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Protease treatment affects both invasion ability and biofilm formation in *Listeria monocytogenes*

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ABSTRACT

Listeria monocytogenes is a notably invasive bacterium associated with life-threatening food-borne disease in humans. Several surface proteins have been shown to be essential in the adhesion of *L. monocytogenes*, and in the subsequent invasion of phagocytes. Because the control of the invasion of host cells by *Listeria* could potentially hinder its spread in the infected host, we have examined the effects of a protease treatment on the ability of *L. monocytogenes* to form biofilms and to invade tissues. We have chosen serratiopeptidase (SPEP), an extracellular metalloprotease produced by *Serratia marcescens* that is already widely used as an anti-inflammatory agent, and has been shown to modulate adhesin expression and to induce antibiotic sensitivity in other bacteria. Treatment of *L. monocytogenes* with sublethal concentrations of SPEP reduced their ability to form biofilms and to invade host cells. Zymograms of the treated cells revealed that Ami4b autolysin, internalinB, and ActA were sharply reduced. These cell-surface proteins are known to function as ligands in the interaction between these bacteria and their host cells, and our data suggest that treatment with this natural enzyme may provide a useful tool in the prevention of the initial adhesion of *L. monocytogenes* to the human gut.

1. Introduction

Listeria monocytogenes is a Gram-positive organism that is a facultative intracellular pathogen of humans and animals, and is responsible for severe food-borne infections, primarily in immunocompromised individuals [1–3]. Following ingestion, bacteria cross the intestinal barrier by penetrating the intestinal cell epithelium, translocate into the liver, spleen, lymph nodes, brain, and, in pregnant women, cross the fetoplacental barrier leading to infection of the foetus. Several surface proteins expressed by this intracellular pathogen interact with abiotic and biotic surfaces and are essential in bacterial adhesion to and invasion of different cell types [4–7]. In fact, *L. monocytogenes* must first adhere to host cells by binding different eukaryotic receptors through several surface ligands [8]. Among these, internalin A (InlA), which binds E-cadherin [9], and internalin B (InlB), that interacts with gC1q-R [10] or activates the tyrosine kinase MET receptor [11], initiate contact with eukaryotic cells. Many other bacterial surface proteins are involved in adherence to and invasion of numerous intestinal cultured cell lines, such as the extracellular protein p60 [12], the fibronectin-binding protein ActA responsible for actin tail formation [13,14], the murein hydrolase activity-autolysins, P45, Ami, MurA, and Auto [15,16], and the 104-kDa *Listeria* adhesion protein, named LAP [17]. Recently, listeriolysin-O, a pore-forming haemolysin [18], already known to play an important role in bacterial escape from the primary phagosome, has been identified as a further factor capable of modulating *L. monocytogenes* adhesion to eukaryotic cells [19].

L. monocytogenes also possesses the ability to adhere to and colonize abiotic surfaces. Bacteria form biofilms on food-processing surfaces which can be a source of contamination and may represent a reservoir that is more resistant to disinfectants and sanitizing agents than planktonic cells [20]. Another particularly interesting feature of this organism is represented by its resistance to acidic conditions (acid tolerance response, ATR). ATR is a complex phenomenon, involving multiple systems for the maintenance of intracellular pH homeostasis [21,22], which has been correlated to the induction or down-regulation of up to 53 proteins [23] and to

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modulation of *in vitro* and *in vivo* adhesive and invasive *L. monocytogenes* behaviour [24–26].

The Gram-negative opportunistic human and insect pathogen *Serratia marcescens* produces many extracellular enzymes and metalloproteases. Serratiopeptidase (SPEP), a 50-kDa extracellular metalloprotease, is produced by the reference strain ATCC 27117 (originally designated as strain E-15 by Miyata et al. [27] and isolated from insect gut), and it is a potent protease in which a zinc atom is essential for proteolytic activity [28,29]. SPEP is widely used as an anti-inflammatory agent [30,31] and has been shown to modulate adhesin expression in some bacterial species and to enhance antibiotic efficacy towards biofilm-forming bacteria [32,33].

The aim of this study was to evaluate the antimicrobial properties of SPEP in impairing virulence-related listerial associations, such as adhesion to and infection of intestine lining cells, attachment to inert surfaces, and acid adaptation in the external environment and in the host. For this purpose we have investigated the effect of non-bacteriostatic and non-bactericidal concentrations of the SPEP enzyme on the human enterocyte-like cell invasion pathway, haemolysis activity, acid resistance response and biofilm formation by different *L. monocytogenes* strains. We have demonstrated a dose-dependent inhibition by the SPEP enzyme of *L. monocytogenes* invasiveness, haemolysis and biofilm formation. Proteomic investigation after SPEP treatment identified various surface cell membrane proteins affected by the enzyme. Among the proteins negatively affected by SPEP treatment, we identified some members of the adhesion superfamily, such as internalin B (InlB), Ami4b autolysin, and the actin assembly-inducing protein ActA.

2. Results

2.1. SPEP is not cytotoxic and does not affect the growth of L. monocytogenes

Preliminary experiments were carried out to assess the effect of SPEP on the multiplication of *Listeria* strains (Table 1). Logarithmically grown clinical and food isolates of *L. monocytogenes* and the non-pathogenic non-invasive *L. innocua* isolate were added to various amounts of SPEP (serial 1:2 dilutions starting from 1000 U/mL) in 96-well plates at time 0, and the growth was monitored over 24 h. SPEP did not influence *L. monocytogenes* and *L. innocua* growth up to 1000 U/mL concentration. Subsequently, cytotoxicity experiments were carried out by incubating SPEP concentrations (serial 1:2 dilutions starting from 1000 U/mL) with Caco-2 cells for 1 and 24 h. In these experimental conditions, cell morphology, viability and proliferation were never affected by SPEP.

2.2. SPEP treatment influences the invasion efficiency of L. monocytogenes

To investigate whether SPEP could alter the bacterial invasive pathway, *L. monocytogenes* and *L. innocua* strains were tested using adhesion and invasion assays in the human enterocyte-like cell line Caco-2. These trials were performed with overnight bacterial cultures grown in the presence of a non-cytotoxic and non-bacteriostatic SPEP concentration (200 U/mL), and further subcultured for 1 h at 37 °C in BHI broth with the enzyme. Table 2 shows the adhesion and invasion efficiency of SPEP-treated and untreated *Listeria* strains. The adhesion levels of *L. monocytogenes* strains were generally low (0.3–3%) and were unaffected by overnight incubation with the enzyme. Conversely, all the *L. monocytogenes* strains tested were truly invasive, and the invasion efficiency, i.e. the percent of the inoculated CFU which were internalized, ranged from 0.5 to 7.5. Furthermore, following SPEP treatment, the invasive efficiency was reduced in most strains (between 2- and 6-fold decreases with respect to untreated bacteria). This effect was dose-dependent (results not shown).

