Characterisation of Antilisterial Bacteriocin-Like Substance Produced by *Enterococcus mundtii*

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Abstract

Solichová K., Složilová I., Jebavá I., Uhrová B., Plocková M. (2012): **Characterisation of antilisterial bacteriocin-like substance produced by *Enterococcus mundtii*.** Czech J. Food Sci., **30**: 89–97.

The antilisterial activity of *E. mundtii* 1282 strain and its cell-free neutralised supernatant was observed against five persistent *L. monocytogenes* strains using the agar spot method. The enterococcal metabolite was consequently characterised as a proteinaceous substance – the bacteriocin-like substance, which was heat-stable at heating to 100°C for 30 min, stable at pH 2–12, and still active after eight-week long storage at 6°C and –20°C. The bacteriocin-like substance reached the highest activity of 6400 AU/ml after ten hours of cultivation of *E. mundtii* 1282 strain, when it was at the stationary phase of its growth. *E. mundtii* 1282 strain produced the bacteriocin-like substance: in BHI broth with pH 4–6, at the cultivation temperature 12–45°C, and in BHI broth with 1–6% (w/w) NaCl.

Keywords: *Enterococcus; enterocin*-like substance; *Listeria monocytogenes*; persistent

Listeria monocytogenes is known to contaminate food processing plants for prolonged periods of time, e.g. in a meat processing plant for 4 years (Nesbakken *et al*. 1996), in a slaughtering plant for 16 months (Rørvik *et al*. 2003), in a fresh sauce industry for 17 months (Pourshaban *et al.* 2000). Significantly extended periods of contamination are typical of dairy industry, e.g. in a cheese plant for 7 years (Unnerstad *et al*. 1996), in an ice cream plant for 7 years (Miettinen *et al*. 1999). This prolonged contamination is often caused by a few dominating strains or persistent strains (Unnerstad *et al*. 1996; Pourshaban *et al*. 2000; Hoffman *et al.* 2003). The occurrence of persistent strains coming from dairy and non-dairy materials is common in the dairy plant environment and, on the contrary, persistent strains are usually not found in raw materials (Miettinen *et al*. 1999). Persistent strains also show remarkably higher adherence to stainless steel surfaces in contrast to non-persistent strains (Chae & Schraft 2000; Lattore *et al.* 2011), and there are also differences in the susceptibility to disinfectants between the persistent and non-persistent *L. monocytogenes* strains (Lundén *et al.* 2008; Purkrtová *et al*. 2010). Variants resistant to nisin A, nisin Z, pediocin PA-1, divergicin M35, and to a bacteriocin-like compound produced by *Bifidobacterium thermophilum* subsp. *infantis* RBL67 were developed from *L. monocytogenes* LSD530. Moreover, the acquired bacteriocin resistance generally decreased its antibiotic sensitivity (Naghmouchi *et al.* 2007). The potency to inhibit the biofilm-forming listerias is important

Supported by the Ministry of Education, Youth and Sports of the Czech Republic, Projects No. MSM 6046137305 and No. MSM 2B08050.

from the manufacturers point of view, because the persistent strains represent a serious hazard. Unpredictable release of part of the biofilm containing *L. monocytogenes* can contaminate the final product and cause listeriosis after the consumption. This serious disease has emerged as an atypical food-borne illness causing major public health concern because of the severity of the disease (meningitis, septicaemia, and abortion), a high fatality rate (20–30% of cases), a long incubation time, and the predilection for the individuals who have an underlying condition, which leads to the impairment of T-cell-mediated immunity (Allerberger 2003).

Among the bacteriocins produced by lactic acid bacteria (LAB), nisin, a class I bacteriocin, has demonstrated antilisterial activity and has been the first bacteriocin to be characterised and the only one approved for use in food applications. However, the emergence of nisin-resistant *L. monocytogenes* mutants has been reported (Martínez *et al*. 2005; Naghmouchi *et al.* 2007). Thus, novel bacteriocins of LAB are of interest for their potential use.

