re-suspended into 4 mL of BHI and filtered through a 0.22-μm pore size filter (Millipore); the indicator strain (*L. monocytogenes* ATCC 19114) was inoculated at a cellular concentration of approximately 10^3 CFU/mL. If no growth of *L. monocytogenes* ATCC 19114 occurred in presence of the bacteriocins, the cells were recovered and transferred into BHI to distinguish between bactericidal and bacteriostatic effect. The supernatant of *E. mundtii* PON10063, treated as above described, was used as negative control. Cell suspensions were followed by measuring the OD at 600 nm at T_0, when the supernatants were added, 2-h intervals for the first 10 h and then at 24 h for seven days.

Tests were carried out in triplicate.

2.6. Bacteriocin production at different incubation temperatures and initial growth pH values

To evaluate the effect of temperature and pH on the production of bacteriocins. Cells of the producer strains were cultivated at 15, 30, 37 and 45 °C in MRS. Incubation was for 48 h, except for the assay at 15 °C that was prolonged for five days. The effect of the initial pH of medium was evaluated by adjusting MRS to pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 with 5 M NaOH or 5 M HCl. Incubation was at 30 °C for 48 h. Tests were carried out in triplicate.

2.7. Evaluation of cellular toxicity

Cellular toxicity of the three active supernatants was assayed following the methodology reported by Xian-guo and Ursula (1994). Each sample (0.8 mL) was placed in a microcentrifuge tube and the final volume of 1 mL was reached adding human erythrocytes. Phosphate buffer saline (PBS) (Oxoid) and tap water were used as negative and positive control, respectively. The tubes were incubated at 37 °C for 30 min and hemolysis was observed after centrifugation at 3000×*g* for 5 min. Hemolysis was scored positive when the erythrocytes did not form a pellet after centrifugation.

2.8. Evaluation of antibiotic resistance

The antibiotic resistance of *E. mundtii* strains was tested according to the guidelines of the Clinical and Laboratory Standards Institute (2011) (CLSI) for enterococci, applying the Performance Standards for Antimicrobial Susceptibility Testing. According to those recommendations, the following antibiotics were assayed by the disk diffusion test: penicillin (10 units) and ampicillin (10 μg) for the "group A primary test and report"; quinupristin–dalfopristin (15 μg), linezolid (30 μg) and vancomycin (30 μg) for the "group B primary test report selectively".

2.9. Amplification, cloning and sequencing of bacteriocin-coding genes

The structural genes for bacteriocin production were analyzed by PCR amplification using primers mapping on the nucleotide sequence of *E. mundtii* bacteriocin-coding genes. Genomic DNA from *E. mundtii* WFE3, WFE20 and WFE31 were used as templates for amplification with the primer pair Mnt-1F (5′-TGAGAGAAGGTTTAAGTTTTGAAGAA-3′)/Mnt-1R (5′-TCCACTGAAATCCATGAATGA-3′) mapping upstream of the coding sequence of known bacteriocins KS (Kawamoto et al., 2002), CRL35 (Saavedra, Minahk, de Ruiz Holgado, & Sesma, 2004), QU2 (Zendo et al., 2005) and MunL (Feng, Guron, Churey, & Worobo, 2009), applying the conditions described by Zendo et al. (2005). DNA from *E. mundtii* PON10063 was used as negative control in PCRs. PCR products were analyzed on 1.5% (*w*/*v*) agarose gel and visualized as above reported.

Amplicons generated with the primer pair Mnt-1F/Mnt-1R were purified by QIA-quick purification kit (Qiagen) and cloned into the pGEM®-T Easy Vector (Promega, Milan, Italy) following manufacturer's instructions. Ligation products were transformed into *E. coli* JM109 high efficiency competent cells and these were plated onto Luria–Bertani (LB) agar (Oxoid) containing 100 μg/mL ampicillin (Sigma–Aldrich), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 80 μg/mL) and IPTG (isopropyl-β-D-thiogalactopyranoside, 0.5 mM) (Eppendorf, Milan, Italy). Recombinant white colonies were screened by colony-PCR using vector specific primers SP6 (5′-ATTTAGGTGACACTATAGAATAC-3′) and T7 (5′-TAATACGACTCACTATAGGG-3′) in a 25-μL reaction mix applying the following amplification program: 94 °C for 5 min, 4 °C for 4 min, 35 cycles at 94 °C for 30 s, 52 °C for 30 s and 72 °C for 50 s, followed by a final extension at 72 °C for 5 min. Insert integrity was confirmed by a nested-amplification with Mnt-1F and Mnt-1R primers using purified colony-PCR amplicons as templates and the previously described amplification program. In addition, T7/Mnt-1F and T7/Mnt-1R primer pairs were alternatively employed in a different nested-PCR (35 cycles at 94 °C for 30 s, 52 °C for 30 s and 72 °C for 40 s, followed by a final extension at 72 °C for 2 min) to determine the orientation of fragments into the cloning vector. PCR products were separated by electrophoresis in a 1.5% (*w*/*v*) agarose gel, stained with SYBR® safe DNA gel.

Sequencing reactions were performed by PRIMM and the sequences were compared by a BLAST search in GenBank/EMBL/DDBJ database. The prediction of the open reading frame (ORF) was performed with the softwares ChromasPro v1.6 (Copyright 2003–2012 Technelysium Pty Ltd. Biotech Works Inc.) and pDRAW32 v1.1.114 (www.acaclone.com). The ClustalX program (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997) was used for nucleotide sequence analysis. Sequence alignments were analyzed and adjusted by GeneDoc program v2.5.000 (K.B. Nicholas and H.B. Nicholas, unpublished data).

2.10. Amplification of enterocin CRL35 biosynthetic cluster

The DNA from the strains WFE3, WFE20 and WFE31 were amplified with the primer pair mun1F (5′-GCAAACCGATAAGAA TGTGGGAT-3′)/mun7R (5′-TATACATTGTCCCCACAACC-3′) (Saavedra et al., 2004), designed to amplify the biosynthetic cluster of enterocin CRL35 that has been shown to share high sequence identity with the cluster of mundticin KS. The amplification program included: denaturation at 94 °C for 3 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 3 min and 40 s, followed by a final extension at 72 °C for 4 min. DNA from *E. mundtii* PON10063 was used as negative control in PCRs. PCR products were analyzed on 1% (*w*/*v*) agarose gel and visualized.