Table 1
Listeria spp. utilized in this study

Species	Strain	Origin	Serotype	Culture collection
L. monocytogenes	ATCC 7644	Clinical	1	ATCC
	LmE1	Clinical	4	IMUR
	LmE8	Clinical	1	IMUR
	LmE9	Clinical	4	IMUR
	LmE10	Clinical	4	IMUR
	Lm2	Clinical	1	IMUR
	LmCTT1	Food	4	UN
	LmFI	Food	4	UN
	Lm7	Food	1	UN
	Lm29	Food	1	UN
L. innocua	HI	Food	–	IPHR

ATCC: American Culture Collection, Rockville, MD, USA; IMUR: Institute of Microbiology, Department of Public Health Sciences University of Rome "La Sapienza"; UN: II University of Naples; and IPHR: Institute of Public Health, Rome.
Serotype determined by agglutination assay.

2.3. SPEP treatment decreases the listeriolysin-O haemolytic activity

Haemolysis induced by the pore-forming toxin listeriolysin-O, that is an essential virulence factor of *L. monocytogenes* [34], was assayed in supernatants from bacteria grown overnight at 37 °C in BHI in the presence or absence of 200 U/mL SPEP. The haemolytic activity titre of *Listeria* strains grown with or without SPEP, expressed in minimal haemolysis units, is shown in Table 2. The ability of bacteria to lyse sheep blood cells was strongly reduced following enzyme treatment (from 5- to 56-fold haemolysis reduction with respect to untreated bacteria). No haemolysis was detectable in supernatants from the non-haemolytic, non-pathogenic *L. innocua* strain.

2.4. SPEP treatment does not modify the L. monocytogenes survival at acidic pH

The effect of SPEP on another interesting feature of *Listeria* spp., the resistance to extremely acidic conditions, was tested. For this purpose *Listeria* strains were screened for acid resistance to lethal pH (pH 3.5) following pre-adaptation of bacterial cultures at mild acidic pH (pH 5.1), as described in Section 4. All *L. monocytogenes* isolates, as well as the *L. innocua* strain, exhibited an acid tolerant response which was strain dependent (Table 3). When acid resistance was tested with bacteria grown overnight in presence of SPEP, and then acid pre-adapted, their survival at lethal pH was not significantly modified, and was similar to that of untreated bacteria.

2.5. SPEP treatment hinders biofilm formation by L. monocytogenes

Since it is known that *L. monocytogenes* has the ability to adhere to inert surfaces and form biofilms, the effect of SPEP on this colonizing property was tested. *Listeria* strains, after overnight culture in the presence or in the absence of 200 U/mL of SPEP, were inoculated in BHI and grown in microtitre polystyrene wells at 37 °C. All strains attained the stationary phase after 24 h. Unattached bacteria were removed and the remaining biofilm was stained. No significant relationship was found between biofilm formation and final cell density of the bacterial supernatants at 24 h. All *L. monocytogenes* strains tested in this study were capable of producing biofilms and could be classified as moderate or strong biofilm producers, according to the classification parameters described in

Table 2
Adhesion, invasion and haemolytic activity of SPEP-treated (200 U/mL) and untreated *L. monocytogenes* strains

Strains	Untreated			SPEP-treated		
	Adhesion[a] (%)	Invasion[b] (%)	Haemolysis[c] (MHU)	Adhesion[a] (%)	Invasion[b] (%)	Haemolysis[c] (MHU)
ATCC 7644	0.5 ± 0.1	3.2 ± 0.3	320	0.4 ± 01	2.0 ± 0.2	25
LmE1	0.6 ± 0.2	0.5 ± 0.05	112	0.4 ± 0.0	0.2 ± 0.1	8
LmE8	2.7 ± 0.5	6.9 ± 0.3	64	2.7 ± 0.7	5.9 ± 0.2	14
LmE9	3.0 ± 0.3	4.7 ± 0.4	56	2.9 ± 0.4	1.8 ± 0.2	1
LmE10	1.7 ± 0.5	1.4 ± 0.2	40	1.9 ± 0.4	0.5 ± 0.0	2
Lm2	1.2 ± 0.2	7.5 ± 0.4	128	0.9 ± 0.1	3.2 ± 0.1	18
LmCTT1	1.9 ± 0.5	0.5 ± 0.0	80	1.7 ± 0.6	0. 4 ± 0.2	5
LmFI	0.9 ± 0.3	0.5 ± 0.1	68	1.0 ± 0.1	0.2 ± 0.1	5
Lm7	0.7 ± 0.4	5.9 ± 0.3	80	0.6 ± 0.2	0.9 ± 0.4	14
Lm29	0.3 ± 0.1	0.5 ± 0.3	48	0.1 ± 0.1	0.1 ± 0.0	4
L. innocua HI	0.1 ± 0.05	0.2 ± 0.0	–	0.15 ± 0.0	0.2 ± 0.0	–

[a] Adhesion is expressed as the percentage of the initial inoculum of bacteria that adhered to intestinal-like cells 1 h post-infection at 4 °C.
[b] Invasion efficiency is expressed as the percentage of the initial inoculum of bacteria that were gentamicin resistant 1 h post-infection.
[c] The ability of bacteria to lyse sheep blood cells was tested in culture supernatants grown overnight in BHI at 37 °C. The haemolytic titre was expressed in minimal haemolysis units (MHU), corresponding to the reciprocal of the highest dilution at which a complete haemolysis was detected. Data represent the means ± standard error of three independent experiments.

Section 4. *L. monocytogenes* LmATCC, LmE8, LmE9 and Lm7 strains were the strongest biofilm formers ($OD_{570} = 0.168$, 0.166, 0.393, and 0.160, respectively), while the non-pathogenic *L. innocua* strain was a non-biofilm former (OD_{570} 0.035) (Table 4). When *Listeria* strains were treated with SPEP, prior and during the assay, the development of biofilms was completely inhibited or significantly reduced. Shorter pre-incubation periods with SPEP showed that, already after 3 h, biofilm formation lowered by about 90%. In order to assess the efficacy of SPEP towards biofilm formation at lower temperatures, further assays we performed. These experiments were carried out at 37 °C, 25 °C and 4 °C with a strong biofilm producer (*L. monocytogenes* Lm7), a moderate biofilm producer (*L. monocytogenes* Lm29) and the ATCC 7644 strain, in the same above reported experimental conditions. Results obtained showed the *L. monocytogenes* strains were able to form biofilm at 25 and 4 °C, even than at a lower extent, and that the protease treatment was able to induce a decrease in the adhesion levels to plastic surfaces in all the experimental conditions tested (data not shown).

