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INCREASED SALT- AND NISIN-SENSITIVITY OF PRESSURE-INJURED BIOLUMINESCENT *LISTERIA MONOCYTOGENES**

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Suspensions of a bioluminescent (*lux*AB) transformant of *Listeria monocytogenes* in pH 7.0 phosphate buffer were pressurised and the effect of the pressure treatment was monitored by plate counting. When the bacteria were suspended in NaCl- and nisin-free buffer the number of colony forming units (CFU) decreased by 3 and 6 log cycles after 300 MPA for 10 and 30 min, respectively. Supplementing the plating medium with 5% NaCl did not influence the colony forming capacity of non-pressurised cells, however, CFU of residual populations after respective treatments of 300 MPa for 10 and 30 min were reduced by a further 2 and 3.5 log cycles in case of salt containing plates. Nisin-addition to the plating medium caused less than one log unit decrease in the CFU of the non-pressurised population. However, the CFU of 10 min-pressurised sample was 4 log cycles less in the nisin-containing plates than in the nisin-free ones, whereas no colonies were formed in the nisin-containing plates even when 1 ml was inoculated from the originally 10^{10} CFU/ml population after 300 MPa for 30 min. The luciferase activities (bioluminescence intensities) decreased concomitant with the reduction of the viable cell counts, however, they were approx. 0.6–0.8 log units less in the presence of 5% NaCl in the pressurised suspension than those expected from the previously established linear correlation between the logarithmic light outputs and the logarithmic viable cell counts.

Keywords: *Listeria monocytogenes,* high hydrostatic pressure, sensitisation, NaCl, nisin, bioluminescence

Introduction

There is an interest in producing high hydrostatic pressure (HHP) preserved foods. HHP treatment is an emerging non-thermal preservation technique which is energy efficient and it can kill vegetative bacteria and fungi without altering the

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flavour and nutrient content of a food. This is because HHP is able to modify large macromolecular assemblies, while it has very little or no effect on small molecules. The effectiveness of hydrostatic pressure pasteurisation on the destruction of several food-borne pathogens in high-water-activity environments has been reported (e.g. [1] and [2]). However, to ensure the safety and shelf life of food products, and to make the process economically feasible, it is necessary to achieve the needed reduction (at least log 10^6) at moderate hydrostatic pressure and preservation time and temperature.

Being a ubiquitous environmental contaminant, and due to its relatively high salt- and acid-tolerance, *Listeria monocytogenes* is considered as the critical non-sporeforming food-borne pathogenic bacterium determining microbiological safety of quality-friendly "minimal processing methods" of food pasteurisation [3]. Therefore, it is important to learn more about the anti-listerial efficiency of HHP treatment, and the tolerance against environmental stress factors of populations of this bacteria surviving such treatment. This paper reports on our preliminary-type experiments in this relation.

Materials and methods

Growth of bacteria and preparation of cell suspensions for pressurisation

A bioluminescent (*lux*AB*)* transformant, *Listeria monocytogenes lux*AB NCTC (pGC4) has been used as test organism. The bacterium was grown by sub-culturing from our stock collection in Brain Heart Infusion Broth (Merck 1.10493) at 30 ºC for 24 hours. The stationary phase cells were harvested by centrifugation at 9000 rpm for 20 min at 5 ºC, washed with sterile, pH 7.0 Sörensen's phosphate buffer (1/15 M) and after repeated centrifugation were re-suspended in the same buffer to a population density of about 10^{10} CFU/ml. For studying the direct effect of NaCl or nisin on the pressure resistance of the test organisms, in a part of the experiments the suspending buffer was supplemented either with 5% NaCl or 2 μg/ml nisin (applied as Nisaplin^{TM} preparation obtained from Aplin and Barrett Ltd., Trowbridge, U.K.). This bacteriocin is approved as a preservative in some foods such as processed cheese, but is not used in medical treatment of humans or animals or for animal growth promotion.

Hydrostatic pressurisation

The cell suspensions were dispensed in 5 ml portions in sterile plastic pouches, sealed after exclusion of air bubbles and exposed to a desired level of hydrostatic pressure in a Stansted "Food-Lab 9000" type high pressure isostat apparatus at room temperature according to Patterson et al. [1]. Non-pressurised pouches containing the cell suspensions served as controls.