The class IIa (or pediocin-like) bacteriocins are a large group of heat-stable antibacterial peptides produced by LAB typified by their potent antilisterial activity (Drider *et al*. 2006), thus the current attention is especially focused on their study and application. Enterocins are bacteriocins produced by enterococci and according to the traditional classification usually belong to the class IIa bacteriocins. The ability of enterocins to inhibit *Listeria* spp. is explained by a close relationship between enterococci and *Listeria* (Moreno *et al*. 2006) and is well-known. Most enterocins are produced by *E. faecium* and *E. faecalis* strains isolated from different food sources (Veljovic *et al.* 2009), but are produced by other species as well, e.g. *E. mundtii* (Campos *et al.* 2006; Ferreira *et al*. 2007; Settani *et al.* 2008), *E. casseliflavus* (Sabia *et al*. 2002). Some enterococcal bacteriocins can be grouped with typical bacteriocins produced by LAB according to the traditional classification, whereas others are atypical and structurally distinct from the general classes of bacteriocins. These atypical enterocins recently played an important role and prompted reclassification of the class II bacteriocins into a new scheme as suggested by Franz *et al*. (2007).

The aim of this study was to determine the antilisterial activity of an enterocin-like substance (ELS), produced by *Enterococcus mundtii* 1282 strain, against *Listeria monocytogenes* persistent strains, and to test the effects of extrinsic parameters (cultivation temperature, pH, and NaCl addition) on its production, as well as to characterise its technologically important traits (pH and thermal stability, influence of enzymes, stability during storage).

MATERIAL AND METHODS

Microorganisms and media. All microorganisms used are summarised in Table 1 and were cultivated aerobically in BHI broth (Himedia Pvt Ltd, Mumbai, India) at 37°C for 18 hours.

Preparation of culture supernatant. The strain with antilisterial activity was grown in accordance with the optimal conditions of its cultivation as given above. The culture (5 ml) was centrifuged at 3680× g for 15 min at 4°C, the cell-free supernatant was neutralised to pH 6.0–6.5 using NaOH (100 g/l solution) and heated at 90°C for 10 min to inacti-

Table 1. Origin of selected microorganisms

Strain	Source	Characterisation
E. mundtii 1282	VFU Brno, CR	raw goat milk
L. monocytogenes L-2296	Milcom a.s., Prague, CR	persistent, raw cow milk, serovar 1/2a
L. monocytogenes L-2299	Milcom a.s., Prague, CR	persistent, raw cow milk, serovar 1/2a
L. monocytogenes L-2300	Milcom a.s., Prague, CR	persistent, raw cow milk, serovar 1/2a
L. monocytogenes L-2297	Milcom a.s., Prague, CR	persistent, raw cow milk, serovar 1/2a
L. monocytogenes Lm-31*	Milcom a.s., Prague, CR	persistent, manufacturing dairy plant equipment
L. innocua Ln-03	DBM, ICT Prague, CR	bovine brain, NCTC 11288, serovar 6a
L. innocua Ln-06*	DBM, ICT Prague, CR	salad

*serovar not determined yet

vate the remaining cells and enzymes. The cell-free neutralised supernatant (CFNS) was used in further experiments concerning the bacteriocin-like substance characterisation (Franz *et al.* 1996).

Screening of antilisterial activity was performed according Kučerová *et al.* (2007); all experiments were done in BHI soft (7 g/l) agar.

Growth and ELS production. An overnight culture was inoculated (1% v/v) into BHI broth and incubated at 37°C for 18 hours. Every two hours samples were taken and the changes in pH, optical density (615 nm), and counts (CFU/ml) were recorded. The ELS activity (AU/ml) against *L. innocua* Ln-06 in CFNS was determined, as described by Chumchalová *et al.* (1998).

