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Bacterial inactivation by high-pressure homogenisation and high hydrostatic pressure

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Abstract

The resistance of five gram-positive bacteria, *Enterococcus faecalis*, *Staphylococcus aureus*, *Lactobacillus plantarum*, *Listeria innocua* and *Leuconostoc dextranicum*, and six gram-negative bacteria, *Salmonella enterica* serovar *typhimurium*, *Shigella flexneri*, *Yersinia enterocolitica*, *Pseudomonas fluorescens* and two strains of *Escherichia coli*, to high-pressure homogenisation (100–300 MPa) and to high hydrostatic pressure (200–400 MPa) was compared in this study. Within the group of gram-positive bacteria and within the group of gram-negative bacteria, large differences were observed in resistance to high hydrostatic pressure, but not to high-pressure homogenisation. All gram-positive bacteria were more resistant than any of the gram-negative bacteria to high-pressure homogenisation, while in relative to high hydrostatic pressure resistance both groups overlapped. Within the group of gram-negative bacteria, there also existed another order in resistance to high-pressure homogenisation than to high hydrostatic pressure. Further it appears that the mutant *E. coli* LMM1010, which is resistant to high hydrostatic pressure is not more resistant to high-pressure homogenisation than its parental strain MG1655. The preceding observations indicate a different response of the test bacteria to high-pressure homogenisation compared to high hydrostatic pressure treatment, which suggests that the underlying inactivation mechanisms for both techniques are different. Further, no sublethal injury could be observed upon high-pressure homogenisation of *Y. enterocolitica* and *S. aureus* cell population by using low pH (5.5–7), NaCl (0–6%) or SDS (0–100 mg/l) as selective components in the plating medium. Finally, it was observed that successive rounds of high-pressure homogenisation have an additive effect on viability reduction of *Y. enterocolitica* and *S. aureus*.

Keywords: High-pressure homogenisation; High hydrostatic pressure; Inactivation; Sublethal injury

1. Introduction

In the pharmaceutical, cosmetic, chemical and food industries, high-pressure homogenisation is used for the preparation or stabilization of emulsions and suspensions, or for creating physical changes, such as viscosity changes, in products. For some applications that require a high degree of disperion, pressures up to 200 MPa or higher are currently being investigated. Another application is cell disruption of yeasts or bacteria in order to release intracellular products such as recombinant proteins.

Although the range of products that undergo high-pressure homogenisation is quite diverse, their microbiological quality is invariably an important parame-

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ter. Many pharmaceutical products need to be sterile, but also for most products in the food industry a partial or complete inactivation of the microbial population is desired. Heat treatment is one of the most efficient and economical processes to achieve this inactivation, but it cannot be used on heat-labile components or heat-labile dispersions. Therefore, there is a growing interest in mild non-thermal processes, which combine efficient germ reduction with a maximal retention of the chemical and physicochemical product properties.

Since high-pressure homogenisation can be applied for cell disruption of dense microbial cultures (Kelemen and Sharpe, 1979; Engler, 1990; Harrison et al., 1991; Siddiqi et al., 1997; Shirgaonkar et al., 1998), we anticipated that high-pressure homogenisation during the production of emulsions or suspensions will also cause a partial inactivation of the microbial population. In the literature, there are indeed a few studies indicating this. Popper and Knorr (1990) reported that a homogenisation treatment at 100 MPa reduces *Escherichia coli*, *Streptococcus lactis* and *Bacillus subtilis* cell populations, respectively, with approximately 3, 1 and 3 log units. Lanciotti et al. (1994) observed that a high-pressure homogenisation treatment of around 100 MPa causes a reduction of *Listeria monocytogenes*, *Yersinia enterocolitica* and *Yarrowia lipolytica* cell population of approximately 2, 3 and 6 log units, respectively. Guerzoni et al. (1999) observed the reduction of, respectively, 2 and 3 log units of the naturally occurring psychrotrophic aerobic cells and the entrococci in goat milk upon high-pressure homogenisation at 100 MPa. Bacterial spores on the other hand seem to be very resistant to high-pressure homogenisation, at least at temperatures below 50 °C (Feijoo et al., 1997). This is not surprising since spores are also very resistant to heat and high hydrostatic pressure (Larson et al., 1918; Basset and Macheboeuf, 1932; Setlow, 1994; Nakayama et al., 1996). Therefore, it will not be possible to sterilize products with high-pressure homogenisation at low temperatures. However, a reduction of vegetative microorganisms only is also potentially interesting since this may extended shelf-life and improve microbiological safety of foods and other products, and may reduce the need of other process steps (heat sterilisation, irradiation) that are carried out for reduction of microorganisms. This may influence product quality and process cost in a beneficial way. The objectives of this study were to investigate the potential of high-pressure homogenisation at pressures up to 300 MPa to reduce microbiological burden and to compare efficiency and mechanism of high-pressure homogenisation and high hydrostatic pressure treatment, using a variety of gram-positive and gram-negative bacteria, including relevant food pathogens and high hydrostatic pressure resistant *E. coli* (LMM1010) (Hauben et al., 1997). In both techniques, microorganisms are subjected to high pressures; however, in high-pressure homogenisation this is only for a very short time in the order of a second or less, while in high hydrostatic pressure treatment the exposure time is in the order of minutes or more. On the other hand, cells in high-pressure homogenisation are exposed to hydrodynamic cavitation, impingement against static surfaces, high turbulence and fluid shear, and this is not the case in hydrostatic pressure treatment.

