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**Experimental Physiology**

# Extremely low electrical current generated by porcine small intestine smooth muscle alters bacterial autolysin production

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The effect of extremely low electrical currents, identical to those generated by the gut smooth muscle, on bacterial autolysin production *in vitro* was tested in the present study. When stimulated with these electrical currents, the bacteria *Pediococcus pentosaceus* 16:1 produced groups of peptidoglycan hydrolases that differed from those produced by the unstimulated (control) bacteria. The autolysins synthesized by the *P. pentosaceus* 16:1 under extremely low electrical currents were effective against peptidoglycans from the cell walls of various lactic acid bacteria strains, whereas the autolysins from the control bacteria acted exclusively against *P. pentosaceus* 16:1 cell walls. Thus, it can be predicted that *in vivo* the electrical currents generated by the intestinal smooth muscles, which can be recorded as the myoelectrical migrating complexes, could regulate lactic acid bacteria strain growth in the gut.

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Lactic acid bacteria (LAB) are now widely used as probiotics. Autolysins, which are endogenous enzymes in these bacteria, hydrolyse specific bonds in peptidoglycans within the bacterial wall, and in this way can regulate their growth and that of other bacteria. A wide variety of these enzymes has been identified, including *N*-acetylmuramidases (lysozymes), *N*-acetylglucosaminidases, *N*-acetylmuramyl-L-alanine amidases, endopeptidases and transglycosylases (Schleifer & Kandler, 1972). The beneficial health effects of the LAB used as probiotics should be exhibited after the colonization of the small intestine. However, during the colonization process the LAB, particularly those adhering to the enterocytes, are exposed to the extremely low electrical currents (ELEC) generated by the intestinal smooth muscle. It has been shown that ELEC similar to those generated by the smooth muscle affect bacterial growth *in vitro* (Grzesiuk *et al.* 2001; Wojcik-Sikora *et al.* 2001). The smooth muscle myoelectrical activity precedes its mechanical action, which in turn will influence the flow

(mixing and propelling) of the digesta. The myoelectric activity consists of the electrical control activity (ECA or slow waves) and the electrical response activity (ERA or spike activity). The ECA is an omnipresent oscillation of membrane potential, generated by a network of the interstitial cells of Cajal and spread electronically to the smooth muscle cells. This controls the excitability of the smooth muscles and largely controls the frequency of contraction, whereas the ERA is under the influence of regulatory mechanisms and determines the contractile force (Tomita, 1981; Sarna, 1989; Sanders, 1996; Laerke *et al.* 2000).

The main objective of this study was to test whether ELEC, having characteristics which are the same as those of the ECA and ERA, and manifesting as the myoelectrical migrating complexes (MMC) of the pig small intestine, under *in vitro* conditions could affect the production of peptidoglycan hydrolases by *Pediococcus pentosaceus* 16:1, a member of the LAB able to colonize the intestine.

## Methods

### Preliminary preparation of bacteria

**Preparation of bacterial cultures and cell walls.** Four species of LAB, namely *P. pentosaceus* 16:1, *Lactobacillus plantarum* 2592, *Lactobacillus paracasei* ssp. *paracasei* F19 and *Leuconostoc mesenteroides* 77:1, were used in this study. *P. pentosaceus* 16:1 and *L. mesenteroides* 77:1 were derived from fermented organically cultured rye, whereas the lactobacillae were isolated from human large intestinal mucosa (from the collection of Lund University, Department of Medical Microbiology, Dermatology and Infection; Kruszevska *et al.* 2002). The LAB isolates were cultured in de Man Rogosa-Sharpe (MRS) broth (Oxoid) for 24 h at 37°C under microaerobic conditions.

The cell walls of *P. pentosaceus* 16:1, *L. plantarum* 2592, *L. paracasei* ssp. *paracasei* F19 and *L. mesenteroides* 77:1 were separated after growth in the MRS broth, supplemented with 0.03 M glucose at 35°C to an optical density (OD) at 625 nm of 1.0 (exponential phase). The cells were harvested by centrifugation at 10 000g for 15 min at 4°C, and the pellet obtained was washed twice with ice-cold 0.01 M potassium phosphate buffer (PBS; pH 6.5) and resuspended in 2 ml of ice-cold distilled water (pH 5.5–6.0). In order to prepare cell wall extracts, 2 ml of 8% of SDS was added to these cell suspensions and the mixture was boiled for 30 min at 100°C. After boiling, the suspension was adjusted to 16 ml with 4% SDS. The extraction was continued overnight at room temperature (RT) with gentle stirring. After centrifugation at 10 000g for 15 min at 4°C the supernatant was harvested. The pellets were resuspended as described above and SDS extraction was repeated. The pellets were washed (a minimum of 10 times) with 0.01 M Tris-HCl (pH 6.5) and then dialysed against frequent changes of distilled water at 4°C and suspended in 0.6 ml of distilled water (Massidda *et al.* 1996).