Enumeration of colony forming units

The effect of treatments was followed by plate counting of appropriate serial dilutions with 0.1% peptone solutions in duplicate plates of Brain Heart Infusion Agar (Merck 1.11925). It was also studied, how the numbers of colony forming units were influenced by supplementing not only the suspending buffer but also the plate-counting medium with 5% NaCl, or, 2 μg ml/nisin.

Measurement of bioluminescence intensities

The bioluminescent transformant of *L. monocytogenes* used as test organism requires an exogeneous long-chain aldehyde substrate for its bacterial luciferase to express nondestructive and "real-time" its bioluminescent phenotype [4]. Luciferase activities of the cell suspensions were estimated by a Turner Mod. 20 luminometer integrating the arbitrary light output values for the first 10-sec intervals during light emission as an effect of addition of 50 μl of 1% nonanal solution (in EtOH) as substrate to 0.3 ml aliquots of the cell suspensions [5]. The minimum number of viable cells required for bioluminescence detection is 10^3–10^4 CFU/ml [5].

Leakage measurements

Leakage of pressure-treated bacteria was compared to that of untreated ones by testing the supernatants of suspensions by an ATP-kit, and U.V. absorptions photometry with a Unicam "Spectronic Helios-β" spectrophotometer where the reference was always the cell-free suspending liquid. For determining ATP leakage of cells in the untreated and pressurised suspensions the ATP Assay Kit number 1243-107 from BioOrbit Oy, Turku, Finland, was used applying the bioluminescence technique. The assay is based upon the quantitative measurement of a stable level of light produced as a result of an enzyme reaction catalysed by firefly luciferase:

$$\text{ATP} + \text{luciferin} + O_2 \xrightarrow{\text{luciferase}} \text{oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{light.}$$

The firefly luciferase and luciferin contained in the ATP Monitoring Reagent are so formulated as to provide a time independent light output over the concentration range 10^{-11}–10^{-4} M ATP. The ATP Standard, which contains a known amount of ATP, is used to calibrate the assay system [6]. These luminometric measurements were performed also with the Turner Mod. 20 luminometer with 10-sec integration time.

Results

Inactivation of L. monocytogenes *by HHP and its sensitisation to NaCl and nisin*

The presence of NaCl or nisin in the suspending buffer in which the bacteria were pressurised at 300 MPa isostatic pressure did not influence their colony counts in BHI agar. The viable cell count decreased by 3, 5, and 6 log units after 300 MPa for 10, 20, and 30 min treatments, respectively, if the dilutions of the treated suspensions were plated with salt- and nisin-free medium (Table I). However, supplementing the plating medium with 5% NaCl, the CFU of pressurised populations were approx. 2 and 3.5 log units less after the respective treatments of 300 MPa for 10 min and 30 min as compared to those of the same pressurisation levels but without NaCl addition to the plating medium. The nisin-addition of 2 μg/ml in the plating medium caused in itself 0.6–0.8 log units decrease in the colony count of the non-pressurised population, whereas the CFU was 4 log units less after 10 min treatment at 300 MPa in the nisin-containing nutrient plates than in the nisin-free ones, and no colonies were formed after a pressure treatment of 300 MPa for 30 min.

Table I

The effect of the presence of 5% NaCl, or, 2 μg/ml nisin in the plating medium (BHI Agar) on the colony forming ability of non-pressurised and HHP-treated *L. monocytogenes lux*AB populations

Sample/treatment	$\log_{10}$ CFU/ml			
	Non-pressurised	300 MPa, 10 min	300 MPa, 20 min	300 MPa, 30 min
Additive-free control	10.0	6.9	5.3	4.1
5% NaCl	10.2	5.1	1.1	0.7
2 μg/ml nisin	9.4	2.8	1.7	< 0.0

In a further experiment, when NaCl or nisin was present at the above-mentioned respective concentrations both in the pressurised and the recovery media (Table II), no additional effect of NaCl could be noted as compared to those shown in Table I, however, the nisin addition decreased the colony-forming ability of the non-pressurised, and to a smaller extent in case of the HHP-treated cells.