All experiments dealing with the effects of different extrinsic parameters were done in BHI medium and in triplicate, and *L. innocua* Ln-03 and Ln-06 strains were used as indicator strains. The effects of the cultivation temperature (12, 20, 30, 37, and 45°C) and medium pH (pH 2, 3, 4, 5 and 6) were determined according to Ogunbanwo *et al.* (2003a) and the influence of NaCl addition (1, 2, 3, 4, 5, and 6% w/w) into the cultivation medium was estimeted as published by Chumchalová *et al.* (1998).

ELS was characterised by its sensitivity to different enzymes, pH, heat treatment, and storage stability. All experiments were carried out in BHI medium and in triplicate, and *L. innocua* Ln-03 and Ln-06 strains were used as indicator strains. The influence of enzymes was established according to Kang and Lee (2005), the enzymes tested were: catalase, proteinase K (both Sigma Aldrich, St. Louis, USA), protamex (protease; Novo Nordisk A/S, Bagsvaerd, Denmark), flavourzyme (aminopeptidase; Novozymes A/S, Bagsvaerd, Denmark), protease, α-chymotrypsi, lysozyme (all Sigma Aldrich, St. Louis, USA), and validase (a mixture of fungal lipase and proteases; Valley Research, South Bend, USA). Their concentrations in CFNS were 0.1 and 1.0 mg/ml. Heat stability (5, 15, 20, 30, 40, 50, 60, 70, 80, 90, and 100°C for 10, 30, 60 min, and 121°C/15 min) was determined according to Jennes *et al.* (2000). The influence of the pH (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12) treatment was tested according to Schillinger *et al.* (1993). The enterocin stability during its storage (–20°C and 4°C) was estimated according to Ogunbanwo *et al.* (2003b).

Table 2. Inhibition of persistent *L. monocytogenes* strains by *E. mundtii* 1282

Indicator strain	Diameter of inhibition zone (mm)	
	live cells	CFNS
L-2296	20 ± 1	13 ± 3
L-2299	18 ± 2	13 ± 2
L-2300	21 ± 4	12 ± 1
L-2297	19 ± 2	13 ± 1
Lm-31	20 ± 2	11 ± 1
**L. innocua* Ln-03	20 ± 1	10 ± 1
**L. innocua* Ln-06	20 ± 1	14 ± 1

*results published by Kučerová *et al.* (2009)

RESULTS AND DISCUSSION

The *E. mundtii* 1282 strain isolated from goat raw milk had previously shown antilisterial activity against *L. innocua* (12 strains) and *L. monocytogenes* CCM5576 due to the production of ELS (Kučerová *et al.* 2009). Thus, this strain was selected for further experiments. It was revealed that this ELS has a potential to inhibit also five persistent *L. monocytogenes* strains (Table 2 and Figure 1). Due to this fact, this enterocin-like substance could be a promising natural tool for ensuring the safety of hazardous food with typical incidence of *L. monocytogenes* taking into consideration the demand of consumers for chemically untreated foods. The inhibition zones obtained in the tests against both *L. monocytogenes* and *L. innocua* species were almost the same. Other authors also

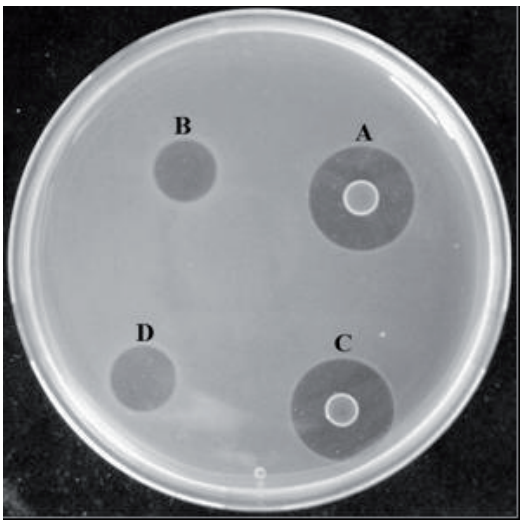

Figure 1. Antilisterial activity of *E. mundtii* 1282 strain against *L. monocytogenes* L-2292 strain by: A, C – live cells of *E. mundtii* 1282; B, D – CFNS of *E. mundtii* 1282