**Crude peptidoglycan preparation.** Crude cell walls were treated with 0.1 N HCl at 60°C for 16 h. The suspension was neutralized with 2.5 N NaOH. The peptidoglycan was collected after centrifugation at 20 000g for 20 min, and the pellet was washed five times with 0.01 M Tris-HCl (pH 6.5). The detailed procedure for the determination of muramic acid is described elsewhere (Sebastian & Larsson, 2003). In brief, samples were heated in 2 M methanolic HCl at 85°C overnight, after which the internal standard (C13-labelled MuAc in a methanolysate of C13-labelled algal cells) was added. The mixture was extracted with heptane, and the lower phase was further purified using propylsulphonic acid columns, evaporated to dryness under nitrogen and further dried under vacuum in a desiccator (2 h). The samples were then acetylated by heating in a mixture of pyridine and acetic anhydride at 60°C for 1 h. The reaction

mixture, dissolved in dichloromethane, was subsequently washed, first with a 0.05 M HCl solution and thereafter with water. The samples were then purified using silica gel columns, evaporated to dryness, dissolved in chloroform and analysed using AN ion trap gas chromatograph/mass spectrometer (GC-MSMS). Sample preparation resulted in methylation of the carboxyl and anomeric hydroxyl groups and acetylation of the three remaining hydroxyl groups. Fragmentation of mass to charge ( $m/z$ ) 187 led to a high intensity of the product ion  $m/z$  145, which was therefore monitored in the MSMS mode.

### Electrical stimulation of *P. pentosaceus* 16:1

**ELEC treatment and characteristics.** The experiments were carried out in glass bacteriological tubes of 15 mm internal diameter containing 10 ml of bacterial suspension ( $10^6$  colony forming units (cfu) ml<sup>-1</sup>). The facing parallel platinum plate electrodes, 5 mm wide and 0.5 mm thick, were inserted from the top to the bottom of the tubes 10 mm apart and connected to the output of a GGP-3 MMC Simulator (Flow Instruments, Warsaw, Poland) which was used to generate a typical ELEC (both ECA and ERA) of the porcine intestine. The strength of the generated electric field was  $E_{\text{peak}} = \pm 83 \mu\text{V mm}^{-1}$ , average conductivity of the medium  $\sigma = 15 \text{ mS cm}^{-1}$  (Gu & Justiz, 2002) and average sample resistance  $R = 10 \Omega$ . The calculated maximal current density during a mean simulated spike potential was  $j_{\text{peak}} = \pm 8.3 \mu\text{A cm}^{-2}$ . The characteristic output voltages of the device, which are the first derivatives of the spreading transmembrane potential changes of the porcine ileum (Jimenez *et al.* 1999), are presented in Figs 1 & 2. The upper trace in Fig. 1 shows the typical output voltage of the simulator during the slow waves of the phase I of the myoelectrical migrating complexes (MMC; ECA only, no spikes), and the lower trace shows the typical output voltage during the slow waves with the superimposed action potentials of the smooth muscles (ECA + ERA) of phase III of the MMC (Lammers & Slack, 2001). The long-term periodical changes of stimulated ERA activity forming the typical pattern of the porcine MMC are shown in Fig. 2 (Bueno *et al.* 1982; Fleckenstein *et al.* 1982).

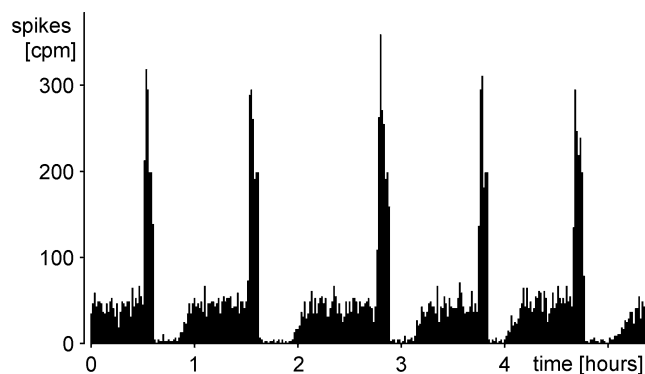
**Cellular autolysis.** The autolytic phenotype of the *P. pentosaceus* 16:1 strain was evaluated under starvation conditions for 72 h, in 0.01 M PBS (pH 6.8) at 30°C with or without exposure to the ELEC. Culture conditions, such as time, temperature, osmolarity and pH, were experimentally determined.