2. Materials and methods

2.1. Preparation of cell suspensions

Strains used in this study and their growth conditions are listed in Table 1. Cultures (40 ml) in 100-ml Erlenmeyer's were grown to stationary phase by shaking (200 rpm) during 21 h, centrifuged at

Table 1
Microorganisms used in this study and their growth conditions

Species	Growth medium	Incubation temperature (°C)	Count medium
L. plantarum	MRS broth	37	MRS agar
L. innocua	BHI broth	37	PCA
S. aureus	Nutrient broth	37	PCA
E. faecalis	BHI broth	37	PCA
L. dextranicum	MRS broth	30	MRS agar
E. coli MG1655	Nutrient broth	37	PCA
E. coli LMM1010	Nutrient broth	37	PCA
Y. enterocolitica	Nutrient broth	30	PCA
S. flexneri	Nutrient broth	37	PCA
P. fluorescens	TS broth	30	PCA
S. typhimurium	Nutrient broth	37	PCA

BHI: Brain Heart Infusion (CM225 Oxoid).
MRS: de Man Rogosa Sharpe (CM359 Oxoid).
PCA: Plate Count Agar (CM325 Oxoid).
TS: Trypton Soya (CM129 Oxoid).
* Nutrient Broth (CM67 Oxoid).

4000 × *g* for 5 min and the pellets were resuspended to a concentration of 10^8 cfu/ml in sterile PBS buffer (10 mM potassium phosphate buffer (pH 7,0), 8.4 g/l NaCl).

2.2. High-pressure homogenisation treatment

Cell suspensions were homogenised at 25 °C and a process pressure ranging between 100 and 300 MPa. The high-pressure homogenisator was an Emulsiflex C5 (Avestin, Ottawa, Canada) and consists of an inlet reservoir, an air-driven pump, and a ceramic homogenising valve. A heat exchanger was placed immediately after the homogenising valve to withdraw the heat generated during passage of the sample through the homogenising valve. In addition, the homogeniser was immersed in a water bath maintained at 25 °C to improve heat dissipation from the homogenising valve block. The outlet temperature of the sample homogenised at 300 MPa was ± 42 °C without heat exchanger and ± 18 °C with heat exchanger.

For cyclic treatments, the high-pressure homogenisator was sterilized with 70% ethanol after each cycle.

2.3. High hydrostatic pressure treatment

Cell suspensions were pressurised in heat-sealed sterile polyethylene bags during 15 min at 25 °C and a process pressure ranging from 200 to 400 MPa. The high hydrostatic pressure equipment (Resato, Roden, The Netherlands) consisted of a manually operated spindle pump, an 8-ml pressure vessel with an external water jacket for temperature control and the necessary high-pressure valves. The compression rate was approximately 100 MPa/min, while decompression was immediate. It was previously established that sample temperature would not exceed 35 °C under these working conditions (Hauben et al., 1997).