LmATCC, LmE8, LmE9 and Lm7 strains, which were the most efficient biofilm producers, were also tested by the SEM morphological approach. For this purpose PTFE 0.22 μm filters were utilized and incubated up to 24 h with bacteria in presence and absence of SPEP in the experimental conditions described above. SEM analysis further confirmed the findings observed on polystyrene surfaces. After 24 h incubation at 37 °C, untreated bacteria produced typical three-dimensional biofilm structures, whereas SPEP-treated bacteria were unable to efficiently attach to and colonize PTFE surfaces. Fig. 1 shows a typical thick net-like pattern established by untreated *Listeria* cells (Lm7 strain) (Fig. 1A) and the almost complete absence of matrix structures as well as the presence of rare bacteria following SPEP treatment (Fig. 1B).

Table 3
ATR response of SPEP-treated (200 U/mL) and untreated *Listeria* strains

Strains	% of Survival			
	Untreated		SPEP-treated	
	pH 7	pH 5	pH 7	pH 5
ATCC 7644	5.0 ± 1.0	15.0 ± 4.0	4.9 ± 2.0	15.1 ± 5.0
LmE1	19.1 ± 3.0	52.6 ± 10.0	10.4 ± 2.0	61.1 ± 8.0
LmE8	59.8 ± 5.0	79.3 ± 9.0	50.1 ± 2.0	82.9 ± 11.0
LmE9	48.7 ± 4.0	81.2 ± 11.0	68.9 ± 8.0	73.7 ± 10.0
LmE10	3.2 ± 0.5	6.0 ± 1.0	5.2 ± 2.0	6.8 ± 2.0
Lm2	5.6 ± 1.5	52.7 ± 5.0	0.3 ± 1.0	39.0 ± 4.0
LmCTT1	0.1 ± 0.0	29.9 ± 6.0	6.5 ± 1.0	29.8 ± 3.0
LmFI	33.7 ± 4.0	50.9 ± 3.0	24.2 ± 5.0	43.9 ± 4.0
Lm7	0.8 ± 0.3	30.4 ± 6.0	9.5 ± 0.6	45.3 ± 5.0
Lm29	1.2 ± 0.7	45.0 ± 5.0	8.3 ± 0.4	51.7 ± 4.0
L. innocua HI	0.3 ± 0.0	53.5 ± 8.0	6.4 ± 1.6	47.2 ± 5.0

Survival rates are reported for acid-adapted (pH 5.1) and non-adapted (pH 7.0) SPEP-treated (200 U/mL) and untreated bacteria. Bacterial counts were performed after 1 h incubation at 37 °C in BHI acidified at pH 3.5. Data represent the mean ± standard error of three independent experiments.

2.6. SDS analysis of surface proteins in SPEP-treated and untreated L. monocytogenes

To investigate the putative effect of SPEP incubation on the surface protein pattern of bacteria, SDS analysis was performed on *L. monocytogenes* Lm7 strain whose invasiveness and biofilm formation ability were significantly affected by SPEP treatment, and compared with that of the Lm29 strain which was only marginally affected by this treatment. Fig. 2A shows the electrophoretic profiles of the protein mixtures obtained from surface protein extraction stained with colloidal Coomassie blue. Several discrete protein bands corresponding to the surface proteins extracted from Lm29 and Lm7 cells were observed. These profiles were compared to the protein patterns following SPEP treatment. Results indicated that following SPEP treatment various bands of the Lm7 protein profile either disappeared or showed a reduced intensity, whereas only slight modifications were detected in the Lm29 profile. The protein bands occurring in Lm7 protein profile and disappearing upon SPEP treatment were selected for identification. The selected protein bands (letters from A to D in Fig. 2A) were excised from the gel and submitted to identification by mass spectrometric analysis. The corresponding gel slices from SPEP-treated Lm29 surface protein, were also excised and submitted to the identification procedure as a control.

2.7. Identification of L. monocytogenes proteins by mass spectrometry analysis

Structural analyses were performed separately on each band from individual lanes. Proteins excised from the gel were reduced, alkylated and digested *in situ* with trypsin and the resulting peptide mixtures were directly analyzed by MALDI-MS, according to the peptide mass fingerprinting procedure. The corresponding mass signals were used to search a non-redundant sequence database using an in house version of the MASCOT software, taking advantage of the specificity of trypsin digestion and the taxonomic category of the samples. The set of experimental mass values was compared to the theoretically predicted peptides from all the

Table 4
Biofilm formation by SPEP-treated (200 U/mL) and untreated *L. monocytogenes* strains

Strains	Untreated		SPEP-treated	
	OD mean ± SD	Biofilm production	OD mean ± SD	Biofilm production
ATCC 7644	0.168 ± 0.034	Strong	0.024 ± 0.0026	No
LmE1	0.101 ± 0.011	Moderate	0.024 ± 0.006	No
LmE8	0.166 ± 0.036	Strong	0.023 ± 0.002	No
LmE9	0.393 ± 0.078	Strong	0.071 ± 0.018	Weak
LmE10	0.076 ± 0.006	Moderate	0.022 ± 0.002	No
Lm2	0.086 ± 0.005	Moderate	0.022 ± 0.005	No
LmCTT1	0.110 ± 0.011	Moderate	0.028 ± 0.005	No
LmFI	0.100 ± 0.018	Moderate	0.024 ± 0.006	No
Lm7	0.160 ± 0.025	Strong	0.027 ± 0.003	No
Lm29	0.070 ± 0.012	Moderate	0.029 ± 0.004	No
L. innocua HI	0.035 ± 0.007	Weak	0.023 ± 0.004	No

Based on the OD (570 nm) produced by bacterial biofilms, strains were classified into the following categories: biofilm non-producers, weak, and moderate or strong biofilm producers. Data represent the means ± standard error of three independent experiments.

proteins in the database. The results are summarized in Table 5. The results indicated that SPEP treatment led to the disappearance of several proteins belonging to the adhesion family. Among these, Ami 4b autolysin, internalin B and adhesion ActA are known to be involved in the interaction between bacteria and target cells.