The culture of *P. pentosaceus* 16:1 in an exponential phase was rapidly chilled (ice-bath). The cells were harvested by centrifugation at 10 000g for 15 min at 4°C and washed three times with distilled water at 4°C. The bacterial pellet was resuspended in 10 ml of PBS, to an

OD at 625 nm of approximately  $1.0$  ( $10^6$  cfu ml $^{-1}$ ; data not shown), and the suspensions were incubated in a 30°C water bath for 72 h. Six samples were always examined simultaneously. The ELEC was generated exclusively in three tested samples. In the remaining three samples with the same experimental set-up, the current was not generated and these samples served as a control. The studies were repeated six times. In all tested samples, the degree of autolysis was expressed as a decrease in the OD at 625 nm, calculated as the extent of the turbidity in the tested samples. OD values were measured every hour for the first 12 h, and every 3 h thereafter for the remaining 60 h (Lortal *et al.* 1997). Following the above tests, the cultures were centrifuged at 10 000g for 15 min at 4°C, and the cell-free supernatants were concentrated for further studies, as described below.

#### Identification of peptidoglycan hydrolases using renaturing two-dimensional gel electrophoresis (2-DE)

In order to identify the number of peptidoglycan hydrolases released by *P. pentosaceus* 16:1 exposed to starvation stress for 24 h and treated or untreated with the ELEC, the 2-DE was run with one repetition for each supernatant obtained from each of six ELEC-treated and six untreated bacterial suspensions. The *P. pentosaceus* 16:1 peptidoglycan was used as a target for the enzymatic activity (Kornilovs'ka *et al.* 2002; Kaino *et al.* 1998).



**Figure 2. Periodical changes of the ERA activity in the simulated MMCs**

The histogram shows GGP-3 MMC Stimulator output. cpm, counts per minute.

Isoelectric focusing (IEF) was performed using IPGphor<sup>TM</sup> horizontal electrophoresis (Pharmacia Biotech, Stockholm, Sweden). A series of three to ten immobilized pH gradient strips (7 cm long; Amersham Bioscience, USA) were rehydrated in 0.25 ml sample buffer range 3–10 (Amersham Bioscience, USA) containing 8 M urea, 2% 3-((3-cholamidopropyl)-dimethylammonio)-1-propane-sulphonate (CHAPS), 1% dithiothreitol (DTT) and the proteins to be tested. IEF was performed according to the manufacturer's instructions.

Before renaturing, the SDS-PAGE first dimensional immobilized pH gradient (IPG) strips were equilibrated twice in solutions containing mainly DTT and iodoacetamide, respectively. The strips were then applied to 12% gels supplemented with *P. pentosaceus* 16:1



**Figure 1. Oscillograms of the GGP-3 MMC Stimulator output waveforms**

Upper trace shows slow waves with no action potentials during phase I of the MMC. Lower trace shows slow waves with action potential activity during phase III of the MMC. Calibration: 2 s per division and 20 mV per division.

peptidoglycan (0.1% w/v), and analysed under reducing conditions, where separation was conducted at 150 V for 60 min. Following electrophoresis, the substrate-containing gels were incubated in distilled water at RT for 30 min and then placed into the renaturation buffer (0.05 M Tris-HCl buffer, pH 6.8, containing 0.1 M KCl and 0.1% Triton X 100). After a second 30 min incubation at RT, the gels were transferred to fresh renaturing buffer and the process was run for 16 h at 37°C. The lytic activity of the hydrolases was visualized as clear spots against a light blue background after gel staining with 0.1% Methylene Blue in 0.01% KOH for 1 h (Lorca *et al.* 2001).

SDS-PAGE gels were scanned with a GS-710 Imaging Densitometer (Bio-Rad; USA), and the molecular weights and isoelectric potential (pI) of the proteins were estimated by image analysis using the Melanie V3 software package (Bio-Rad, USA).

### Peptidoglycan hydrolases obtained from the autolysis of *P. pentosaceus* 16:1 and bacterial cell walls

The degradative activity of the *P. pentosaceus* 16:1 peptidoglycan hydrolases obtained after treatment was evaluated against the cell walls of the lactic acid bacteria *L. plantarum* 2592, *L. paracasei* ssp. *paracasei* F19 and *L. mesenteroides* 77:1 obtained as described above. Briefly, native cells and cell walls derived from the lactic acid bacteria were autoclaved at 120°C for 15 min. Then, the cells and cell walls were disrupted by ten ultrasonication cycles (20 s each) at 4°C with 20 s intervals. For native cell wall separation, a two step centrifugation procedure was applied.

The pellets containing native cells of *P. pentosaceus* 16:1 (0.4% w/v), *L. plantarum* 2592 (0.4% w/v), *L. paracasei* F19 (0.4% w/v), *L. mesenteroides* 77:1 (0.4% w/v) and *Micrococcus lysodeikticus* ATCC 4698 (0.2% w/v; Sigma, USA; positive control), respectively, were washed with cold water and incorporated into 1% agarose (Litex, Denmark)

in PBS (pH 6.8) on Petri dishes (Strating & Clarke, 2001). The supernatants from the ELEC-treated and untreated suspensions of *P. pentosaceus* 16:1 (20 µl) were poured into agarose gels enriched with the native cell walls obtained from the lactic acid bacteria. Lysosyme was used as the positive control. Hydrolytic activity was measured as the diameter (in mm) of transparency in the agarose gels caused by degradation of the lactic acid bacterial cell walls by *P. pentosaceus* 16:1 peptidoglycan hydrolases.