2.11. Bacteriocin production in food model systems

In order to evaluate the effect of different food components on the inhibitory activity of the *E. mundtii* strains, five food model systems [vegetable broth (VB), meat broth (MB), fish broth (FB), cereal broth (CeB) and cheese broth (ChB)] were developed: VB with carrot (50 g/L), tomato (50 g/L), zucchini (50 g/L) and celery (50 g/L); MB with pork (50 g/L), calf (50 g/L), chicken (50 g/L) and sheep (50 g/L) meat; FB with salmon (50 g/L), octopus (50 g/L), anchovy (50 g/L) and cod (50 g/L); CeB with wheat bran (50 g/L), wheat (50 g/L), barley (50 g/L) and rice (50 g/L) kernels; ChB with Caciocavallo (50 g/L), Parmigiano (50 g/L), Pecorino (50 g/L) and Vastedda (50 g/L) cheeses. The preparation of the five broths was as follows: all four ingredients of each broth were homogenized with a Sorvall Omni-Mixer (Dupont Instruments, Newtown, CT) at the maximum speed for 1 min, transferred to a Schott Duran bottle, added with distilled H_2O (1 L), left under magnetic stirring for 1 h and centrifuged at 10,000×*g* for 5 min. Each supernatant was divided in two aliquots: one was sterilized by autoclaving at 121 °C for 20 min (autoclaved broths), while the other aliquot was filter (0.20-μm pore size filter, Sartorius, Aubagne Cedex, France) sterilized (filtered broths). The broths were subjected to the measurement of pH, determined electrometrically using the pH meter BASIC 20+ (Crison Instrument S.A., Barcelona, Spain), and water activity (a_w), obtained with the AquaLab vapor sorption analyzer (Decagon Devices, Pullman, WA, USA).

Autoclaved and filtered broths were inoculated singly with the strains WFE3, WFE20 and WFE31 as above reported (Section 2.3) at a final concentration of approximately 10^6 CFU/mL, after cell

washing. MRS was also inoculated as control trial. *E. mundtii* PON10063 was used as negative control. Incubation was at 30 °C for 24 h. Tests were carried out in triplicate.

2.12. In situ activity of E. mundtii strains against L. monocytogenes

To evaluate the potential of the bacteriocin producing strains during the fermentation of different foods, the five food model systems were inoculated with dual combinations *Enterococcus*/*L. monocytogenes*. Tests in MRS were carried out for comparison. All strains were prepared as reported in Section 2.3. The *Enterococcus* strains were inoculated at the final concentration of approximately 10^6 CFU/mL to act as starter cultures, while *L. monocytogenes* ATCC 19114 at about 10^4 CFU/mL to simulate a massive contamination. *E. mundtii* PON10063 was used as negative control. Incubation was at 30 °C for five days to mimic a common food fermentation process. Plate counts were performed to enumerate the surviving cells. The broths (1 mL) were subjected to the serial decimal dilution in Ringer's solution and the cell suspensions were spread plated (0.1 mL) and incubated as follows: on kanamycin aesculin azide (KAA) agar (Oxoid), incubated aerobically at 37 °C for 24 h, for *Enterococcus*; on *Listeria* Selective Agar Base (LSAB) (Oxoid) supplemented with SR0140E (Oxford formulation), incubated aerobically at 37 °C for 48 h, for *L. monocytogenes*. In order to verify the specificity of the media employed, the absence of growth of enterococci on LSAB and that of *L. monocytogenes* on KAA were verified prior *in situ* activity determination. Tests were carried out in duplicate.

2.13. Statistical analyses

Data of inhibitory activities, effect of enzyme, pH, temperature and organic solvent treatments on bacteriocins, production of bacteriocins under different pH and temperature conditions and production of bacteriocins in different food models were statistically analyzed using the generalized linear model (GLM) procedure, including the effects of strain, with the program SAS 2008 – version 9.2 (Statistical Analysis System Institute Inc., Cary, NC, USA). The Student "*t*" test was used for mean comparison. The *post-hoc* Tukey method was applied for pairwise comparison.

The *in situ* anti-*Listeria* efficacy of bacteriocins was analyzed by the Student "*t*" test and *post-hoc* Tukey method. Significance level was $P < 0.05$.

3. Results

3.1. Genotypic identification and differentiation of enterococci

The isolates WFE3, WFE20 and WFE31 were identified as *E. mundtii* by 16S rRNA gene sequencing, and the gene sequences were deposited in GenBank under the Acc. No. KC291248–KC291250. Species identification was confirmed by using a specific multiplex PCR system developed to distinguish among *E. mundtii*, *Enterococcus flavescens* and *Enterococcus sulfureus* (results not shown) and with partial sequencing of *pheS* gene for WFE31, which confirmed the identification as *E. mundtii* (data not shown).

RAPD-PCR analysis (Fig. 1) recognized the three isolates as three distinct strains.

3.2. Bacteriocin production

E. mundtii WFE3, WFE20, and WFE31 were active against several bacterial strains (Table 1). All three *Enterococcus* strains inhibited the same sensitive strains, but the inhibitory effect was strain dependent, since different activities ($P < 0.05$), expressed in AU/mL were registered. The highest inhibitions of all three supernatants were detected against *L. monocytogenes* strains: all 25 indicator strains belonging to this species and isolated from different sources resulted sensitive. *E. mundtii* WFE3 and WFE20 were less active than strain WFE31. No big differences in terms of inhibitory power ($P > 0.05$) were found among the three strains against *Enterococcus hirae* DSM 20160^T, *L. innocua* 4202, *Lactobacillus paraplantarum* DSM 10667^T and *Pediococcus acidilactici* LMG 11384^T. Gram-negative bacteria were not inhibited by any of the active supernatants.

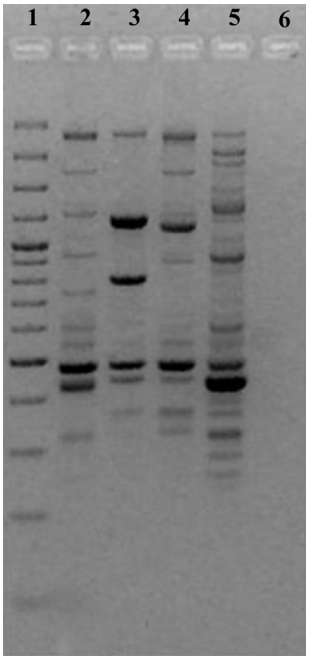

Fig. 1. RAPD-PCR profiles obtained with primer M13. Lanes: 1, GeneRuler 100 bp Plus DNA ladder (M·Medical); 2, *E. mundtii* WFE3; 3, *E. mundtii* WFE20; 4, *E. mundtii* WFE31; 5, *E. mundtii* PON10063; 6, PCR negative control.