determined similar sensitivity of different *Listeria* species to a particular enterococcal bacteriocin (Sabia *et al*. 2002; Ferreira *et al.* 2007). Considering these circumstances, *L. innocua* Ln-03 and Ln-06 strains, being less problematic to handle with respect to safety, were used in this study as model strains for specific characterisation of the bacteriocin-like substance instead of pathogenic *L. monocytogenes* strains.

The factors influencing bacteriocin production are most important when using bacteriocinogenic cultures. Bacteriocin production *in situ* may, among others, depend greatly on physicochemical factors (e.g. pH, temperature, a_w, CO_2, O_2, redox potential, time of incubation) as well as on the food-related factors (e.g. the food structure, buffering capacity, composition, additives, antimicrobials, and processing conditions that may indirectly damage bacterial cells, as well as thermal treatments and other treatments intended to reduce the microbial load). Sometimes, at least ten-fold higher bacteriocin concentrations must be added to foods in order to achieve an equivalent inhibitory effect. The efficacy of bacteriocins in foods will greatly depend on the number of food-related factors (e.g. food processing conditions, storage temperature, food pH, and bacteriocin instability at pH changes, inactivation by food enzymes, limited stability of bacteriocin during food shelf life, etc.) that in most cases involve the interaction with the food components, precipitation, inactivation, or uneven distribution of bacteriocin molecules in the food matrix (Gálvez *et al.* 2007).

Table 3. Effect of selected extrinsic parameters on the production of ELS produced by *E. mundtii* 1282 when tested against *L. innocua* Ln-03 and Ln-06 strains

Growth condition		Inhibition zone (mm)			
		Ln-03		Ln-06	
		live cells	CFNS	live cells	CFNS
Temperature (°C)	12	13 ± 2	6 ± 0	12 ± 1	7 ± 1
	20	19 ± 0	12 ± 1	19 ± 0	12 ± 0
	30	18 ± 1	13 ± 1	20 ± 0	12 ± 1
	37	18 ± 0	12 ± 1	19 ± 0	10 ± 1
	45	12 ± 2	3 ± 2	15 ± 1	7 ± 1
pH	4	0 ± 0	0 ± 0	4 ± 3	3 ± 2
	5	17 ± 0	10 ± 1	18 ± 0	10 ± 2
	6	18 ± 0	12 ± 2	18 ± 1	12 ± 0
NaCl (%, w/w)	1	19 ± 1	9 ± 2	19 ± 0	11 ± 1
	2	16 ± 0	6 ± 0	18 ± 0	7 ± 2
	3	17 ± 0	6 ± 0	18 ± 0	7 ± 0
	4	18 ± 0	7 ± 1	18 ± 0	7 ± 1
	5	18 ± 0	6 ± 0	18 ± 0	8 ± 0
	6	17 ± 0	6 ± 0	18 ± 0	7 ± 0