Changes in the luciferase activity

To estimate the HHP stability of cellular as cytoplasmic reporter molecule of our bioluminescent test organism, the viable cell counts estimated by plating with additive-free BHI agar and the luciferase activities of respective samples were compared as a function of HHP treatment and addition of NaCl or nisin in the

Table II

The effect of the presence of 5% NaCl, or, 2 μg/ml nisin in both the suspending buffer and the plating medium (BHI Agar) on the colony forming ability of non-pressurised and HHP-treated *L. monocytogenes lux*AB populations

Sample/treatment	$\log_{10}$ CFU/ml	
	Non-pressurised	300 MPa, 10 min
Additive-free control	10.1	7.2
5% NaCl	10.2	5.1
2 μg/ml nisin	8.0	2.4

Table III

Comparison of "viable cell counts" ($\log_{10}$ CFU/ml) and luciferase activities ($\log_{10}$ mV, 10 sec) of non-pressurised and pressurised *L. monocytogenes* populations[1] (mean values ± S.D. of triplicate measurements)

Sample and treatment	$\log_{10}$ CFU/ml	$\log_{10}$ mV, 10 sec
Non-pressurised, in pH 7.0 buffer	10.0	3.22±0.1
Non-pressurised, in pH 7.0 buffer +5% NaCl	10.2	2.58±0.05
300 MPa, 10 min in pH 7.0 buffer	6.9	1.48±0.03
300 MPa, 20 min in pH 7.0 buffer	5.3	0.89±0.02
300 MPa, 10 min in pH 7.0 buffer + 5% NaCl	5.1	0.10±0.03
300 MPa, 10 min in pH 7.0 buffer + 2 μg/ml nisin	7.1	1.65±0.06

[1] NaCl and nisin were present in the suspending buffer.

pressurised suspensions. The results are summarised in Table III. One can note from these data that if the cells were pressurised in additive-free or nisin-containing buffered suspensions the relation between bioluminescence intensity (luciferase activity) and the concentrations of colony forming units was similar to that established previously for untreated populations of the same test organism [5], i.e. the luciferase activity decreased concomitant with the reduction of the viable cell count. However, luciferase activities in the presence of 5% NaCl in the pressurised medium were approx. 0.6–0.8 log units less than expected from the previously published calibration.

Leakage of cell components

The ATP content was estimated from the supernatants of the non-pressurised control suspension as well as from suspensions treated at 200 MPa for 10 min and 300 MPa for 10 min, respectively. The ATP assay procedure resulted in the following ATP concentrations:

Non-pressurised suspension: 2.5×10^{-5} μmol,
200 MPa for 10 min: 3.2×10^{-5} μmol,
300 MPa for 10 min: 3.6×10^{-5} μmol.

The absorbance value of the 5-fold diluted supernatant of the cell suspension pressurised at 300 MPa for 10 min showed only a 6% higher value at 258 nm wavelength than that of the non-pressurised suspension.

Discussion and conclusions

The pressure-resistance of our test organism was within the range of HHP resistance of *L. monocytogenes* strains investigated by Patterson et al. [1].

The HHP treatment causing significant inactivation of populations of *L. monocytogenes* resulted in an increased NaCl- and nisin-sensitivity of the pressure-injured survivor fraction of the test organism. The increased ATP content and the increased UV-absorbances of supernatants of pressure-treated suspensions as compared to those of untreated suspensions revealed an increased leakage of the HHP-treated cells.

These results support and complement earlier findings on sensitisation of bacteria by HHP to anti-microbial peptides [7–11]. They are also in agreement with the consideration that the primary site of action of nisin against vegetative cells is the cytoplasmic membrane [12]. Pressure-induced outer membrane damage and periplasmic leakage of *E. coli* under high hydrostatic pressure has been observed also by Hauben et al. [13]

It is likely that the pressure-sensitivity of bioluminescence and its enhancement with NaCl (i.e. increase of ionic strength) was due to denaturation of the luciferase enzyme rather than to a more general effect reflecting the metabolism of the cells. Thus, use of bioluminescent test organisms provides an additional measure to examine the mechanisms of microbes and the role of the intra- and extracellular environments in stability of cell components [14].