3.3. Effect of different treatments on the antibacterial activity

The antibacterial compounds were all inactivated by proteolytic enzymes (Table 2), confirming their proteinaceous nature. The three presumptive bacteriocins were insensitive to α-amylase and lipase. Heat treatment progressively reduced the inhibitory activities of the supernatants; the inhibition was completely lost after the sterilization of supernatants. All three bacteriocins retained almost the full activity at the different pH and concentration of ethanol tested. After treatment with α-amylase and lipase and exposure at pH 10.0 and 11.0, the supernatants from the strains WFE3 and WFE20 showed comparable activities ($P < 0.05$) against *L. monocytogenes* ATCC 19114, while the treatment at 100 °C determined a decrease of WFE31 activity which was at the same level ($P > 0.05$) to that of WFE3.

3.4. Absorption to the producing cells and effect of the bacteriocins

The bacteriocins of *E. mundtii* WFE3, WFE20, and WFE31 were not absorbed by the cell surface. The effect of the active supernatants was followed for seven days (Fig. 2). During this period the growth of *L. monocytogenes* ATCC 19114 was completely inhibited by the addition of all three supernatants. After test, the cells were recovered from the bacteriocin-containing broths and transferred into bacteriocin-free BHI. Since no growth of *L. monocytogenes* ATCC

Table 2
Effect of enzymes, heat treatment, pH, and organic solvent on the inhibitory activity of *E. mundtii* WFE3, WFE20, and WFE31.

Treatment	*Enterococcus* strains			
	PON10063	WFE3	WFE20	WFE31
Control (supernatant not treated)	–	273,066 ± 118,241A	51,200 ± 0B	819,200 ± 0C
Enzymes:				
Proteinase K	–	–	–	–
Protease B	–	–	–	–
Trypsin	–	–	–	–
α-amylase	–	85,333 ± 29,560A	42,667 ± 14,780A	819,200 ± 0B
Lipase	–	85,333 ± 29,560A	51,200 ± 0A	682,667 ± 236,483B
Statistical significance:				
Strains		***	***	***
Heat treatment:				
100 °C for 20 min	–	68,267 ± 29,560A	25,600 ± 0B	170,667 ± 59,120A
100 °C for 60 min	–	25,600 ± 0A	10,667 ± 3695B	42,667 ± 14,780C
121 °C for 15 min	–	–	–	–
Statistical significance:				
Strains		**	***	**
pH:				
3.0	–	170,667 ± 59,120A	25,600 ± 0B	682,667 ± 236,483C
4.0	–	204,800 ± 0A	25,600 ± 0B	819,200 ± 0C
5.0	–	170,667 ± 59,120A	21,333 ± 7390B	819,200 ± 0C
6.0	–	102,400 ± 0A	25,600 ± 0B	819,200 ± 0C
7.0	–	102,400 ± 0A	21,333 ± 7390B	682,667 ± 236,483C
8.0	–	51,200 ± 0A	25,600 ± 0B	819,200 ± 0C
9.0	–	42,667 ± 14,780A	17,067 ± 7390B	682,667 ± 236,483C
10.0	–	25,600 ± 14,780A	12,800 ± 0A	409,600 ± 0B
11.0	–	25,600 ± 0A	12,800 ± 0A	341,333 ± 118,241B
Statistical significance:				
Strains		***	**	***
Organic solvent:				
C_2H_5OH 5%	–	102,400 ± 0A	25,600 ± 0B	341,333 ± 118,241C
C_2H_5OH 10%	–	85,333 ± 29,560A	25,600 ± 0B	204,800 ± 0C
C_2H_5OH 15%	–	85,333 ± 29,560A	25,600 ± 0B	204,800 ± 0C
Statistical significance:				
Strains		ns	ns	ns

The results are expressed in activity units (AU)/mL and indicate mean value ± SD of three replicates. The activity was measured in MRS supernatants.
–, no inhibition.
All assays were carried out against *L. monocytogenes* ATCC 19114.
P value: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns = not significant.
Uppercase letters indicate different statistical significances (overall $P < 0.05$, Tukey's correction). Means within a given column with the same letter are not statistically different from each other.

19114 was observed after incubation at the optimal temperature, the effect of the three bacteriocins, at the tested concentrations, was assumed to be bactericidal. However, these observations cannot exclude a bacteriostatic behavior at lower concentrations of bacteriocins. The supernatant from *E. mundtii* PON10063 did not show inhibitory effect, in fact, in its presence, the growth curve of the indicator strain was almost superimposable to that obtained without any addition of concentrated supernatant.

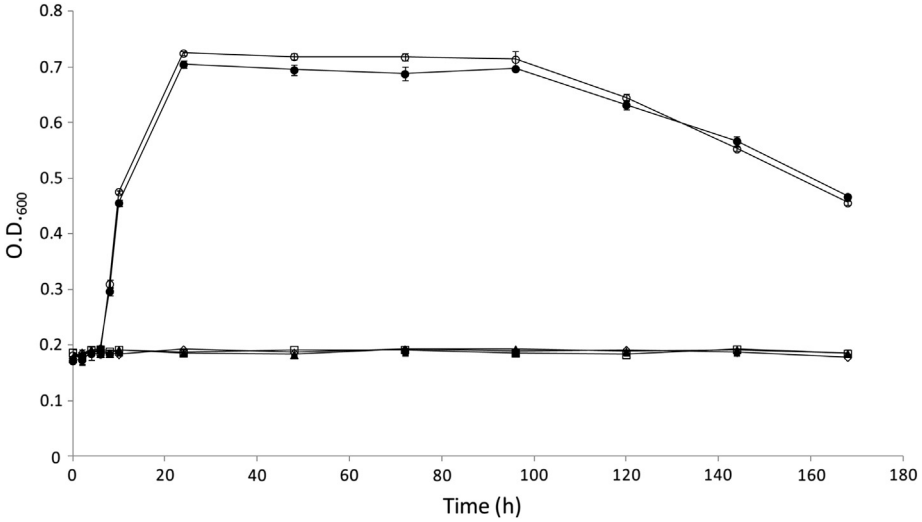

Fig. 2. Effect of bacteriocin produced by *E. mundtii* WFE3, WFE20, and WFE31 evaluated against *L. monocytogenes* ATCC 19114. Symbols: ○, without addition; ●, with supernatant from non bacteriocin producer PON10063; ◇, with bacteriocin WFE3; □, with bacteriocin WFE20; ▲, with bacteriocin WFE31. Bars represent the standard deviation of the mean value. Bars not visible are smaller than symbol size.