2.8. Zymogram analysis of L. monocytogenes autolysins affected by SPEP treatment

Some portions of the cell-surface protein samples from SPEP-treated and untreated cultures of *L. monocytogenes* Lm7 and Lm29 strains were analyzed by SDS-PAGE and parallel zymogram assays. The results are shown in Fig. 2B. In the separation range considered (8% acrylamide/bis-acrylamide), various autolytic bands were detectable for both strains. At least six autolytic bands could be detected for strain Lm7 (black dots), one of which greater than 150 kDa, and only two for strain Lm29. These results clearly indicated that SPEP treatment led to the disappearance and/or downshifting of several protein bands belonging to the autolysins family, for both strains considered.

3. Discussion

We have previously demonstrated that serratiopeptidase from *S. marcescens* is effective in preventing experimental infections caused by biofilm-forming bacteria and enhances the antibiotic efficacy in the treatment of these infections [33]. The present study was undertaken to identify the effect of this metalloprotease on various properties related to *L. monocytogenes* virulence.

Many proteolytic enzymes produced by bacteria, or by animal organs such as the pancreas, have been included in therapeutic agents. SPEP is a proteolytic enzyme widely used as an anti-inflammatory drug and as a mucoactive agent [35]. Animal trials have shown that SPEP is absorbed from the intestinal tract and transferred into the circulation in an enzymatically active form after intraduodenal or oral administration [36]. SPEP has been used as an anti-inflammatory drug for breast engorgement [30], swelling of the ankle joint [37], acute or chronic inflammation in otorhinolaryngology [31], prosthetic device infections [33], chronic airway disease [35], amicrobial chronic prostato-vesiculitis [38], and inflammatory venous disease [39].

In this study we have demonstrated the *in vitro* efficacy of SPEP in affecting different virulence properties related to *L. monocytogenes* interaction with cells and abiotic surfaces. A profusion of evidence obtained in the present experiments demonstrates that *in vitro* treatment of bacteria with non-cytotoxic, non-bactericidal and non-bacteriostatic concentrations of enzyme can modulate the expression of the virulent phenotype of clinical and food *L. monocytogenes* isolates, i.e. bacterial invasion ability, haemolysis and biofilm production. In fact, our results clearly indicate that in most strains, following SPEP treatment, the invasive efficiency in the intestinal-like Caco-2 cell model, the ability of bacteria to lyse sheep erythrocytes, and the development of biofilm on inert surfaces at different temperatures (37, 25 and 4 °C) were significantly reduced or completely inhibited. Scanning electron microscopy showed that untreated cells of a strong biofilm-forming strain of *Listeria* produced a complex three-dimensional community on a PFTE filter, while SPEP-treated cells of the same strain adhered only poorly and failed to produce biofilms. Conversely the acid

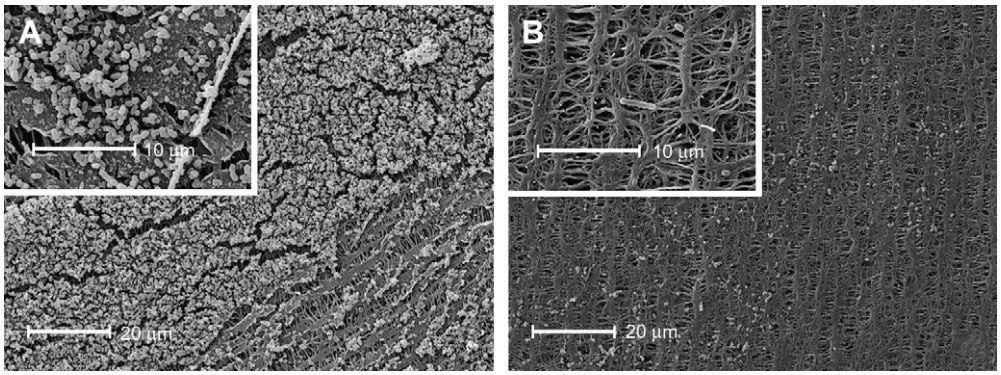

Fig. 1. Scanning electron microscopy analysis of *L. monocytogenes* Lm7 grown overnight in BHI in presence of a PFTE filter as a substrate for sessile growth in absence (A), or in presence (B) of 200 U/mL of SPEP. In the magnification box of panel A, the extracellular matrix is visible and biofilm-embedded bacteria cover the filter surface. In panel B the PTFE filter background is visible and only single cells are present. Bar, 20 µm and magnification box bar, 10 µm.

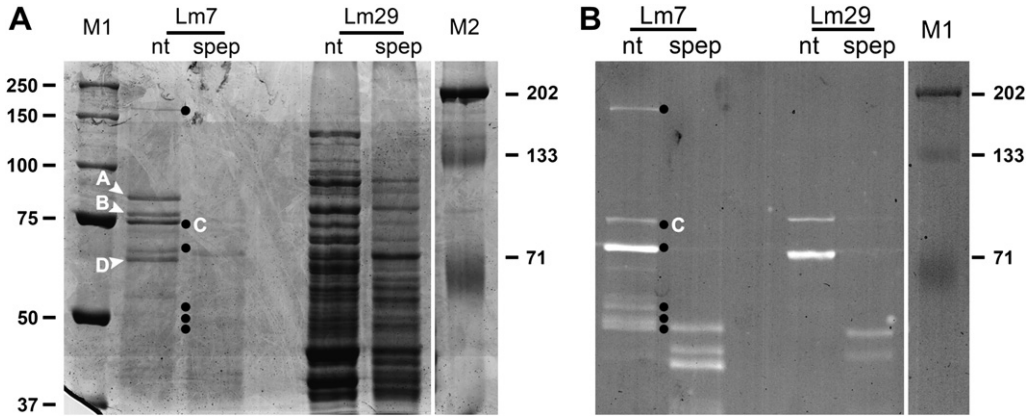

Fig. 2. Crude cell envelope SDS extract from SPEP-treated (SPEP 200 U/mL) and untreated (nt) *L. monocytogenes* Lm7 and Lm29 strains, analyzed by simultaneous (A) SDS-PAGE and (B) zymogram assays (see Section 4.13). The apparent molecular masses of standard M1 (Precision Plus Protein unstained, Bio-Rad) and M2 (Kaleidoscope Prestained, Bio-Rad), indicated are in kDa. Autolysins (black circles) formed translucent areas in the zymogram and appear as white bands in the photographs. Protein bands indicated by A, B, C and D were analyzed by mass spectrometry analysis and the results are summarized in Table 5.

resistance properties of the *Listeria* strains tested, that allow acid-adapted bacteria to survive at lethal pH, were not modified by SPEP treatment.