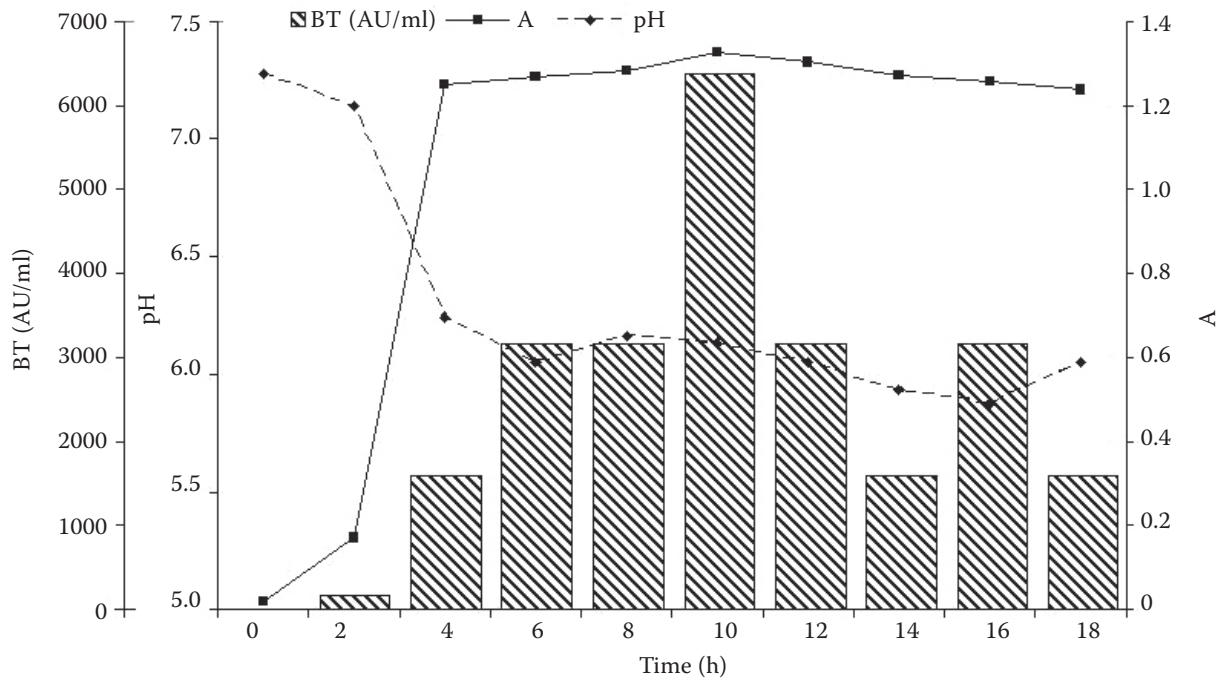

Figure 2. Growth characteristic and bacteriocin titre of *E. mundtii* 1282 strain during its cultivation in BHI broth at 37°C for 18 hours

Table 4. Stability of ELS produced by *E. mundtii* 1282 during its enzyme and pH treatment and storage at temperature –20°C and 6°C when tested against *L. innocua* Ln-03 and Ln-06 strains

Enzyme	Diameter of inhibition zone (mm)			
	Ln-03		Ln-06	
	0.1 mg/ml	1.0 mg/ml	0.1 mg/ml	1.0 mg/ml
Proteinase K	0 ± 0	0 ± 0	0 ± 0	0 ± 0
α-Chymotrypsin	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Protease	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Flavourzyme	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Protamex	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Catalase	7 ± 1	6 ± 1	12 ± 1	11 ± 1
Lysozyme	7 ± 0	6 ± 1	11 ± 1	11 ± 0
Validase	8 ± 1	6 ± 0	11 ± 0	10 ± 0
Days of storage	6°C	–20°C	6°C	–20°C
7	13 ± 0	11 ± 0	11 ± 0	10 ± 0
14	10 ± 0	9 ± 1	10 ± 0	10 ± 0
21	9 ± 1	10 ± 1	11 ± 1	11 ± 0
28	12 ± 0	9 ± 1	11 ± 0	10 ± 0
35	13 ± 1	12 ± 0	11 ± 0	10 ± 1
42	10 ± 0	10 ± 0	14 ± 1	14 ± 0
49	13 ± 0	14 ± 0	13 ± 0	15 ± 1
56	10 ± 1	10 ± 0	14 ± 0	13 ± 0
pH				
2	14 ± 0		17 ± 0	
3	15 ± 1		17 ± 1	
4	17 ± 1		17 ± 1	
5	17 ± 0		17 ± 0	
6	16 ± 1		18 ± 1	
7	17 ± 1		16 ± 0	
8	16 ± 1		17 ± 0	
9	15 ± 1		16 ± 1	
10	15 ± 0		15 ± 0	
11	13 ± 1		14 ± 1	
12	0 ± 0		5 ± 0	
Control*	0 ± 0		0 ± 0	