Table 3
Effect of different growth conditions on the inhibitory activity of *E. mundtii* WFE3, WFE20, and WFE31.

Treatment	*Enterococcus* strains			
	PON10063	WFE3	WFE20	WFE31
Control[a]	–	273,066 ± 118,241A	51,200 ± 0B	819,200 ± 0C
Temperature:				
15 °C	–	204,800 ± 0A	25,600 ± 0B	682,667 ± 236,483C
37 °C	–	204,800 ± 0A	34,133 ± 14,780B	819,200 ± 0C
45 °C	–	170,667 ± 59,121A	25,600 ± 0B	409,600 ± 0C
Statistical significance:				
Strains		ns	**	**
pH:				
4.0	–	170,667 ± 59,121A	17,067 ± 7390B	273,066 ± 118,241A
5.0	–	170,667 ± 59,121A	25,600 ± 0B	409,600 ± 0C
6.0	–	204,800 ± 0A	25,600 ± 0B	819,200 ± 0C
7.0	–	204,800 ± 0A	21,333 ± 7390B	819,200 ± 0C
8.0	–	204,800 ± 0A	25,600 ± 0B	682,667 ± 236,483C
9.0	–	204,800 ± 0A	25,600 ± 0B	409,600 ± 0C
Statistical significance:				
Strains		ns	ns	*

The results are expressed in activity units (AU)/mL and indicate mean value ± SD of three replicates. The activity was measured in MRS supernatants.
–, no inhibition.
All assays were carried out against *L. monocytogenes* ATCC 19114.
P value: *, $P \leq 0.05$; **, $P \leq 0.01$; ns = not significant.
Uppercase letters indicate different statistical significances (overall $P < 0.05$, Tukey's correction). Means within a given column with the same letter are not statistically different from each other.
[a] Growth in MRS at 30 °C for 24 h.

3.5. Effect of different incubation temperatures and initial growth pHs on bacteriocin activity

Growth at temperatures different from 30 °C (Table 3) did not affect ($P > 0.05$) bacteriocin production for the strain WFE3, but determined a consistent decrease ($P < 0.05$) for the activities of supernatants from the strains WFE20 and WFE31. The effect of pH of the MRS medium different from 6.5 on the bacteriocin production was significant ($P < 0.05$) only for the strain WFE31, bacteriocin WFE20 at pH 4.0 and for WFE31 at pH 4.0 and 5.0.

3.6. Evaluation of cytotoxicity and antibiotic sensitivity

Hemolysis of human erythrocytes was negative in PBS and *Enterococcus* supernatants, showing that the metabolites of the tested strains were not hemolytic.

For the evaluation of the effect of the antibiotics, using CLSI breakpoints for enterococci, all *Enterococcus* strains resulted resistant to penicillin and susceptible to ampicillin, quinupristin–dalfopristin, linezolid and vancomycin (results not shown).

3.7. Analysis of bacteriocin determinants

In order to analyze the DNA sequences encoding for bacteriocins, two different PCR amplifications were performed with DNAs extracted from *Enterococcus* strains. By means of the primer pairs Mnt-1F/and Mnt-1R, a 380 bp-long fragment was amplified from WFE3, WFE20 and WFE31 genomic DNA (Fig. 3A), while only WFE3 and WFE31 strains showed a PCR product of approximately 3128 bp with Mun1R/Mun7R (Fig. 3B). No amplification product was obtained from the DNA of the non bacteriocin producer *E. mundtii* PON10063.

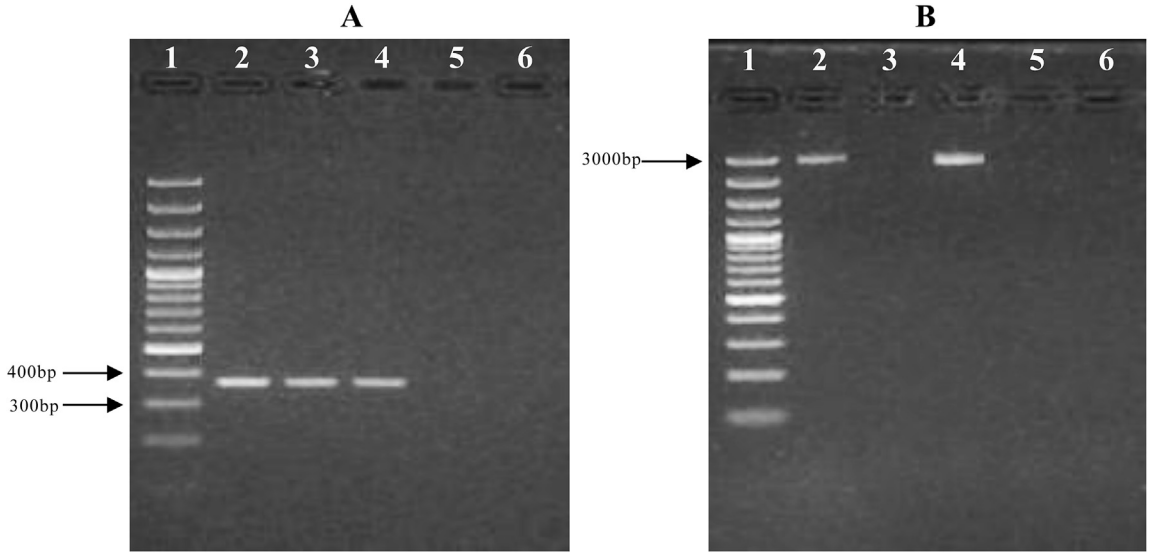

Fig. 3. PCR products for the structural genes of bacteriocins: (A) amplicons from PCR with Mnt-1F/Mnt-1R; (B) amplicons from PCR with Mun1F/Mun7R. Lanes: 1, GeneRuler 100 bp Plus DNA ladder (M·Medical); 2, *E. mundtii* WFE3; 3, *E. mundtii* WFE20; 4, *E. mundtii* WFE31; 5, *E. mundtii* PON10063; 6, PCR negative control.