It is well known that all of the *L. monocytogenes* properties discussed above can be directly or indirectly related to bacterial surface proteins. In fact *Listeria* encodes over 130 surface proteins that are associated with the ability of this bacterium to survive in diverse environments and to interact with different kinds of eukaryotic cells. Among surface proteins, those with murein hydrolase activity-autolysins, such as p60, P45, Ami, MurA and Auto, are involved in numerous cellular processes including anchoring properties and biofilm formation [4–7,15,16,40]. For this reason we used zymograms and proteomic analysis to examine the surface protein profile of the *L. monocytogenes* Lm7 strain, whose invasiveness, haemolysis and biofilm formation were strongly influenced by SPEP, to assess whether an alteration of surface proteins had occurred as a result of enzyme treatment. Comparative proteomic investigation on Lm7 protein profiles before and after protease incubation identified various surface cell membrane proteins affected by SPEP treatment. Zymogram analysis showed that various autolytic bands, corresponding to the autolysin family, disappeared after SPEP treatment. Many autolytic bands were clearly visible for the highly invasive strain Lm7 while in Lm29 only two bands were detected. Among the proteins removed from the surface of *Listeria* cells by SPEP treatment were some members of the adhesion superfamily, such as internalin B (InlB), Ami 4b autolysin, actin assembly-inducing protein ActA, and the autolytic enzyme *N*-acetyl muramoyl-L-alanine amidase. These findings could explain the different interaction of SPEP-treated and untreated bacteria with eukaryotic cells and inert surfaces. Indeed, both InlB and Ami proteins are associated with the bacterial surface by their C-terminal part made of conserved modules containing the dipeptide GW, and they contribute to the fine-tuning of the molecular events that occur in *Listeria*–cell interactions and to the adhesion of bacteria to mammalian cells [4,14,29,38,41]. In addition, InlB cooperates for efficient *L. monocytogenes* entry [41]. ActA protein, the other protein removed by SPEP treatment, is a hydrophobic tail protein critical for virulence in inducing actin polymerization and generating force for the movement [5], well known to be involved in the recognition of heparin sulphate proteoglycan receptors [13].

Table 5
Proteins from *L. monocytogenes* Lm7 SDS analysis, identified by mass spectrometric analysis

Protein band	Identification	Accession number
A	Actin assembly-inducing protein (ActA)	GI/29651044
B	Ami 4b Autolysis	GI/12054356
C	Protein *N*-acetyl muramoyl-L-alanine amidase, Family 4	GI/47092504
D	Internalin b precursor (InlB)	GI/47092504

Concerning the complete inhibition or strong reduction of haemolytic activity by *L. monocytogenes* strains pre-incubated with SPEP, these can be attributed to an effect directed towards listeriolysin-O, the pore-forming haemolysin of *Listeria* [18]. This protein, known to be involved in the delivery of bacteria to the cytoplasm, has been recently identified as a further factor capable of modulating *L. monocytogenes* adhesion to eukaryotic epithelial cells [19]. Thus the decreased invasion of intestinal cells could also be explained by the effect of SPEP on this protein.

L. monocytogenes is an opportunistic Gram-positive intracellular facultative pathogen, responsible for severe food-borne diseases, that causes rare but frequently fatal infections. In recent years the detection of *Listeria* strains isolated from humans, from food production or processing facilities that are resistant to one or more antibiotics is emerging [42,43]. In fact, recent studies demonstrated that *L. monocytogenes* isolated from the environment contained one or more antimicrobial resistance genes [42,44]. Moreover, biofilm-grown cells may express properties distinct from planktonic cells, one of which is an increased resistance to antimicrobial agents [45]. For this purpose the identification of molecules capable of altering the process of *Listeria* interaction with cells or abiotic surfaces, and hindering its entry into susceptible cells appears to be fundamental for defining appropriate preventive and therapeutic strategies. Results of this study allowed the identification of a non-cytotoxic natural compound effective towards *Listeria* virulence. To confirm our *in vitro* findings, additional *in vivo* studies should be developed. The interest of SPEP, besides its being commonly used in human therapy, resides in the fact that, at concentrations not affecting bacterial viability, it could prevent *L. monocytogenes* entry at the initial site of infection in the gut, and hinder the attachment to different abiotic surfaces. Therefore it can be argued that this protease could be developed, as a potential "antipathogenic agent" that would hinder the entry of *Listeria* into human tissues, and also reduce the extent to which these pathogens form biofilms in the food-processing environment.

4. Materials and methods

4.1. Bacterial strains and culture conditions

Clinical and food *L. monocytogenes* isolates as well as a non-pathogenetic *L. innocua* strain used in this study are listed in Table 1. All strains were cultured on Oxford selective medium (Oxoid, UK), and their identity was confirmed using the API *Listeria* kit (Bio Mérieux, France) according to the manufacturer's instructions. Haemolysis on Muller Hinton agar (Oxoid) supplemented with 5% horse blood was used as additional test.

4.2. Chemicals

Serratiopeptidase (SPEP, 2540 U/mg), obtained from Takeda Italia Farmaceutici (Rome, Italy), was dissolved in phosphate-buffered saline pH 7.2 (PBS) at a stock concentration of 20 000 U/mL and stored at −20 °C. All reagents and solvents were of the highest purity available from Sigma Chemicals (St. Louis MO, USA). Trifluoroacetic acid (TFA) HPLC grade was from Carlo Erba (Italy).

4.3. Minimum inhibitory concentration (MIC) assays

MICs by SPEP were determined in 96-well plates measuring the optical density at the wavelength of 630 nm (OD_{630}). BHI was added to all wells and SPEP (1000 U/mL) was added to the first well and serially diluted (1:2 dilutions). Logarithmic-phase cultures of *L. monocytogenes* and *L. innocua* were added to each well to achieve 10^6 CFU/well. The microtitre plates were incubated at 37 °C and OD_{630} was recorded after 1 and 24 h. The MICs were defined as the lowest concentrations of proteins that completely inhibited growth.

4.4. Cells

Human enterocyte-like cells derived from a colonic carcinoma (Caco-2 cells) were cultured in Eagle's minimal essential medium (MEM), supplemented with 10% foetal calf serum and 2 mM glutamine in an atmosphere of 95% air and 5% CO_2. All media were from Euroclone. All incubations were carried out in a 5% CO_2 atmosphere at 37 °C. Monolayers were used 48 h after seeding.

4.5. Cytotoxicity assays

To establish the maximal non-cytotoxic dose of SPEP Caco-2 cells were seeded two-days prior to the assays into 96-well plates (Falcon) at a density of approximately 10 000 cells per well. Two-fold serial dilutions of the compound were added to the well ($n = 3$). Untreated cells served as negative control (0% cytotoxicity). The plates were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 1 and 24 h. Cell morphology and viability (determined by neutral red staining) were examined by light microscopy, and cell proliferation was quantitatively evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Results were expressed as complete cytotoxicity (++) when at least one of the parameters was affected in 100% of cells, partial cytotoxicity (+) when one parameter was affected in 50% of cells, or absence of cytotoxicity (–) when none of the parameters was affected.