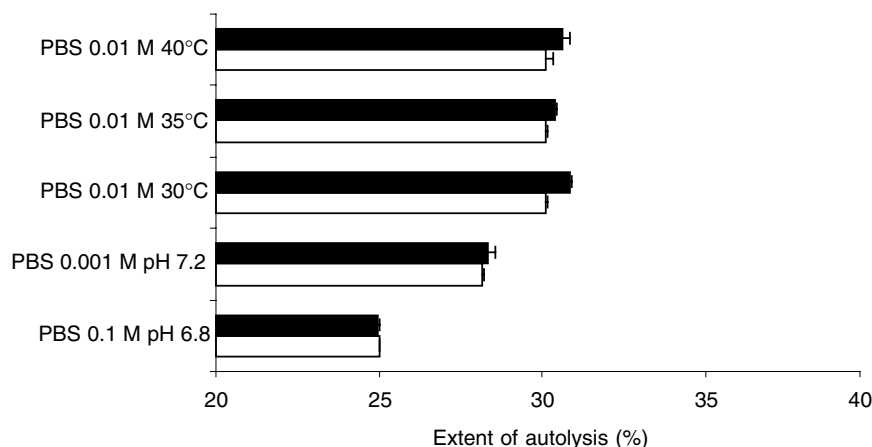
### Statistics

Since the criteria of normality of distribution and the equality of the variability of data were fulfilled (as indicated by a  $\chi^2$  test), parametric tests were used. The results of the hydrolase activity in the supernatant obtained from *P. pentosaceus* 16:1 suspensions treated and untreated with ELEC were compared statistically using Student's unpaired *t* test or a two-way analysis of variance (ANOVA) and Tukey's *post hoc* test (SigmaStat for Windows v2.0, SPSS Science, Chicago, IL, USA).

## Results

### Experimental conditions

A phosphate concentration of 0.01 M at pH 7.2, room temperature (25°C) was found to promote greater autolysis of *P. pentosaceus* 16:1 compared to a phosphate concentration of 0.1 M at pH 6.8 ( $P < 0.01$ ). Temperatures within the range 30–40°C also increased the autolytic responses ( $P < 0.01$ ; Fig. 3). However, a tendency ( $P < 0.057$ ) to a greater degree of autolysis was observed in the ELEC-treated samples when they were incubated at 30°C. The above observations were the main factors determining the experimental conditions for the entire experiment, which were set as phosphate concentration 0.01 M and temperature 30°C.



**Figure 3.** The degree of autolysis of the *P. pentosaceus* 16:1 cells after 10 h incubation with (■) or without ELEC (□) under different osmolar, temperature and pH conditions. Values are means  $\pm$  s.d.;  $n = 6$ .

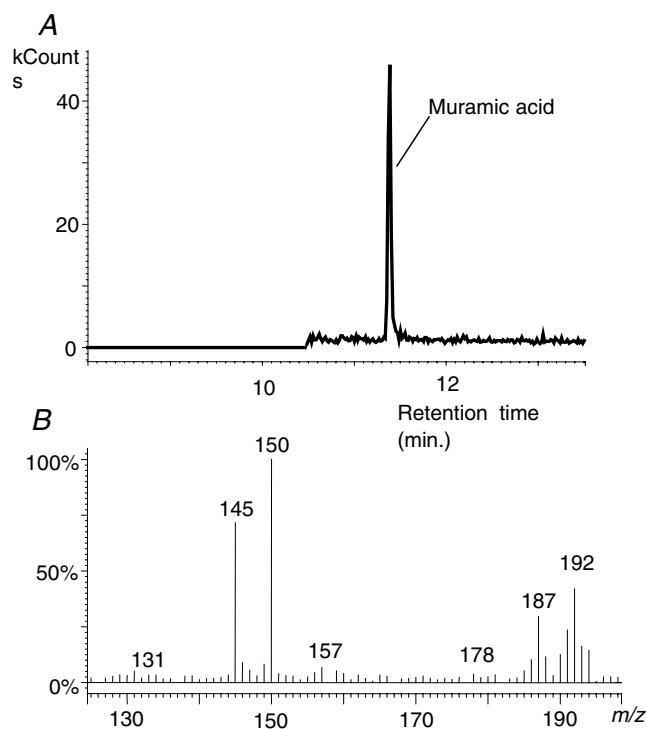
Maximum autolysis was observed when the cells were harvested in the exponential phase and then incubated in PBS. It was found that *P. pentosaceus* 16:1 underwent autolysis at pH 6.8, which is within the optimal pH range for LAB lysis, as has been reported previously (pH 6.2–6.8; Mora *et al.* 2003).