2.4. Determination of loss of viability and sublethal injury

To assess loss of viability caused by a treatment, untreated and treated cell suspensions were serially diluted in PBS and plated on the surface of an appropriate count medium (see Table 1). Plates were incubated 21 h at 30 or 37 °C (see Table 1) and colony forming units (CFU) were determined. The logarithm of the reduction factor (log RF) was calculated as:

$$\log RF = \log\left(\frac{\text{CFU before treament}}{\text{CFU after treament}}\right)$$

To assess sublethal injury caused by a treatment, untreated and treated cell suspensions were serially diluted in PBS and surface-plated on a set of selective agar plates. These were based on Plate Count Agar (PCA Oxoid, Basingstoke, United Kingdom) to which selective agents were added: Sodium Dodecyl Sulphate (SDS 0–100 mg/l) (Merck, Darmstadt, Germany), NaCl (0–6%), or Mc Ilvaine buffer (pH 5.5–7), consisting of a mixture of citric acid (0.1 M)—Na_2HPO_4 (0.2 M). Plates were incubated 21 h at 30 or 37 °C (see Table 1) and colony forming units (CFU) were counted. The logarithms of the reduction factors for the different selective media were calculated.

The data presented are means of three replicate experiments, unless otherwise indicated. Significant differences at 5% level were calculated with the paired Student's *t*-test.

3. Results

3.1. Effect of high-pressure homogenisation on viability of different bacteria

Cell suspensions of the different test bacteria were subjected to different high-pressure homogenisation treatments at a constant temperature of 25 °C and pressures ranging between 100 and 300 MPa. Inactivation of all the bacteria is represented in Fig. 1. A remarkable observation is the clear distinction between gram-positive and gram-negative bacteria. At all pressures, each of the gram-positive bacteria in our test panel was significantly ($P < 0.05$) more resistant than each of the gram-negative bacteria. At a working pressure of 200 MPa, inactivation of all gram-positive bacteria was less than 1.0 log unit, while it is 2.0–4.6 log units for the gram-negative bacteria. Within the group of gram-positive bacteria, *Staphylococcus aureus*, *Enterococcus faecalis* and *Lactobacillus plantarum* were the most resistant, while *Listeria innocua* and *Leuconostoc dextranicum* were slightly more

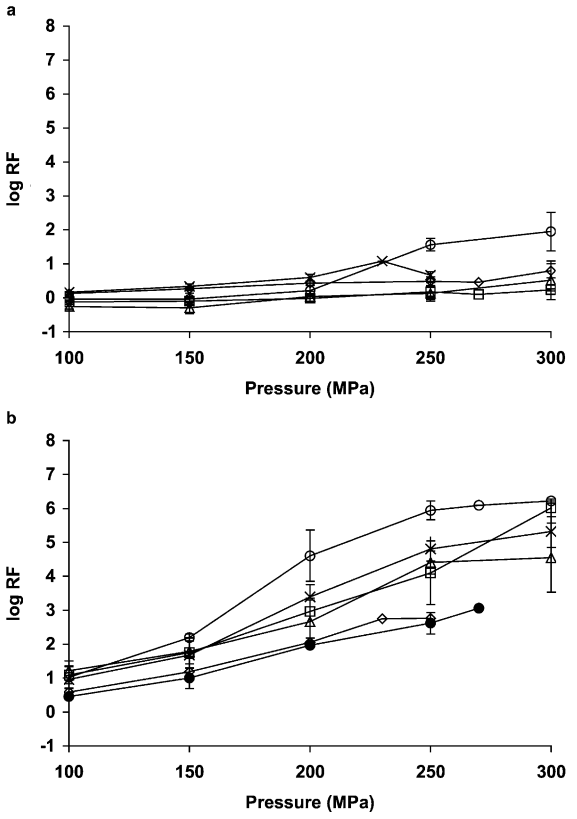

Fig. 1. Inactivation of gram-positive (a) and gram-negative bacteria (b) as a function of the homogenisation pressure. Process temperature 25 °C. logRF = log(N_0/N). The presented data are the mean value of three experiments ± standard deviation. Gram-positive bacteria: *E. faecalis* (◇), *S. aureus* (□), *L. plantarum* (△), *L. innocua* (×), *L. dextranicum* (○). Gram-negative bacteria: *S. typhimurium* (●), *S. flexneri* (×), *E. coli* MG1655 (△), *E. coli* LMM1010 (□), *Y. enterocolitica* (◇), and *P. fluorescens* (○).

sensitive but the observed reduction for the latter remained below 2 log units at 300 MPa.