4.6. Adhesion assays

Bacteria from 18 h cultures in BHI broth, grown in presence or in absence of different SPEP concentrations (50, 100, 200, 400 U/mL), were further subcultured for 1 h at 37 °C in BHI with or without enzyme. Caco-2 cells, cultured in 24-well plates (Falcon) to obtain semi-confluent monolayers (1×10^5 cells/well) were then inoculated with 0.5 mL of bacterial suspensions in logarithmic-phase growth at an MOI of about 100 bacteria per cell. The adhesion assay was carried out by keeping cells and bacteria in contact for 1 h at 4 °C. Loosely bound bacteria were removed from the cell monolayers by five washes with PBS. The cells were then fixed in methanol and stained with Giemsa or alternatively lysed with ice-cold 0.1% Triton X-100 and plated on TSA agar to determine viable adherent bacteria. Adhesion efficiency was expressed as the percentage of the inoculated bacteria that adhered to Caco-2 cells.

4.7. Invasion assays

Caco-2 cells, cultured in 24-well plates, were infected with 0.5 mL of logarithmically grown bacteria in presence or in absence of SPEP as above described. The entry of *L. monocytogenes* was tested by infecting cells for 1 h at 37 °C at an MOI of about 100 bacteria per cell. After this incubation period, the monolayers were extensively washed and 1 mL of fresh medium containing 50 μg/mL of gentamicin to kill extracellular bacteria was added to each well, and maintained for 1 h at 37 °C. Cells were then lysed by the addition of cold 0.1% Triton X-100 and plated on TSA to determine viable intracellular bacteria. Invasion efficiency was expressed as the percentage of the inoculated CFUs that were internalized.

4.8. Haemolytic activity

L. monocytogenes strains grown overnight at 37 °C in BHI with or without 200 U/mL SPEP were tested for their ability to lyse sheep blood cells. Briefly, samples of 1 mL of the cultures were centrifuged at $10\,000 \times g$ for 10 min at 4 °C and the clear supernatants were supplemented with 10 mM dithiotreitol. Aliquots of the supernatants were serially diluted in microtiter U-plates (Falcon) to determine the haemolytic activity according to Dominguez Rodriguez et al. [34]. The haemolytic titre was expressed in minimal haemolysis units (MHU), defined as the reciprocal of the highest dilution at which haemolysis was detected.

4.9. Acid tolerant response (ATR) measurements

L. monocytogenes strains were screened for acid resistance to lethal pH (pH 3.5). ATR measurements were performed as follows: overnight cultures of bacteria in BHI with or without SPEP were diluted (1:30) into fresh medium containing or not containing the enzyme, and grown at 37 °C until the optical density of the culture reached approximately $OD_{600} = 0.15$ (early log phase). Duplicate samples were centrifuged and the pellets were resuspended in an equal volume of BHI, adjusted to pH 5.1 with 1 M lactic acid (acid-adapted bacteria), or to pH 7.2 (non-adapted bacteria), and incubated for 1 h at 37 °C. To determine the ATR, cells were harvested by centrifugation and resuspended in BHI acidified to pH 3.5 with 3 M lactic acid. The acidified cultures were incubated at 37 °C. After 1 h samples were serially diluted in PBS and survival was determined by performing plate counts of viable cells (colony forming units, CFU) on TSA plates.

4.10. Quantification of biofilm formation

Quantification of biofilm production in plastic multi-well plates was based on the method described by Stepanovic et al. [46]. The wells of a sterile 96-well flat-bottomed polystyrene plate (Falcon) were filled with 230 μl of the appropriate medium containing or not containing 200 U/mL SPEP. Twenty microlitres of overnight bacterial cultures grown in BHI broth containing or not containing 200 U/mL of SPEP was added into each well. The plates were incubated aerobically with or without the enzyme for 24 h at 37 °C.

Growth was monitored by measuring the OD_{600}, and after 24 h incubation in presence or absence of the enzyme the ability of the *Listeria* strains to colonize the polystyrene plates was tested. The content of the plates was then poured off and the wells washed three times with 300 μl of sterile distilled water. The remaining attached bacteria were fixed with 250 μl of methanol per well and after 15 min the plates were emptied and air dried. The plates were stained with 250 μl per well of crystal violet for 5 min. Excess stain was rinsed off by placing the plate under running tap water. After the plates were air dried, the dye bound to the adherent cells was resolubilized with 250 μl of 33% (v/v) glacial acetic acid per well. The OD of each well was measured at 570 nm. Based on the OD produced by bacterial films, strains were classified into the following categories: non-producers, weak, moderate or strong biofilm producers. The cut-off OD was defined as three standard deviations above the mean OD of the negative control (ODc). Biofilm production was classified as follows: $OD \leq ODc$ = no biofilm formation, $ODc < OD \leq (2 \times ODc)$ = weak biofilm formation, $(2 \times ODc) < OD \leq (4 \times ODc)$ = moderate biofilm formation, and $(4 \times ODc) < OD$ = strong biofilm formation.

4.11. Electron microscopy

Samples for scanning electron microscopy (SEM) were prepared as follows: strains were grown in BHI broth containing or not containing 200 U/mL of SPEP in presence of a PTFE 0.22 μm filters (Millipore). After 24 h incubation the PFTE filters were washed three times with sterile distilled water and subsequently dehydrated in ethanol–water mixtures, with increasing ethanol concentrations (65%, 75%, 85%, 95%, and 100%). After critical point drying in a Baltec CPD030 dryer, specimens were mounted on SEM discs, coated with gold, and observed with a scanning electron microscope (XL30 ESEM, Philips).

4.12. Surface protein extraction and processing

The surface proteins were extracted according to the method of Tabouret [47]. Briefly, after centrifugation of 10 mL of each bacterial culture ($OD_{600} = 0.6$), pellets were washed twice in PBS and then suspended in 200 μl SDS 1% in PBS. Samples were incubated at 37 °C for 15 min and after centrifugation the supernatants were collected and used for SDS-PAGE analysis. The protein content in the samples was determined by the Bradford procedure [48].