After 72 h starvation, 30% of the *P. pentosaceus* 16:1 cells ( $3.3 \times 10^5$  cfu ml<sup>-1</sup>; data not shown) were lysed, measured as OD at 625 nm, both in the ELEC-stimulated and the unstimulated suspensions (Fig. 4). The curve of autolysis was proportional and slow over 10 h. This is in agreement with earlier observations that bacterial cells undergo autolysis slowly and not completely. Turbidity decreased to a minimum of 30% after 10 h of incubation and then remained constant until the end of the incubation period (72 h). Generally, 30% quantitative lysis of bacteria is recognized as being maximal.

Pure muramic acid (peptidoglycan) of *P. pentosaceus* 16:1, as a substrate for autolysins, was applied on SDS-PAGE gels (2-DE). The amount of this macromolecule to be used in these experiments had been determined by using a methyl ester *O*-methyl acetate derivative (GC-MSMS) (Fig. 5).

#### Effect of ELEC on the autolysis of *P. pentosaceus* 16:1

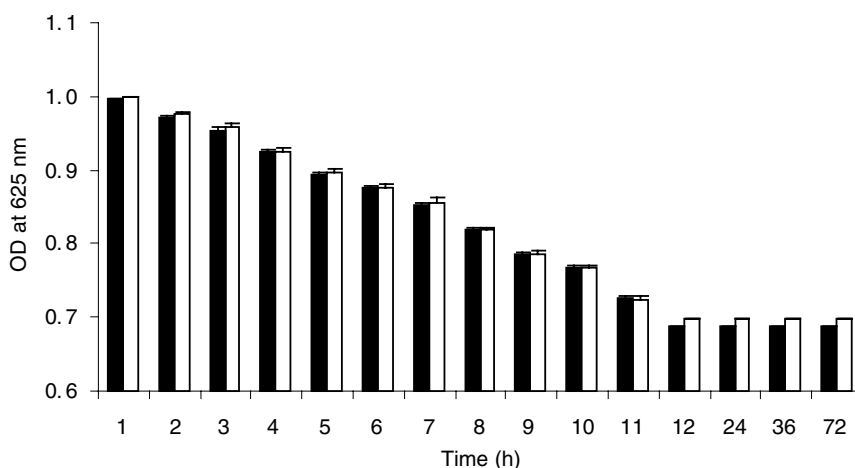
Stimulation by the ELEC caused qualitative changes in the autolysin production, as shown by comparison with the autolysis present in untreated samples of *P. pentosaceus* 16:1 after incubation. The pI of the proteins varied between 7.86 and 8.7 ( $8.03 \pm 0.69$ ,  $n = 12$ ) with relative molecular masses ( $M_r$ ) ranging from 28 to 230 kDa ( $n = 12$ ). A representative example of the autolysin profile obtained is presented in Fig. 6. The molecular mass of autolysins ranged from 28 to 230 kDa when the samples were treated with the ELEC (Fig. 6B and Table 1). The profile of autolysins from the control, ELEC-untreated samples had molecular masses ranging between 60 and



**Figure 5. Chromatographic profile of muramic acid derivatives** Methyl ester *O*-methyl acetate muramic acid derivative of *P. pentosaceus* (A) and spectrum obtained from GC-MSMS (B). kCount, relative amount of the muramic acid.

180 kDa (Fig. 6A). The number of autolytic proteins in the supernatant from the ELEC-treated suspensions of *P. pentosaceus* 16:1 was  $36 \pm 5$  ( $n = 6$ ) and in untreated suspension it was  $14 \pm 3$  ( $n = 6$ ,  $P < 0.001$ ; Table 1).

The activity spectrum of autolysins from untreated *P. pentosaceus* 16:1 varied according to the starvation conditions. It was also possible to identify enzymes with a low  $M_r$  (under 45 kDa). These unique hydrolases were detected in a region between  $M_r$  41, 44 and 50 kDa and had a different pI. In these studies, the major autolysin spots



**Figure 4. Kinetics of the autolysis of the *P. pentosaceus* 16:1 cells treated with ELEC (■) or untreated (□)**

Values are means  $\pm$  s.d.;  $n = 6$ .