Among the tested gram-negative bacteria *S. typhimurium* and *Y. enterocolitica* were the most resistant to high-pressure homogenisation, followed by *E. coli* and *S. flexneri*, while *P. fluorescens* was very sensitive to a high-pressure homogenisation treatment.

Another observation concerns the two strains of *E. coli*. Strain LMM1010 is a spontaneous mutant isolated from strain MG1655, which is characterized by an extreme resistance to inactivation by high hydrostatic pressure (Hauben et al., 1997). Our current results however indicate that this mutant has similar sensitivity as the parental strain to high-pressure homogenisation at least up to a pressure of 300 MPa.

3.2. Effect of high hydrostatic pressure on viability of different bacteria

Cell suspensions of all the test bacteria were also subjected to different high hydrostatic pressure treatments at a constant temperature of 25 °C and pressures ranging from 200 to 400 MPa. The inactivation caused by these treatments is represented in Fig. 2. In general, it appears that gram-positive bacteria are more resistant to high hydrostatic pressure than gram-negative bac-

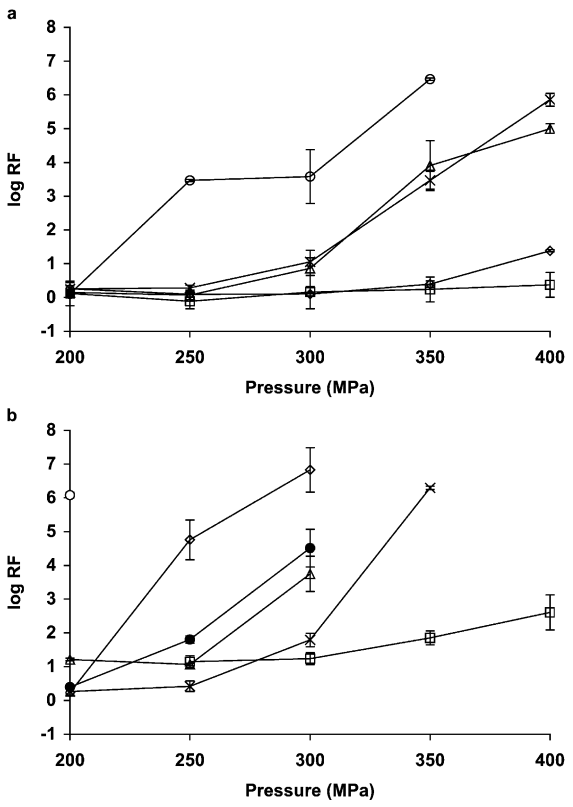

Fig. 2. Inactivation of gram-positive (a) and gram-negative bacteria (b) as a function of high hydrostatic pressure. Process temperature 25 °C and process time 15 min. logRF = log(N_0/N). The presented data are the mean value of three experiments ± standard deviation. Gram-positive bacteria: *E. faecalis* (◇), *S. aureus* (□), *L. plantarum* (△), *L. innocua* (×), *L. dextranicum* (○). Gram-negative bacteria: *S. typhimurium* (●), *S. flexneri* (×), *E. coli* MG1655 (△), *E. coli* LMM1010 (□), *Y. enterocolitica* (◇), and *P. fluorescens* (○).

teria. The two most resistant bacteria are gram-positive, and the two most sensitive are gram-negative. However, there is considerable overlap. For instance, *L. dextranicum* is more sensitive to high hydrostatic pressure than several gram negative bacteria (*S. flexneri*, *E. coli* and *S. typhimurium*), and the pressure resistant mutant of *E. coli* (LMM1010), is more resistant than several gram-positive bacteria (*L. dextranicum*, *L. innocua* and *L. plantarum*), particularly at pressures >300 MPa.