4.13. SDS-PAGE and renaturing SDS-PAGE (zymogram)

SDS-PAGE was carried out by standard methods [48] with an SDS–polyacrylamide separating gel (pH 8.8; percent of polyacrylamide varying between 8 and 12) and constant voltage (180 V) at room temperature. Renaturing SDS-PAGE was performed according to the methods of Valence and Lortal [49], with some modifications. SDS–polyacrylamide separating gel (8–12% acrylamide, pH 8.8) containing 0.2% (wt/vol) lyophilized *M. luteus* cells provided by Sigma, was used to detect the lytic activities. After electrophoresis, the gels were soaked (2 times, 15 min) in distilled water at room temperature. The gels were then transferred into the renaturing buffer (50 mM Tris–HCl pH 8.0 containing 1% [vol/vol] Triton X-100) and shaken at 60 rpm for 2 h at 40 °C to allow renaturation. The renatured autolysins appeared as clear translucent bands on opaque background. For each experiment, two gels were prepared from the same stock solution and electrophoresed in the same apparatus at the same time. No difference in the migration of the standards due to the presence of cells of *M. luteus* in the gels was noted.

4.14. In situ digestion

Coomassie blue-stained protein bands were excised from SDS-PAGE gels and washed in deionized MilliQ grade water (2 times, 10 min). The excised spots were then washed first with acetonitrile and then with 0.1 M ammonium bicarbonate (three times, 15 min). Protein samples were reduced by incubation in 10 mM DTT for 45 min at 56 °C; free cysteines were alkylated by incubation in 55 mM iodoacetamide for 30 min at room temperature in the dark. The supernatant of the alkylating solution was discarded and the reaction stopped by washing gel pieces with acetonitrile and digestion buffer. Enzymatic digestion was carried out with trypsin (12.5 μg/mL) in 10 mM ammonium bicarbonate pH 7.8. Gel pieces were incubated at 4 °C for 2 h. Trypsin solution was then removed and a new aliquot of the digestion solution was added; samples were incubated for 18 h at 37 °C. A minimum reaction volume was used in order to obtain the complete rehydratation of the gel. Peptides were then extracted by washing the gel particles with 10 mM ammonium bicarbonate and 0.1% TFA in 50% acetonitrile at room temperature.

4.15. Protein identification by mass spectrometry

MALDI-TOF mass spectra were recorded using an Applied Biosystem Voyager DE-STR instrument. A mixture of analyte and matrix solution (alfa-cyano-hydroxycinnamic acid 10 mg/mL in 66% ACN, 0.1% TFA, in MilliQ water) was applied to the metallic sample plate and dried down at room temperature. Mass calibration was performed using external peptide standards. Raw data were analyzed using the computer software provided by the manufacturer and reported as monoisotopic masses. Peptide masses of each digested protein were used to search for protein databases using an in house version of the MASCOT software from Matrix Science.

4.16. Statistical analysis

All tests were carried out in triplicate and the results were averaged. Data were expressed as the mean ± standard error. The statistical significance between different experimental conditions was determined by the paired Student's *t*-test (p values of <0.05 were considered significant).

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References

[1] Farber JM, Peterkin PI. *Listeria monocytogenes*, a food-borne pathogen. Microbiol Rev 1991;55:476–511.
[2] Lorber B. Listeriosis. Clin Infect Dis 1997;24:1–9.
[3] McLauchlin J. *Listeria* and listeriosis. Clin Microbiol Infect 1997;3:484–92.
[4] Cabanes D, Dussurget O, Dehoux P, Cossart P. Auto, a surface associated autolysin of *Listeria monocytogenes* required for entry into eukaryotic cells and virulence. Mol Microbiol 2004;51:1601–14.
[5] Cossart P, Pizarro-Cerda J, Lecuit M. Invasion of mammalian cells by *Listeria monocytogenes*: functional mimicry to subvert cellular functions. Trends Cell Biol 2003;13:23–31.
[6] Hamon M, Bierne H, Cossart P. *Listeria monocytogenes*: a multifaceted model. Nat Rev Microbiol 2006;4:423–34.
[7] Pizarro-Cerda J, Cossart P. Subversion of cellular functions by *Listeria monocytogenes*. J Pathol 2006;208:215–23.
[8] Vázquez-Boland JA, Kuhn M, Berche P, Chakraborty T, Domínguez-Bernal G, Goebel W, et al. *Listeria* pathogenesis and molecular virulence determinants. Clin Microbiol Rev 2001;14:584–640.
[9] Mengaud J, Ohayon H, Gounon P, Mege RM, Cossart P. E-cadherin is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into epithelial cells. Cell 1996;84:923–32.