**Table 1. Example of the characteristics of the autolysins (as protein spots) in supernatants obtained from *P. pentosaceus* 16:1 suspensions treated with electrical current (+ELEC) and untreated (–ELEC)**

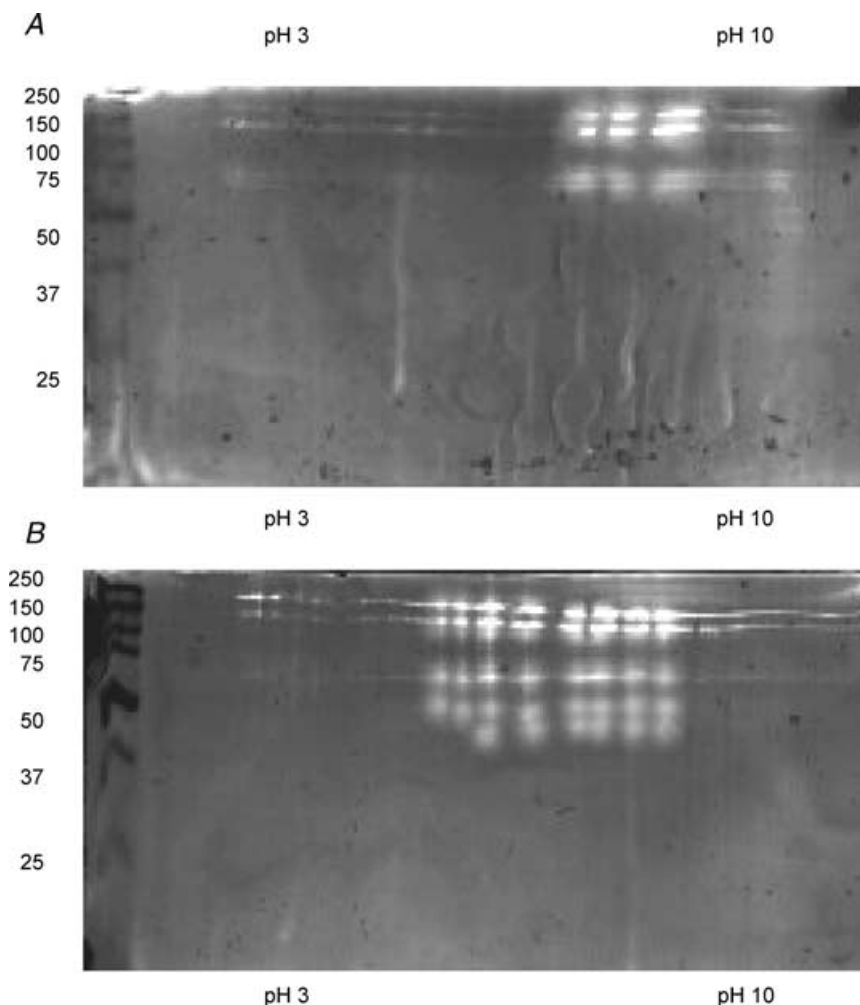
+ELEC			–ELEC		
Spot no.	M <sub>r</sub>	pI	Spot no.	M <sub>r</sub>	pI
1	41	5.8	4	60	6.5; 6.9; 7.5; 8.6
5	44	6.5; 6.9; 7.2; 7.4; 7.6	4	67	6.5; 6.9; 7.5; 8.6
8	50	5.3; 5.5; 5.9; 6.5; 6.9; 7.2; 7.4; 7.6	4	130	6.5; 6.9; 7.4; 8.3
8	61	5.3; 5.5; 5.9; 6.4; 7.0; 7.1; 7.4; 7.6	4	180	6.5; 6.9; 7.4; 8.3
8	110	5.3; 5.5; 5.9; 6.4; 7.0; 7.1; 7.4; 7.6			
8	170	5.3; 5.5; 5.9; 6.4; 7.0; 7.1; 7.4; 7.6			
2	230	7.8; 8.1			

pI, isoelectric point; M<sub>r</sub>, relative molecular mass.

were noted in the region of 110 and 170 kDa (Fig. 6B). The autolysis profile obtained was characteristic and similar to that presented by Mora *et al.* (2003) for *P. acidilactici*, which showed autolytic activity of molecules ranging between 45 and 110 kDa. It should be mentioned that technically, the process of autolysis and the presence of metal ions in the buffer of bacterial cells was crucial for the zymogram visualization (Strating & Clarke, 2001).

### Effect of peptidoglycan hydrolases from ELEC-treated *P. pentosaceus* 16:1 on LAB strains

The peptidoglycans isolated from the different LAB strains used in this study were submitted to the action of lysozyme, as a control (Table 2), and their stable and identical sensitivity to the action of this enzyme indicated that, although the tested cell walls were acetylated, there was



**Figure 6. Pattern of the supernatant protein bands after 24 h starvation of *P. pentosaceus* 16:1 resolved by isoelectrofocusing and separated with renaturing 12% SDS-PAGE**

The zymograms show autolysis of the bacteria untreated (A) or treated with ELEC stimulation (B). Values of M<sub>r</sub> are shown on the left of the figure.