Within the gram-positive and gram-negative bacteria, the order of resistance to high hydrostatic pressure and to high-pressure homogenisation is also different. Among the tested gram-positive bacteria, *S. aureus* and *E. faecalis* show the highest resistance to high hydrostatic pressure, while *L. innocua* and *L. plantarum* have intermediate resistance and *L. dextranicum* is most sensitive. The tested gram-negative bacteria in descending order of resistance to high hydrostatic pressure, are *E. coli* LMM1010, *S. flexneri*, *E. coli* MG1655, *S. typhimurium*, *Y. enterocolitica* and *P. fluorescens*.

3.3. Sublethal injury caused by high-pressure homogenisation

The gram-negative bacterium and gram-positive bacterium showing the highest resistance to high-pressure homogenisation, respectively, *Y. enterocolitica* and *S. aureus*, were chosen to study sublethal injury caused by high-pressure homogenisation by plating treated bacterial suspensions on PCA modified with different selective factors, such as low pH, NaCl or SDS. The results are presented in Fig. 3. For *Y. enterocolitica*, the maximum NaCl concentration that allowed growth of the untreated cells was 2%. None of the selective components caused an increase in the reduction factor of *Y. enterocolitica* and *S. aureus* compared to the reduction factor on the non-selective medium, indicating that a high-pressure homogenisation treatment causes little or no sublethal injury.

3.4. Cyclic high-pressure homogenisation treatment

The effect of cyclic high-pressure homogenisation treatment on the viability of *Y. enterocolitica* and *S. aureus* was determined, by subjecting cell suspensions to several successive high-pressure homogenisation

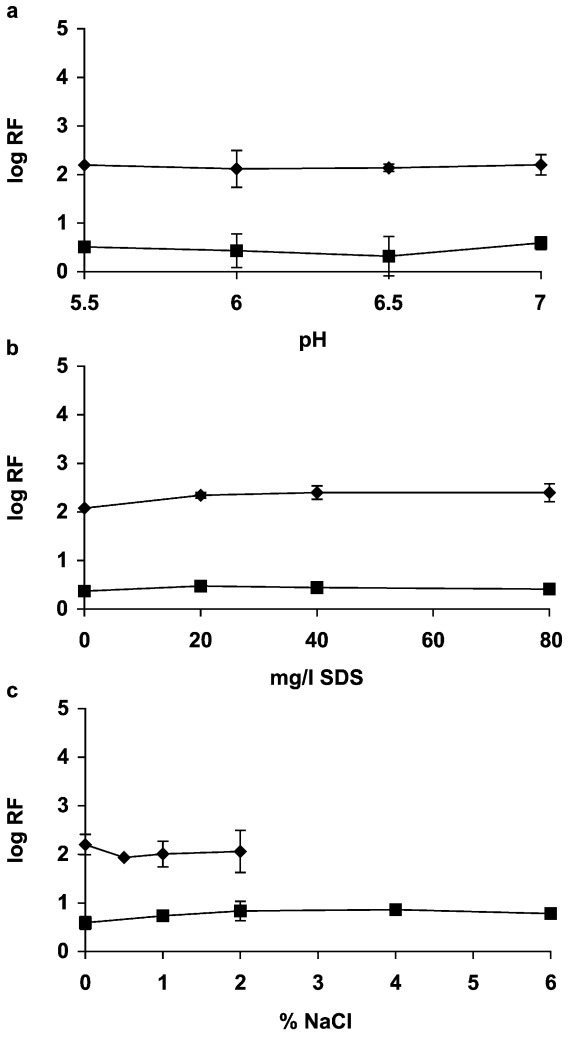

Fig. 3. Viability reduction of *S. aureus* (■) and *Y. enterocolitica* (♦) caused by a high-pressure homogenisation treatment at, respectively, 300 and 200 MPa and determined on PCA modified with different selective factors: low pH (a), SDS (b) or NaCl (c). $\log RF = \log(N_0/N)$. The presented data are the mean value of three experiments ± standard deviation.

treatments, always at 150 MPa and 25 °C for *Y. enterocolitica* and 250 MPa and 45 °C for *S. aureus*. This higher temperature was chosen for *S. aureus* because it allowed somewhat higher inactivation levels. After each round, the viability reduction was determined (Fig. 4). For both microorganisms, it can be seen that each round causes approximately the same