The sensitising effects suggest a potential combination of HHP treatment with other anti-microbial stress factors in order to enhance non-thermally the microbiological safety of food. Considering the multifactorial nature of food systems, however, an item-by-item approach is needed for the optimisation of any new technologies. Better understanding the mechanism of the pressure inactivation of viable cells and the combined effects requires also further studies. Our results, nevertheless, support findings on importance of the damage of cell membranes as well as on the effects of increased ionic strength on inactivation of food-borne bacteria.

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References

1. Patterson, M. F., Quinn, M., Simpson, R., Gilmour, A.: Sensitivity of vegetative pathogens to high hydrostatic pressure treatment in phosphate-buffered saline and foods. J. Food Protection **58,** 524–529 (1995).
2. Knorr, D.: Hydrostatic pressure treatment of food: microbiology. In: Gould, G. W. (ed.): New methods of food preservation. Blackie Academic & Professional, Glasgow, Scotland, 1995, pp. 159–175.
3. Martens, T. (ed.): Harmonization of safety criteria for minimally processed foods. Inventory Report, FAIR Concerted Action, FAIR CT9-1020. Food linked agro-industrial research, European Commission, 1997.
4. Stewart, G. S. A. B., Aldsworth, T. G., Sharman, R. L., Gibson, P. T., Dodd, C. E. R.: Bioluminescence: *lux* as an enabling tool for the microbiological analysis of food. In: Tortorello, M. L., Gendel, S. M. (eds): Food microbiological analysis: new technologies. Marcel Dekker, New York, 1997, pp. 265–288.
5. Farkas, J., Andrássy, É., Beczner, J., Vidács, I., Mészáros, L.: Utilizing luminometry for monitoring growth of *Listeria monocytogenes* in its liquid and gelified monocultures and cocultures with "acid-only" *Lactococcus lactis.* Int J Food Microbiol **73,** 159–170 (2002).
6. Thore, A.: Technical aspects of the bioluminescent firefly luciferase assay of ATP. Science Tools **26,** 30–34 (1979).
7. Kalchayanand, N., Sikes, T., Dunne, C. P., Ray, B.: Hydrostatic pressure and electroporation have increased bactericidal efficiency in combination with bacteriocins. Appl Environ Microbiol **60,** 4174–4177 (1994).
8. Kalchayanand, N., Sikes, A., Dunne, C. P., Ray, B.: Interaction of hydrostatic pressure, time and temperature of pressurization and pediocin AcH on inactivation of foodborne bacteria. J Food Protection **61,** 425–431 (1998).
9. Ponce, E., Pla, R., Sendra, E., Guamis, B., Mor-Mur, M.: Combined effect of nisin and high hydrostatic pressure on destruction of *Listeria innocua* and *Escherichia coli* in liquid whole egg. Int J Food Microbiol **43,** 15–19 (1998).
10. Yuste, J., Mor-Mur, M., Capellas, M., Guamis, B., Pla, R.: Microbiological quality of mechanically recovered poultry meat treated with high hydrostatic pressure and nisin. Food Microbiol **15,** 407–414 (1998).
11. García-Graells, C., Masschalck, B., Michiels, C. W.: Inactivation of *Escherichia coli* in milk by high-hydrostatic pressure treatment in combination with antimicrobial peptides. J Food Protection **62,** 1248–1254 (1999).
12. Cleveland, J., Montville, T. J., Nes, I. F., Chikindas, M. L.: Bacteriocins: safe, natural antimicrobials for food preservation. Int J Food Microbiol **71,** 1–20 (2001).
13. Hauben, J. A., Wuytack, E. Y., Soontjens, C. C. F., Michiels, C. W.: High-pressure transient sensitization of *Escherichia coli* to lysozyme and nisin by disruption of outer-membrane permeability. J Food Protection **59,** 350–355 (1996).
14. Mackey, B. M., Cross, D., Park, S. F.: Thermostability of bacterial luciferase expressed in different microbes. J Appl Bacteriol **77,** 149–154 (1994).