The sequences of the 380 bp amplicons (Fig. 4) revealed that all three *Enterococcus* strains active against *L. monocytogenes* ATCC 19114 possessed bacteriocin-coding genes sharing 99% identity with mundticin KS (Kawamoto et al., 2002). Moreover, a complete open reading frame (ORF) was deduced from the structural gene of the three bacteriocins. It was found to encode the bacteriocin precursor (58 amino acid residues). Mature predicted peptides showed 100% identity to mundticin KS. Additionally, high sequence similarity was observed comparing aminoacidic sequences from strains WFE3, WFE20 and WFE31 to different bacteriocins in the mundticin group of class IIa, such as mundticin L (98%) and enterocin CRL35 (98%). A second partial ORF was also predicted from all three bacteriocin producing strains and it was found to encode for a putative ATP binding cassette (ABC) transporter.

3.8. Bacteriocin production in food model systems

Bacteriocin production in the different autoclaved and filtered food broths is reported in Table 4. A very low residual activity (0.01–4.17%) was recovered from the autoclaved food model systems after the growth of bacteriocin producing *E. mundtii* strains, while the filtration of the food models allowed a general higher retention of inhibitory activities for all strains which reached 33.07% for the strain WFE20 in MB. However, no activity was recovered from filtered ChB inoculated with the strains WFE20 and WFE31. Except autoclaved CeB and filtered ChB, the best results in terms of activity recovery were shown by the strain WFE20. On the contrary, the strongest reduction of the anti-*Listeria* inhibition was registered for the strain WFE31 in all food model systems sterilized both by autoclaving and filtration. The two methodology applied for the sterilization of the model systems did not determine significant differences ($P > 0.05$) of pH and a_w, but the food models were statistically different ($P < 0.001$).

3.9. In situ anti-listerial activity of bacteriocin producing E. mundtii strains

The effects of the active strains against *L. monocytogenes* ATCC 19114 during growth in the different autoclaved and filtered food model systems are shown in Table 5. Tests performed in MRS

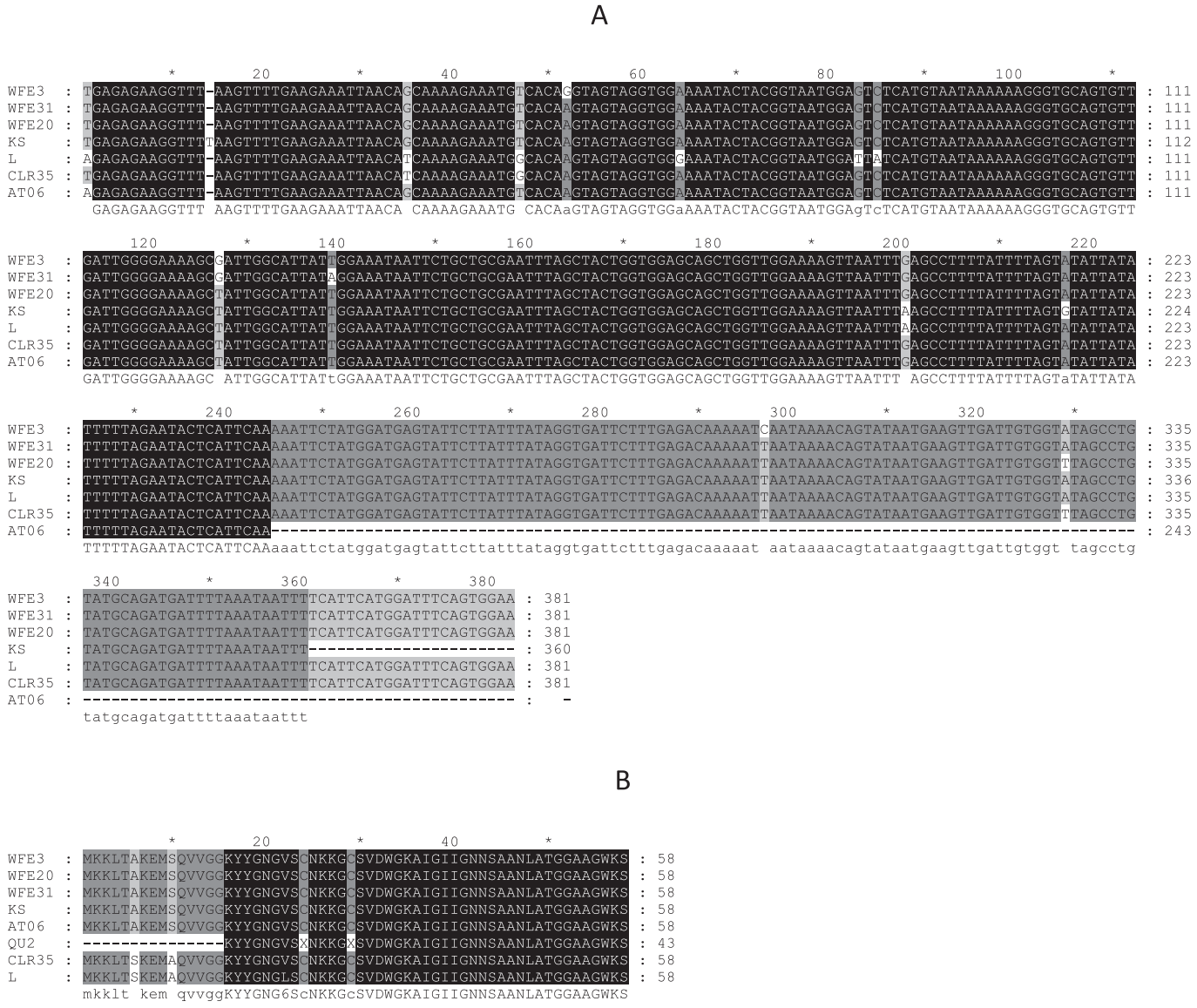

Fig. 4. Alignment of the structural bacteriocin-coding genes (A) and aminoacidic sequences; (B) in *E. mundtii* WFE3, WFE20 and WFE31.

Table 4
Bacteriocin production[a] by *E. mundtii* WFE3, WFE20, and WFE31 in food model systems.