*distill water adjusted to the relevant pH values (2–12)

During the 18 h growth of *E. mundtii* 1282 in BHI broth at 37 °C, the pH decreased from 7.28 to 6.05. The counts increased from 5.0×10^6 CFU/ml to 1.2×10^9 CFU/ml, with maximum count 9.5×10^9 CFU/ml after 10 h of cultivation. Detectable levels of the ELS were observed after 2 h of the growth (approximately 2.0×10^2 AU/ml) against *L. innocua* Ln-06. Maximal production of ELS (6.4×10^3 AU/ml) was reached after 10 h of cultivation in the stationary phase of growth (Figure 2). This finding is in accordance with the fact that bacteriocin production is usually associated with the late exponential/early stationary growth phase (Moreno *et al*. 2002; Van den Berghe *et al*. 2006).

Table 5. Influence of heat treatment on the stability of ELS produced by *E. mundtii* 1282 when tested against *L. innocua* Ln-03 and Ln-06 strains

Temperature (°C)	Time (min)	Diameter of inhibition zone (mm)		Temperature (°C)	Time (min)	Diameter of inhibition zone (mm)	
		Ln-03	Ln-06			Ln-03	Ln-06
	10	13 ± 0	13 ± 0		10	14 ± 1	14 ± 1
5	30	12 ± 0	12 ± 0	60	30	13 ± 0	13 ± 0
	60	12 ± 1	12 ± 1		60	11 ± 1	11 ± 1
	10	13 ± 1	13 ± 1		10	13 ± 0	13 ± 0
15	30	13 ± 1	13 ± 1	70	30	12 ± 0	12 ± 0
	60	12 ± 1	12 ± 1		60	12 ± 0	12 ± 0
	10	13 ± 0	13 ± 0		10	13 ± 1	13 ± 1
20	30	13 ± 1	13 ± 1	80	30	12 ± 0	12 ± 0
	60	14 ± 1	14 ± 1		60	10 ± 1	10 ± 1
	10	12 ± 0	12 ± 0		10	12 ± 0	12 ± 0
30	30	16 ± 0	16 ± 0	90	30	11 ± 1	11 ± 1
	60	13 ± 0	13 ± 0		60	10 ± 1	10 ± 1
	10	10 ± 0	10 ± 0		10	12 ± 0	12 ± 0
40	30	11 ± 0	11 ± 0	100	30	8 ± 0	8 ± 0
	60	10 ± 1	10 ± 1		60	0 ± 0	0 ± 0
	10	11 ± 0	11 ± 0	121	15	0 ± 0	0 ± 0
50	30	11 ± 1	11 ± 1	Control		10 ± 1	14 ± 0
	60	13 ± 0	13 ± 0				