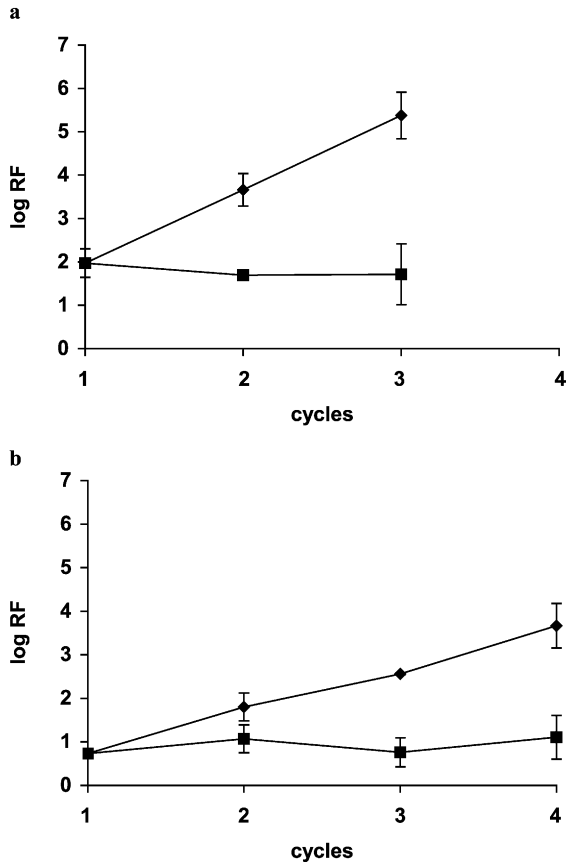

Fig. 4. Viability reduction of *Y. enterocolitica* (a) and *S. aureus* (b) caused by successive high-pressure homogenisation treatments at respectively 150 MPa and 25 °C and 250 MPa and 45 °C. Expressed as cumulative inactivation of all cycles (♦) or as inactivation caused by each cycle (■). logRF = log(N_0/N). The presented data are the mean value of three experiments ± standard deviation.

viability reduction, indicating that the different rounds have an additive effect.

4. Discussion

The first objective of this study was to investigate the resistance of different gram-positive and gram-negative bacteria to high-pressure homogenisation. Within our group of five gram-positive and five gram-negative test bacteria we found that, without any exception, the gram-positive bacteria were more resistant to high-pressure homogenisation than the gram-negative bacteria (Fig. 1). Together with some older published work, these data provide convincing evidence to generalize the statement that gram-positive bacteria are more resistant to high-pressure homogenisation than gram-negative bacteria. Kelemen and Sharpe (1979) working with *E. coli*, *S. faecalis*, *B. subtilis*, *Lactobacillus casei* and *S. aureus* (i.e. one gram-negative and four gram-positive bacteria) made the same observation and concluded that composition of the cell wall and more particularly peptidoglycan, determines the resistance to high-pressure homogenisation. Peptidoglycan is the skeletal structure of bacterial cells, which gives to the cell their strength. Middelberg and O'Neill (1993) also believed that peptidoglycan structure has an important role in the resistance to high-pressure homogenisation and developed a model for disruption of *E. coli* by high-pressure homogenisation in which one of the parameters, the mean effective strength, is correlated with the peptidoglycan cross-linkage. It is known that gram-positive bacteria have a thicker peptidoglycan (about 40 layers) than gram-negative bacteria (1 up to 5 layers) (Lengeler et al., 1999). The tight correspondence between peptidoglycan structure and high-pressure homogenisation resistance suggests that high-pressure homogenisation kills vegetative bacteria mainly through mechanical destruction of the cell integrity, caused by the spatial pressure and velocity gradients, turbulence (Doulah et al., 1975), impingement (Engler and Robinson, 1981; Keshavarz-Moore et al., 1990) and/or cavitation (Save et al., 1994; Shirgaonkar et al., 1998), that occur in a liquid during high-pressure homogenisation. In line with this hypothesis, Kelemen and Sharpe (1979) also postulated cell shape to be an additional factor determining bacterial resistance to high-pressure homogenisation, rods predicted to be more easily disrupted than cocci. Our results do not confirm this postulate; for example the coccus *L. dextranicum* is much more sensitive to high-pressure homogenisation than the rod *L. plantarum* (Fig. 1).