Food model systems	Characteristics of food models[b]		*Enterococcus* strains			
	pH	a_w	PON10063	WFE3 (residual activity %)	WFE20 (residual activity %)	WFE31 (residual activity %)
Autoclaved CeB	6.10 ± 0.02	0.957 ± 0.001	–	4267 ± 1867 (1.56)A	533 ± 231 (1.04)B	200 ± 0 (0.02)B
Filtered CeB	6.37 ± 0.02	0.933 ± 0.001	–	4266 ± 1847 (1.56)A	800 ± 0 (1.56)B	8533 ± 3695 (1.04)A
Autoclaved ChB	5.12 ± 0.01	0.960 ± 0.001	–	1600 ± 0 (0.59)A	2133 ± 924 (4.17)A	533 ± 231 (0.06)B
Filtered ChB	5.60 ± 0.02	0.918 ± 0.001	–	50 ± 0 (0.02)A	0 (0)B	0 (0)B
Autoclaved FB	6.62 ± 0.03	0.946 ± 0.001	–	50 ± 0 (0.02)A	200 ± 0 (0.39)B	50 ± 0 (0.01)A
Filtered FB	6.61 ± 0.01	0.980 ± 0.001	–	12,800 ± 0 (4.69)A	8533 ± 3695 (16.67)AC	4266 ± 1847 (0.52)BC
Autoclaved MB	6.42 ± 0.01	0.958 ± 0.001	–	1067 ± 462 (0.39)A	400 ± 0 (0.78)A	50 ± 0 (0.01)B
Filtered MB	5.86 ± 0.03	0.903 ± 0.001	–	12,800 ± 0 (4.69)A	16,933 ± 7159 (33.07)A	8533 ± 3695 (1.04)A
Autoclaved VB	5.33 ± 0.03	0.953 ± 0.001	–	100 ± 0 (0.04)A	533 ± 231 (1.04)B	400 ± 0 (0.04)B
Filtered VB	5.05 ± 0.02	0.929 ± 0.001	–	2133 ± 924 (0.78)A	1600 ± 0 (3.13)AB	800 ± 0 (0.10)AC
Statistical significance:						
Treatment (*T*)	ns	ns	ns	*	*	***
Broth (*B*)	***	***	ns	ns	ns	ns
T * *B*	ns	***	ns	***	***	***

–, no inhibition.
All assays were carried out against *L. monocytogenes* ATCC 19114.
Abbreviations: CeB, cereal broth; ChB, cheese broth; FB, fish broth; MB, meat broth; VB, vegetable broth.
P value: *, $P \leq 0.05$; ***, $P \leq 0.001$; ns = not significant.
Uppercase letters indicate different statistical significances (overall $P < 0.05$, Tukey's correction). Means within a given column with the same letter are not statistically different from each other.
[a] The results are expressed in activity units (AU)/mL and indicate mean value ± SD of three replicates.
[b] Evaluated before inoculation.

confirmed the bactericidal effect of the three bacteriocinogenic strains against *L. monocytogenes* ATCC 19114. The growth of the indicator strain reached the maximal level generally at the second day of incubation, when a concentration of about 10^7 CFU/mL was registered in MRS and in almost all food models except filtered ChB and filtered MB for which a decrease was recorded from the first day on. Thus, for these two food models the inhibition of *L. monocytogenes* ATCC 19114 may be mainly imputable to the low a_w (below 0.92). All levels of concentration registered for the indicator strain in absence of *E. mundtii* were comparable ($P > 0.05$) to those showed in presence of the non bacteriocin producer *E. mundtii* PON10063. No differences ($P > 0.05$) were registered for the three bacteriocins among autoclaved and filtered MRS, filtered CeB, filtered ChB, autoclaved FB, autoclaved MB, autoclaved VB and filtered VB. The growth of *L. monocytogenes* ATCC 19114 was completely inhibited within the first 24 h of co-culturing in MRS, filtered CeB, filtered ChB and autoclaved VB, while the highest *Listeria* survival was observed in autoclaved MB and in presence of all three *E. mundtii* strains.

4. Discussion

In the present work, the bacterial isolates WFE3, WFE20 and WFE31 of wheat flour origin, presumptively allotted into the group of enterococci on the basis of growth on a kanamycin containing agar medium and preliminarily screened as producers of antimicrobial compounds (work in preparation), were characterized for their potential application as bio-preservative agents.

The three isolates were identified by a multiple genotypic approach as three distinct *E. mundtii* strains. Their supernatants were tested against pro-technological, spoilage and pathogen bacteria. Several strains of LAB were used as indicators, since they are commonly used as starter cultures in food fermentations. A bacteriocin active against starter LAB may have detrimental effects and, for this reason, not applicable in fermented food systems. *E. mundtii* WFE3, WFE20 and WFE31 were all found to be particularly active against *Listeria* spp. Within this group, *L. monocytogenes* is a difficult pathogen to control because of its ubiquitous distribution, tolerance to high levels of salt and its stability to grow at a relatively low pH and at refrigeration temperatures (Guinane, Cotter, Hill, & Ross, 2005). The anti-listerial effects of LAB are a wanted characteristic, since it strongly contributes to the safety of the final foods (Deegan, Cotter, Hill, & Ross, 2006). The three strains behaved similarly and they inhibited only a few species within the pro-technological bacterial group, but they were ineffective against Gram-negative bacteria, at least in the non concentrated form. Our findings showed the common characteristics of bacteriocins, because they are active mostly on strains closely related to the producer strains (Cotter, Hill, & Ross, 2005). The activity of some bacteriocins from LAB against Gram-negative bacteria is an unusual phenomenon; only a few bacteriocins possessed this behavior (Kuwano et al., 2005; Todorov & Dicks, 2005).

Proteolytic enzymes determined the loss of inhibitory activity for all three supernatants, proving their proteinaceous nature, a general characteristic of bacteriocins (Jack, Tagg, & Ray, 1995). Treatment with α-amylase and lipase did not alter the antibacterial activity of the active supernatants, suggesting that the active compounds did not contain a sugar or lipid moiety. A resistance of the bacteriocins to the heat treatments was registered. According to the classification of Nes et al. (1996), the bacteriocins WFE3, WFE20 and WFE31 were considered members of class II.

The retention of inhibitory power of the *E. mundtii* active supernatants in the wide pH range considered and in presence of different percentages of ethanol provided evidences of their possible application in several food ecosystems. From this perspective, when a bacteriocinogenic strain is applied during fermentation it is also important to evaluate the production of bacteriocin in different conditions that may characterize the food environment during growth (Settanni et al., 2008).

Generally, bacteriocin production by LAB is reported as a temperature-sensitive process, whereby the optimal temperature for bacteriocin production does not necessarily coincide with the optimal growth temperature (Leroy & De Vuyst, 1999). It has been suggested that bacteriocin production by LAB is enhanced by sub-optimal temperatures (Delgado, Brito, Peres, Noé-Arroyo López, & Garrido-Fernández, 2005). However, in the present study, as already observed in a previous study by Settanni et al. (2008), the results showed that temperatures different from those in the range

Table 5
In situ anti-listerial activity[a] of bacteriocin producing *Enterococcus* strains.