Control – untreated CFNS

The effects of the selected extrinsic parameters on the ELS production were tested (Table 3) using the indicator strains *L. innocua* Ln-03 and Ln-06. ELS was produced in BHI broth at all temperatures tested. During the cultivation at extreme temperatures (12°C and 45°C), the growth rate of *E. mundtii* 1282 strain decreased whereby ELS was produced in lower amounts in comparison to the cultivation at optimal temperature. In spite of this decline in the production of ELS during cultivation at lower temperatures, the *E. mundtii* 1282 strain could be utilised as a protective agent of ripened cheese in using it as a bacteriocinogenic culture. The growth of *E. mundtii* 1282 strain in BHI broth at pH adjusted to 2 and 3 was completely suppressed. The cultivation in BHI broth at pH 4 significantly reduced the production of ELS. In our case, the highest ELS production was observed in BHI broth at pH 5.0 and 6.0. As regards the salt tolerance, the addition of NaCl (1–6% w/w) into the cultivation medium did not affect the ELS production at all; higher concentrations of NaCl in the medium only slowed down the growth rate. The highest production of ELS in broth at pH 5.0 and 6.0 and minimal salt influence on its production make *E. mundtii* 1282 strain eligible for the protection of both hard and mould cheeses. ELS produced by *E. mundtii* 1282 was completely inactivated by all proteolytic enzymes tested (proteinase K, α-chymotrypsin, protease, flavourzyme, and protamex) at both enzyme concentrations followed. The antagonistic activity of its CFNS was not inhibited by catalase, which indicated that the inhibition observed was not due to hydrogen peroxide. CFNS was also minimally inactivated by lysozyme and validase, which suggests that the peptide did not contain or require a lipid or carbohydrate moieties for the activity, however, more lipolytic and saccharolytic enzymes have to be tested to confirm this finding (Table 4). The complete loss of this enterocin inhibitory potential after the proteolytic enzymes treatment can complicate its utilisation as a biopreservative in food in view of its protein/proteolytic enzymes content. The bacteriocins sensitivity to proteolytic enzymes is common (Kang & Lee 2005; Ghrairi

et al. 2008; Pinto *et al*. 2009) and is related with bacteriocins definition. On the other hand, our ELS could be probably inactivated in human alimentary tract after the consumption of biopreserved foods with no impact on natural enteric microflora. The ELS activity produced by *E. mundtii* 1282 was not affected by the pH treatment (Table 4) at the values of 2–11. Only the extreme pH 12 caused a decrease of its activity against *L. innocua* Ln-06 and complete loss of its activity against *L. innocua* Ln-03. This significant pH-stability allows the application of our ELS as biopreservative in the wide range of foods, and especially the fermented food. As far as the storage-stability is concerned, the stability of *E. mundtii* 1282 ELS was not affected by eight-week long storage at –20°C and 4°C (Table 4). The ability of our ELS to retain its activity during storage suggests it as a suitable biopreservative. The ELS of *E. mundtii* 1282 was not inhibited by any of the applied heat treatments except for 100°C/60 min and 121°C/min (Table 5). After the heat treatment at 100°C/30 min, the ELS activity only decreased. This finding enables e.g. the addition of our ELS directly into the milk for cheese manufacture before pasteurisation without any effect on its activity.

The antilisterial ELS produced by *E. mundtii* 1282 showed typical properties of enterocins, e.g. maximal production during the cultivation of producing strains at their optimal temperature (Aymerich *et al*. 2000; Annamalai *et al.* 2009) and neutral pH (Du Toit *et al*. 2000). Also the noticeable pH stability (Ghrairi *et al*. 2008; Pinto *et al.* 2009), heat-stability (Kawamoto *et al.* 2002; Campos *et al*. 2006), and storage-stability (Sabia *et al*. 2002; Ferreira *et al*. 2007) are typical traits of enterocins. Taking into consideration the attributes of this new ELS produced by *E. mundtii* 1282 strain, the crucial benefit resides in its activity against the persistent *L. monocytogenes* strains and its suitability for the protection of smear-cheese that are often contaminated by this pathogen.

CONCLUSIONS

E. mundtii 1282 strain with previously described antilisterial activity inhibited five persistent *L. monocytogenes* strains. This strain produced a long storage-heat- and pH-stable ELS, which was deactivated by all proteolytic enzymes tested. The tested extrinsic parameters, that allowed simultaneous growth of the strain, had minimal influence on its production. Based on the results obtained, this compound will be purified in the future and used in laboratory conditions for studying its protective effect in smear-cheese model system with persistent *L. monocytogenes* strains as indicators.

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Received for publication July 21, 2010
Accepted after corrections October 5, 2011

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