The second objective of this study was to compare bacterial resistance to high-pressure homogenisation and to high hydrostatic pressure. In both techniques, the cells are subjected to high pressures in a similar range. Therefore, it was possible that the inactivation mechanisms for both techniques would be similar, at least in the overlapping pressure range (up to 300 MPa). This appears not to be the case since high-pressure homogenisation results in a completely dif-

ferent inactivation pattern of the test bacteria than high hydrostatic pressure. First, within each group (gram-positive or gram-negative bacteria) there are large differences in resistance to high hydrostatic pressure (Fig. 2), but not to high-pressure homogenisation (Fig. 1). Second, it is possible to completely discern gram-positive and gram-negative bacteria on the basis of their resistance to high-pressure homogenisation, while based on resistance to high hydrostatic pressure (Fig. 2) both groups overlap. Third, within the group of gram-negative bacteria there also exists another order in resistance to high-pressure homogenisation than to high hydrostatic pressure. For instance, *Y. enterocolitica* is very resistant to high-pressure homogenisation while it is very sensitive to high hydrostatic pressure. Another example is *E. coli* mutant LMM1010, which is much more resistant to high hydrostatic pressure than its parental strain, but equally resistant to high-pressure homogenisation. Fourth and final, high-pressure homogenisation does not cause sublethal injury as opposed to high hydrostatic pressure (Hauben et al., 1996; García-Graells et al., 1998; Kalchayanand et al., 1994, 1998; Ritz et al., 2001). All those observations together suggest that the inactivation mechanisms of high-pressure homogenisation differ from those of high hydrostatic pressure, at least over the pressure ranges studied. Presumably, the high pressure developed during high-pressure homogenisation is not to any major extent responsible for inactivation of microorganisms because the bacteria are only exposed to this high-pressure for a very short time in the order of a second or less. For comparison, exposure time to high hydrostatic pressure treatment in this work was 15 min.

The results relating to sublethal injury deserve some further discussion. The finding that high-pressure homogenisation does not cause sublethal injury reflects the 'all or nothing' impact of this technique on bacteria. Bacteria are either disrupted or not; if they are disrupted they are dead, and if they are not disrupted they are indistinguishable from untreated cells. Such behaviour can be well explained by a mechanism in which the peptidoglycan is the sole vital target, as discussed above. This is different from high hydrostatic pressure treatment, which can cause an accumulation of sublethal injury ultimately leading to death. In this case, the existence of multiple non-vital targets is a more likely explanation, and cell death occurs only after inactivation of a certain number of these targets. High hydrostatic pressure has indeed been reported to interfere with various cellular structures or functions, such as cytoplasmic membrane (Isaacs et al., 1995; Chilton et al., 1997; Pagan and Mackey, 2000), ribosomes (Isaacs et al., 1995; Niven et al., 1999) and specific enzymes (Isaacs et al., 1995; Simpson and Gilmour, 1997; Ritz et al., 2000).

The inability of high-pressure homogenisation to cause sublethal injury may limit applications of this technique in hurdle technology. If the cells surviving high-pressure homogenising treatment are not injured, it can be anticipated that they will not be sensitised to other treatments or to unfavourable conditions such as low pH, high NaCl concentration, or antimicrobials. On the other hand, lytic enzymes such as lysozyme, or other treatments that weaken peptidoglycan, may increase bacterial sensitivity to high-pressure homogenisation.

Finally, it has been observed that several successive rounds of high-pressure homogenisation have an additive effect on viability reduction (Fig. 4). This could be a promising approach to increase the microbicidal efficiency of the treatment. Indeed, under the conditions used in this work, i.e. homogenisation pressure up to 300 MPa and treatment temperature 25 or 45 °C, inactivation of the most resistant organisms (*S. aureus*) by a single homogenisation treatment remained under 1 log unit, which is far insufficient for applications such as food pasteurisation. The level of inactivation increased to almost 4 log units after four rounds of homogenisation, and can probably be further increased by applying additional treatment rounds.

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