Growth media	Time	Growth of *L. monocytogenes* ATCC 19114 (Log CFU/mL) in co-culture with *Enterococcus mundtii*				
		Without *Enterococcus* strains	Strain PON10063	Strain WFE3	Strain WFE20	Strain WFE31
Autoclaved MRS	T_0	4.65 ± 0.40A	4.61 ± 0.26A	4.75 ± 0.26A	4.62 ± 0.18A	4.66 ± 0.36A
Autoclaved MRS	1 d	6.88 ± 0.38A	6.30 ± 0.32A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B
Autoclaved MRS	2 d	7.70 ± 0.30A	6.70 ± 0.28A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B
Autoclaved MRS	5 d	6.10 ± 0.25A	5.70 ± 0.29A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B
Filtered MRS	T_0	4.70 ± 0.30A	4.52 ± 0.30A	4.81 ± 0.40A	5.00 ± 0.30A	4.56 ± 0.40A
Filtered MRS	1 d	6.71 ± 0.30A	6.70 ± 0.40A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B
Filtered MRS	2 d	7.76 ± 0.32A	7.52 ± 0.36A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B
Filtered MRS	5 d	5.23 ± 0.13A	5.60 ± 0.41A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B
Autoclaved CeB	T_0	4.60 ± 0.30A	4.75 ± 0.34A	4.66 ± 0.34A	4.77 ± 0.24A	4.75 ± 0.34A
Autoclaved CeB	1 d	6.84 ± 0.40A	6.81 ± 0.52A	0.00 ± 0.00B	0.00 ± 0.00B	2.00 ± 0.40C
Autoclaved CeB	2 d	6.60 ± 0.34A	6.65 ± 0.52A	0.00 ± 0.00B	0.00 ± 0.00B	1.70 ± 0.30C
Autoclaved CeB	5 d	5.40 ± 0.27A	5.65 ± 0.50A	0.00 ± 0.00B	0.00 ± 0.00B	0.70 ± 0.30B
Filtered CeB	T_0	4.54 ± 0.25A	4.68 ± 0.39A	4.58 ± 0.18A	4.95 ± 0.41A	4.54 ± 0.26A
Filtered CeB	1 d	6.45 ± 0.32A	6.91 ± 0.52A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B
Filtered CeB	2 d	6.20 ± 0.36A	6.55 ± 0.54A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B
Filtered CeB	5 d	5.29 ± 0.22A	5.36 ± 0.50A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B
Autoclaved ChB	T_0	4.61 ± 0.39A	4.77 ± 0.39A	4.72 ± 0.31A	4.75 ± 0.21A	4.72 ± 0.31A
Autoclaved ChB	1 d	6.27 ± 0.29A	6.58 ± 0.25A	2.18 ± 0.29B	2.48 ± 0.39B	2.40 ± 0.39B
Autoclaved ChB	2 d	7.09 ± 0.36A	7.26 ± 0.26A	0.00 ± 0.00B	0.00 ± 0.00B	2.70 ± 0.20C
Autoclaved ChB	5 d	6.21 ± 0.34A	6.26 ± 0.33A	0.00 ± 0.00B	0.00 ± 0.00B	1.70 ± 0.20C
Filtered ChB	T_0	4.82 ± 0.39A	4.71 ± 0.30A	4.52 ± 0.31A	4.35 ± 0.21A	4.75 ± 0.31A
Filtered ChB	1 d	3.81 ± 0.30A	3.90 ± 0.17A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B
Filtered ChB	2 d	3.78 ± 0.26A	3.88 ± 0.22A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B
Filtered ChB	5 d	3.85 ± 0.30A	3.95 ± 0.35A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B
Autoclaved FB	T_0	4.50 ± 0.29A	4.70 ± 0.44A	4.70 ± 0.24A	4.65 ± 0.44A	4.62 ± 0.24A
Autoclaved FB	1 d	7.03 ± 0.25A	6.49 ± 0.45A	2.70 ± 0.15B	2.70 ± 0.35B	3.00 ± 0.25B
Autoclaved FB	2 d	7.70 ± 0.38A	7.95 ± 0.35A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B
Autoclaved FB	5 d	6.82 ± 0.30A	6.95 ± 0.44A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B
Filtered FB	T_0	4.64 ± 0.20A	4.68 ± 0.29A	4.76 ± 0.45A	4.59 ± 0.33A	4.69 ± 0.36A
Filtered FB	1 d	6.60 ± 0.34A	6.48 ± 0.48A	2.70 ± 0.32B	0.00 ± 0.00C	0.00 ± 0.00C
Filtered FB	2 d	7.18 ± 0.40A	7.27 ± 0.27A	2.08 ± 0.30B	0.00 ± 0.00C	0.00 ± 0.00C
Filtered FB	5 d	6.65 ± 0.35A	6.90 ± 0.35A	2.00 ± 0.36B	0.00 ± 0.00C	0.00 ± 0.00C
Autoclaved MB	T_0	4.30 ± 0.25A	4.62 ± 0.24A	4.68 ± 0.14A	4.65 ± 0.34A	4.76 ± 0.44A
Autoclaved MB	1 d	6.88 ± 0.32A	6.86 ± 0.20A	2.40 ± 0.20B	2.00 ± 0.20B	2.18 ± 0.20B
Autoclaved MB	2 d	6.70 ± 0.31A	7.11 ± 0.42A	2.40 ± 0.12B	2.00 ± 0.52B	2.53 ± 0.42B
Autoclaved MB	5 d	5.94 ± 0.26A	6.11 ± 0.34A	1.40 ± 0.21B	1.00 ± 0.42B	1.53 ± 0.21B
Filtered MB	T_0	4.68 ± 0.30A	4.58 ± 0.25A	4.53 ± 0.27A	4.69 ± 0.29A	4.81 ± 0.26A
Filtered MB	1 d	3.59 ± 0.30A	3.70 ± 0.30A	0.00 ± 0.00B	0.70 ± 0.40B	0.00 ± 0.00B
Filtered MB	2 d	3.58 ± 0.26A	3.28 ± 0.26A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B
Filtered MB	5 d	3.55 ± 0.30A	3.85 ± 0.30A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B
Autoclaved VB	T_0	4.80 ± 0.40A	4.70 ± 0.25A	4.70 ± 0.23A	4.72 ± 0.33A	4.59 ± 0.23A
Autoclaved VB	1 d	6.48 ± 0.28A	6.18 ± 0.36A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B
Autoclaved VB	2 d	6.15 ± 0.25A	6.30 ± 0.23A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B
Autoclaved VB	5 d	5.00 ± 0.22A	5.30 ± 0.21A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B
Filtered VB	T_0	4.60 ± 0.40A	4.80 ± 0.25A	4.70 ± 0.35A	4.67 ± 0.25A	4.78 ± 0.26A
Filtered VB	1 d	6.88 ± 0.28A	6.68 ± 0.36A	1.00 ± 0.21B	1.00 ± 0.29B	2.18 ± 0.30B
Filtered VB	2 d	6.25 ± 0.33A	6.40 ± 0.23A	0.00 ± 0.00B	0.00 ± 0.00B	0.70 ± 0.40B
Filtered VB	5 d	5.30 ± 0.45A	5.33 ± 0.21A	0.00 ± 0.00B	0.00 ± 0.00B	0.00 ± 0.00B