[10] Braun L, Ghebrehiwet B, Cossart P. gC1q-R/p32, a C1q-binding protein, is a receptor for the InlB invasion protein of *Listeria monocytogenes*. Embo J 2000; 19:1458–66.
[11] Shen Y, Naujokas M, Park M, Ireton K. InIB-dependent internalization of *Listeria* is mediated by the Met receptor tyrosine kinase. Cell 2000;103:501–10.
[12] Park JH, Lee YS, Lim YK, Kwon SH, Lee CU, Yoon BS. Specific binding of recombinant *Listeria monocytogenes* p60 protein to Caco-2 cells. FEMS Microbiol Lett 2000;186:35–40.
[13] Alvarez-Dominguez C, Vazquez-Boland JA, Carrasco-Marin E, Lopez-Mato P, Leyva-Cobian F. Host cell heparan sulfate proteoglycans mediate attachment and entry of *Listeria monocytogenes*, and the listerial surface protein ActA is involved in heparan sulfate receptor recognition. Infect Immun 1997;65:78–88.
[14] Suarez M, Gonzalez-Zorn B, Vega Y, Chico-Calero I, Vazquez-Boland JA. A role for ActA in epithelial cell invasion by *Listeria monocytogenes*. Cell Microbiol 2001;3:853–64.
[15] Milohanic E, Jonquieres R, Cossart P, Berche P, Gaillard JL. The autolysin Ami contributes to the adhesion of *Listeria monocytogenes* to eukaryotic cells via its cell wall anchor. Mol Microbiol 2001;39:1212–24.
[16] Popowska M. Analysis of the peptidoglycan hydrolases of *Listeria monocytogenes*: multiple enzymes with multiple functions. Pol J Microbiol 2004;53(Suppl.):29–34.
[17] Kim KP, Jagadeesan B, Burkholder KM, Jaradat ZW, Wampler JL, Lathrop AA, et al. Adhesion characteristics of *Listeria* adhesion protein (LAP)-expressing *Escherichia coli* to Caco-2 cells and of recombinant LAP to eukaryotic receptor Hsp60 as examined in a surface plasmon resonance sensor. FEMS Microbiol Lett 2006;256:324–32.
[18] Geoffroy C, Gaillard JL, Alouf JE, Berche P. Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin-O from *Listeria monocytogenes*. Infect Immun 1987;55:1641–6.
[19] Krawczyk-Balska A, Bielecki J. *Listeria monocytogenes* listeriolysin O and phosphatidylinositol-specific phospholipase C affect adherence to epithelial cells. Can J Microbiol 2005;51:745–51.
[20] Pan Y, Breidt Jr F, Kathariou S. Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. Appl Environ Microbiol 2006;72:7711–7.
[21] Cotter PD, Gahan CG, Hill C. A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. Mol Microbiol 2001;40:465–75.
[22] Gahan CG, O'Driscoll B, Hill C. Acid adaptation of *Listeria monocytogenes* can enhance survival in acidic foods and during milk fermentation. Appl Environ Microbiol 1996;62:3128–32.
[23] Phan-Thanh L, Mahouin F. A proteomic approach to study the acid response in *Listeria monocytogenes*. Electrophoresis 1999;20:2214–24.
[24] Conte MP, Petrone G, Di Biase AM, Ammendolia MG, Superti F, Seganti L. Acid tolerance in *Listeria monocytogenes* influences invasiveness of enterocyte-like cells and macrophage-like cells. Microb Pathog 2000;29:137–44.
[25] Conte MP, Petrone G, Di Biase AM, Longhi C, Penta M, Tinari A, et al. Effect of acid adaptation on the fate of *Listeria monocytogenes* in THP-1 human macrophages activated by gamma interferon. Infect Immun 2002;70:4369–78.
[26] O'Driscoll B, Gahan CG, Hill C. Adaptive acid tolerance response in *Listeria monocytogenes*: isolation of an acid-tolerant mutant which demonstrates increased virulence. Appl Environ Microbiol 1996;62:1693–8.
[27] Miyata K, Maejima K, Tomoda K, Isono M. Serratia protease: part. I purification and general properties of the enzyme. Agric Biol Chem 1970;34:310–8.
[28] Salamone PR, Wodzinski RJ. Production, purification and characterization of a 50-kDa extracellular metalloprotease from *Serratia marcescens*. Appl Microbiol Biotechnol 1997;48:317–24.
[29] Baumann U. Crystal structure of the 50 kDa metallo protease from *Serratia marcescens*. J Mol Biol 1994;242:244–51.
[30] Kee WH, Tan SL, Lee V, Salmon YM. The treatment of breast engorgement with Serrapeptase (Danzen): a randomised double-blind controlled trial. Singapore Med J 1989;30:48–54.
[31] Mazzone A, Catalani M, Costanzo M, Drusian A, Mandoli A, Russo S, et al. Evaluation of Serratia peptidase in acute or chronic inflammation of otorhinolaryngology pathology: a multicentre, double-blind, randomized trial versus placebo. J Int Med Res 1990;18:379–88.
[32] Maheshwari M, Miglani G, Mali A, Paradkar A, Yamamura S, Kadam S. Development of tetracycline-serratiopeptidase-containing periodontal gel: formulation and preliminary clinical study. AAPS PharmSciTech 2006;7:76.
[33] Selan L, Berlutti F, Passariello C, Comodi-Ballanti MR, Thaller MC. Proteolytic enzymes: a new treatment strategy for prosthetic infections? Antimicrob Agents Chemother 1993;37:2618–21.
[34] Dominguez Rodriguez L, Vazquez Boland JA, Fernandez Garayzabal JF, Echalecu Tranchant P, Gomez-Lucia E, Rodriguez Ferri EF, et al. Microplate technique to determine hemolytic activity for routine typing of *Listeria* strains. J Clin Microbiol 1986;24:99–103.
[35] Nakamura S, Hashimoto Y, Mikami M, Yamanaka E, Soma T, Hino M, et al. Effect of the proteolytic enzyme serrapeptase in patients with chronic airway disease. Respirology 2003;8:316–20.
[36] Moriya N, Nakata M, Nakamura M, Takaoka M, Iwasa S, Kato K, et al. Intestinal absorption of serrapeptase (TSP) in rats. Biotechnol Appl Biochem 1994; 20(Pt 1):101–8.
[37] Esch PM, Gerngross H, Fabian A. Reduction of postoperative swelling. Objective measurement of swelling of the upper ankle joint in treatment with serrapeptase – a prospective study. Fortschr Med 1989;107:67–8. 71–2.
[38] Vicari E, La Vignera S, Battiato C, Arancio A. Treatment with non-steroidal anti-inflammatory drugs in patients with amicrobial chronic prostato-vesiculitis: transrectal ultrasound and seminal findings. Minerva Urol Nefrol 2005;57:53–9.
[39] Bracale G, Selvetella L. Clinical study of the efficacy of and tolerance to seaprose S in inflammatory venous disease. Controlled study versus serratiopeptidase. Minerva Cardioangiol 1996;44:515–24.
[40] Milohanic E, Jonquières R, Glaser P, Dehoux P, Jacquet C, Berche P, et al. Sequence and binding activity of the autolysin–adhesin Ami from epidemic *Listeria monocytogenes* 4b. Infect Immun 2004;72:4401–9.
[41] Braun L, Ohayon H, Cossart P. The InIB protein of *Listeria monocytogenes* is sufficient to promote entry into mammalian cells. Mol Microbiol 1998;27:1077–87.
[42] Gandhi M, Chikindas ML. *Listeria*: a foodborne pathogen that knows how to survive. Int J Food Microbiol 2007;113:1–15.
[43] Poros-Gluchowska J, Markiewicz Z. Antimicrobial resistance of *Listeria monocytogenes*. Acta Microbiol Pol 2003;52:113–29.
[44] Srinivasan V, Nam HM, Nguyen LT, Tamilselvam B, Murinda SE, Oliver SP. Prevalence of antimicrobial resistance genes in *Listeria monocytogenes* isolated from dairy farms. Foodborne Pathog Dis 2005;2:201–11.
[45] Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 2001;9:34–9.
[46] Stepanovic S, Cirkovic I, Ranin L, Svabic-Vlahovic M. Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. Lett Appl Microbiol 2004;38:428–32.
[47] Tabouret M, de Rycke J, Dubray G. Analysis of surface proteins of *Listeria* in relation to species, serovar and pathogenicity. J Gen Microbiol 1992;138:743–53.
[48] Sambrook J, Russell DW. Molecular cloning: a laboratory manual. 3rd ed. New York: Cold Spring Harbor Laboratory Press; 2001.
[49] Valence F, Lortal S. Zymogram and preliminary characterization of *Lactobacillus helveticus* autolysins. Appl Environ Microbiol 1995;61:3391–9.