**Table 2.** The effect of *P. pentosaceus* 16:1 autolysins expressed as the diameter of the transparent zones (mean  $\pm$  S.D,  $n = 6$ ) obtained before and after ELEC treatment on the degradation of the host and different species of LAB cell walls

Native cell walls isolated from:	Diameter of transparent zone (mm)		
	Supernatant from <i>P. pentosaceus</i> 16:1		Hen egg lysozyme (control)
	Starved cells +ELEC	Starved cells -ELEC	
<i>P. pentosaceus</i> 16:1	9.0 $\pm$ 1.5 <sup>b</sup>	11.0 $\pm$ 1.2	7.0 $\pm$ 0.9 <sup>a</sup>
<i>L. plantarum</i> 2592	8.6 $\pm$ 1.6 <sup>b</sup>	Not detectable	6.6 $\pm$ 0.8 <sup>a</sup>
<i>L. paracasei</i> ssp. <i>paracasei</i> F19	6.5 $\pm$ 0.9 <sup>a</sup>	Not detectable	6.8 $\pm$ 0.7 <sup>a</sup>
<i>L. mesenteroides</i> 77:1	7.3 $\pm$ 1.1 <sup>ab</sup>	Not detectable	6.4 $\pm$ 0.6 <sup>a</sup>
<i>M. lysodeikticus</i> ATCC 4698	6.4 $\pm$ 0.4 <sup>a</sup>	Not detectable	6.5 $\pm$ 1.0 <sup>a</sup>

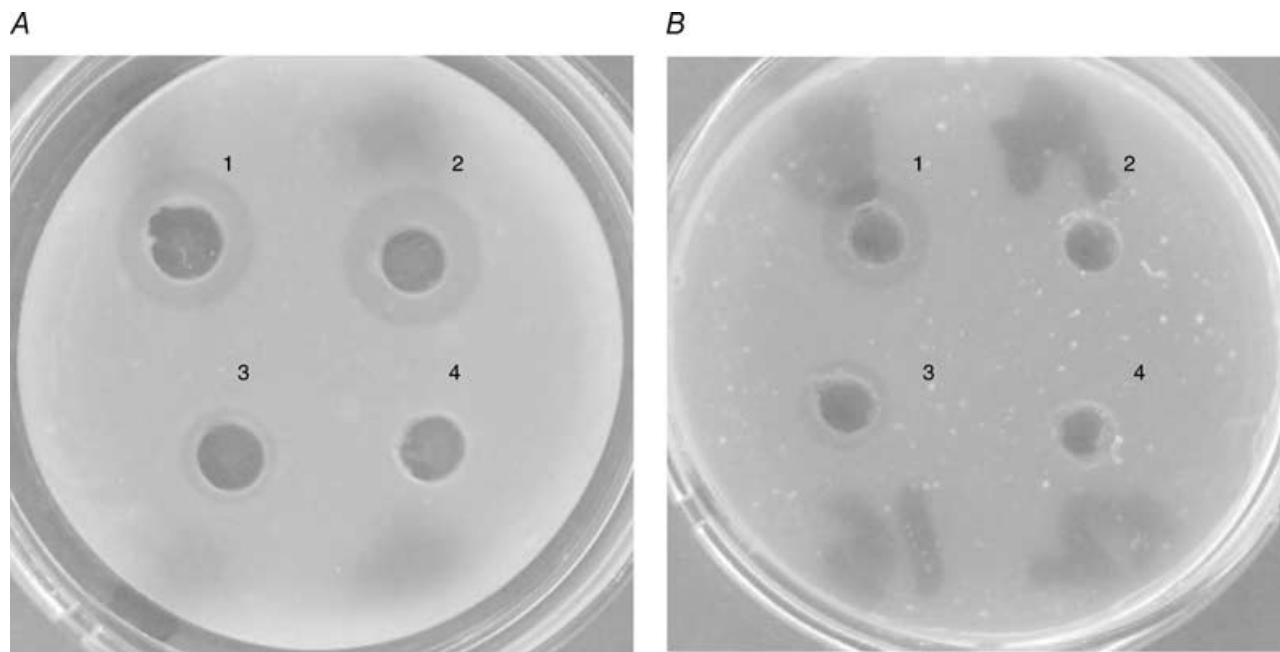
Different superscripts indicate statistical significance at  $P < 0.05$ .

still a substantial effect of the autolysin. It has generally been considered that acetylation of the peptidoglycans would essentially limit their degradation by autolysins, and the degree of peptidoglycan *O*-acetylation corresponded to the degree of resistance to lysozyme hydrolysis (Clarke, 1993). Thus, acetylation might block the action of specific groups of autolysins while not affecting others. The physiological role of peptidoglycan *O*-acetylation is not understood, but it might belong to a control system of autolysin action.

The degradation of the peptidoglycans obtained from the different bacteria by the cell-free supernatant of 72 h-

starved *P. pentosaceus* 16:1 bacteria treated and untreated with the ELEC was significantly different  $P < 0.05$  (Table 2). An example is shown of the lysis, caused by the autolysins produced by the ELEC-treated and untreated *P. pentosaceus* 16:1, of the peptidoglycans suspended in agar gels and isolated from *P. pentosaceus* 16:1 (Fig. 7A) and *L. plantarum* 2592 (Fig. 7B).