Results indicate mean value ± SD of two replicates.
–, no growth.
Abbreviations: CeB, cereal broth; ChB, cheese broth; FB, fish broth; MB, meat broth; VB, vegetable broth.
Uppercase letters indicate different statistical significances (overall $P < 0.05$, Tukey's correction). Means within a given column with the same letter are not statistically different from each other.
[a] Evaluated against *L. monocytogenes* ATCC 19114.

(30–37 °C) optimal for the growth of *E. mundtii* determined a lower production of the inhibitory compounds. Also the effect of the initial pH of the growth medium was in agreement with the previous findings (Settanni et al., 2008), showing a higher bacteriocin activity in the range 6.0–8.0. The neutral pH range is known to be optimal for bacteriocin production by enterococci (Leroy & De Vuyst, 2002; Van den Berghe, De Winter, & De Vuyst, 2006).

Although *E. mundtii* has not been reported as the cause of human outbreaks, as being a member of the group of enterococci its use in food applications needs to be validated by the absence of risks for consumer: e.g. absence of cytotoxicity and sensitivity to antibiotics. Hemolysis of human erythrocytes by the active supernatants was negative, proving that the whole metabolic production of *E. mundtii* WFE3, WFE20 and WFE31, including the bacteriocins, was not dangerous for the consumers' health. Our strains were sensitive to the antibiotics suggested by the CLSI, except to penicillin to which they resulted resistant. The resistance to penicillin is common to many enterococci (Murray, 1990), thus, the strains *E. mundtii* WFE3, WFE20, and WFE31 may be considered suitable for food application.

The three bacteriocins were genetically investigated. The structural genes were analyzed using primers mapping on the nucleotide sequence of *E. mundtii* bacteriocin-coding genes. All three strains were positive for the presence of a fragment of the known mundticin KS (Kawamoto et al., 2002), CRL35 (Saavedra et al., 2004), QU2 (Zendo et al., 2005) and MunL (Feng et al.,

2009), while only *E. mundtii* WFE20 was negative when analyzed with the primers designed for the biosynthetic cluster for enterocin CRL35. The nucleotide sequences of the three 381 bp DNA fragments obtained after the first PCR amplification showed only five nucleotides different among the three strains. Furthermore, the aminoacidic sequences did not show any difference among the bacteriocins produced by *E. mundtii* WFE3, WFE20, and WFE31 and they were identical to mundticin KS (Kawamoto et al., 2002). The absence of amplification product from WFE20 total DNA with the primer pairs Mun1F/Mun7R could be due to the sequence polymorphisms among the three *E. mundtii* strains. This result reflects also the genetic differences highlighted by RAPD analysis.

Mundticin KS belongs to the class IIa (Kawamoto et al., 2002) which contains a consensus YGNGV amino acid motif near the N terminus. These bacteriocins are active against *L. monocytogenes* (Ennahar, Sashihara, Sonomoto, & Ishizaki, 2000), a characteristic that determines their importance in industrial applications. Further analyses are necessary to characterize the entire bacteriocin genetic loci of *E. mundtii* WFE3, WFE20, and WFE31. Some mundticins have been reported to be encoded by gene clusters carried by plasmids (Feng et al., 2009; Kawamoto et al., 2002), but some *E. mundtii* strains have been found to express bacteriocins by chromosomal genes (Settanni et al., 2008). A character residing on chromosomal DNA is more stable than plasmid encoded information and may be significant for future applications of these strains at industrial level.

The influence of food components on bacteriocin production and activity was evaluated in five different food model systems obtained with fresh vegetables, cereals, cheeses, meats and fishes and subjected to two different sterilization procedures: autoclaving and filtration. Bacteriocin activity was recovered from almost all broths except filtered cheese broth inoculated with the strains WFE20 and WFE31. The production was strain dependent, but the best results were registered in filtered meat and fish broths. The different production may be imputable to the different presence and concentration of the food components, as well as to the different pH and a_w of the food models. Type and concentration of carbon and nitrogen sources are relevant for bacteriocin production (Delgado et al., 2007). Several authors reported that higher bacteriocin activities are observed with increased nitrogen concentrations (Aasen et al., 2000; Kim et al., 1997).

Although with some differences, nine of the ten food model systems allowed bacteriocin production. Hence, all broths were used to test the *in situ* efficacy of the *E. mundtii* strains against *L. monocytogenes* during the simulation of a common fermentation. The inhibition produced by the growing cultures was often good. This because, in addition to the bacteriocin production, the competition for nutrients, initial pH and a_w might have been limiting for the development of *L. monocytogenes*. In particular, filtered ChB and filtered MB were characterized by a_w below 0.92, which is reported to be the limit for the growth of *L. monocytogenes* in presence of NaCl and sucrose (Nolan, Chamblin, & Troller, 1992). Some *E. mundtii* have already been found effective *in situ* against *L. monocytogenes* in vegetable, cheese and fish products (Bennik et al., 1999; Bigwood et al., 2012; Vera Pingitore et al., 2012), but their bacteriocins have not been characterized for nucleotide or amino acid sequences.

This work was mainly performed to evaluate the effect of several foods (different in composition and nutrient concentrations) on the expression and activity of bacteriocins produced by three *E. mundtii* strains. The results showed that all three strains were able to produce the antimicrobial compounds in different food matrices and to control the growth of *L. monocytogenes in situ* during the fermentation process. Works will be prepared to follow the bacteriocin expression in the different food matrices.

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