Similarly to lysozyme, the supernatant from the starved pediococcal suspension treated with ELEC degraded peptidoglycans both from its own cell walls (*P. pentosaceus* 16:1) and from those of the other bacterial strains (*L. plantarum* 2592, *L. paracasei* ssp.



**Figure 7.** Example of the lysis of the peptidoglycans suspended in agar gels and isolated from *P. pentosaceus* 16:1 (A) and *L. plantarum* 2592 (B) as a result of the action of the peptidoglycan hydrolases (autolysins) produced by *P. pentosaceus* 16:1

The extent of the hydrolase activity is indicated by the diameter of the transparency around the application point: 1, supernatant from *P. pentosaceus* 16:1 treated with ELEC during starvation stress; 2, supernatant from *P. pentosaceus* 16:1 untreated with ELEC during starvation stress; 3, lysozyme as positive control; and 4, supernatant from *P. pentosaceus* 16:1 culture before exposure to starvation stress.



*paracasei* F19, *L. mesenteroides* 77:1 and the control bacteria *M. lysodeikticus* ATCC 4698). In contrast, the supernatant from starved pediococci (*P. pentosaceus* 16:1) untreated with ELEC acted exclusively against the host peptidoglycan (*P. pentosaceus* 16:1). As noted above, the ELEC-treated *P. pentosaceus* 16:1 produced both low- and high-molecular autolysins, while the untreated *P. pentosaceus* 16:1 produced exclusively the high-molecular weight ones.

## Discussion

Generally considered as safe (GRAS) pediococci can be isolated from meat and gut digesta, fermented vegetables and dairy products, and they are commonly used as probiotics. As for other Gram-positive bacteria, the cell wall of the pediococci is composed of peptidoglycan, with a matrix consisting of polysaccharides, teichoic acids and proteins (Govindasamy-Lucey *et al.* 2000; Konings *et al.* 2000). Peptidoglycan (muramic acid), the substrate used in the present studies, is a polymer of amino sugars cross-linked by short peptides which forms a covalent matrix that surrounds the cytoplasmic membrane and constitutes the major skeletal component of the Gram-positive bacteria cell wall. Cell-wall peptidoglycan is strong and highly dynamic; the structure expands as the cell grows and is reshaped when it divides or differentiates. Autolysins are involved in the selective hydrolysis of peptidoglycans and thus can contribute to cellular processes, including proliferation and death (Shandholm & Sarimo, 1981; Tomasz, 1984).

The absolute numbers of autolysins counted on the gels probably did not reflect the true intracellular autolysin profile. It should be noted that about 70% of the bacterial cells which survived the stress of 72 h incubation (two periods of starvation, and treatment with the ELEC) still had their autolysins localized intracellularly. In addition, during bacterial growth, it has been observed that peptidoglycan hydrolases can be processed during their transport through the cytoplasmic membrane, and multiple forms of the same active autolysin can be generated (Leclerc & Asselin, 1989). Thus, it could not be excluded that the ELEC not only affected autolysin production (i.e. number and type), but also may have participated in autolysin remodelling during the transport of these enzymes through the bacterial walls.

The results obtained clearly showed that the ELEC, having characteristics similar to those currents produced by the intestinal wall, qualitatively affected the autolysin production by *P. pentosaceus* 16:1. In addition, it could be speculated that the profile of the liberated autolysins depended on the susceptibility of the bacteria to the ELEC under the present experimental conditions. ELEC treatment *in vitro* allowed for the further identification of

high- and low-autolytic *P. pentosaceus* 16:1 cells. Finally, the profile of autolysins liberated from the cells was associated with the action of ELEC. Considering that autolysins are intensively produced where bacterial cells are actively growing and dividing, it was concluded that the ELEC can regulate this process.

However, it was not possible to define the mode of action of the ELEC. Several questions could be addressed in this respect, e.g. is it purely an electrical effect or is the effect related to the slow electrolysis in the medium caused by the ELEC? Another important question is whether direct or alternating currents of comparable amplitude to those employed in the present series of experiments could produce similar changes in autolysin production. Unfortunately, we were not able to find relevant references in the scientific literature to discuss this intriguing suggestion.

The lack of answers to these questions cannot abolish the importance of the fact that the ELEC, having parameters related to the MMC, can affect bacterial autolysin production. Thus, it is likely that *in vivo* electrical currents related to the MMC can qualitatively affect the autolysin production by the probiotic bacteria in the gut, and in this way influence the process of natural colonization of the intestine by LAB and other bacteria. Moreover, the clinical relevance to human medicine of this observation should also be addressed, since there are many studies recommending the use of LAB bacteria in the oral treatment of a long list of diseases.

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