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The lactoperoxidase system increases efficacy of high-pressure inactivation of foodborne bacteria

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Abstract

The inactivation of eight different bacteria comprising *Escherichia coli* LMM1010 and MG1655, respectively a pressure-resistant strain and the corresponding wild-type, *Salmonella* Typhimurium, *Pseudomonas fluorescens*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Listeria innocua* and *Lactobacillus plantarum*, by high hydrostatic pressure in skim milk supplemented with the lactoperoxidase–hydrogen peroxide–thiocyanate (LP) system at naturally occurring concentration was studied. In the absence of pressure treatment, the LP system had either no effect, i.e. on *S.* Typhimurium and *E. coli* LMM1010, a growth inhibiting effect, i.e. on *E. coli* MG1655, *L. innocua*, *S. aureus*, *L. plantarum* and *E. faecalis*, or a bactericidal effect, i.e. on *P. fluorescens*. The presence of the LP system affected inactivation by high pressure in a cell density-dependent manner. At low cell concentration (10^6 cfu/ml), the LP system strongly increased high-pressure inactivation as measured immediately after pressure treatment of all bacteria except the pressure-resistant *E. coli*. At high cell density (10^9 cfu/ml), only inactivation of *L. innocua*, *E. faecalis* and *L. plantarum* were enhanced. For both *E. coli* strains, the fate of the bacteria during 24 h following pressure treatment was also studied. It was found that in the presence of the LP system, considerable further inactivation occurred in the first hours after pressure treatment. The potential of the LP system to improve the bactericidal efficiency of high-pressure treatment for food preservation is discussed.

Keywords: High hydrostatic pressure; Lactoperoxidase system; Nonthermal pasteurisation

1. Introduction

High hydrostatic pressure treatment is a promising technique for the pasteurisation and sterilization of foods and other products. Because the treatment inactivates spoilage and pathogenic microorganisms at lower temperature than a conventional heat treatment, and because the chemical and physical properties of foods are less affected by pressure than by heat, pressure-preserved products are generally of higher quality than heat-preserved products. As for thermal processing, the development of reliable and safe high-pressure processes will require detailed knowledge about the high-pressure inactivation of various food microorganisms, and how this inactivation is affected by relevant process and product parameters such as processing time, pressure and temperature, food type,

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specific food constituents, pH, etc. A vast amount of literature on this topic has accumulated during the past two decades (Cheftel, 1992; Knorr, 1995; Smelt, 1998).

An important insight that emerged recently from these studies is that different strains of a single species can have widely varying pressure resistance. As a result, the efficiency of pressure treatment as a nonthermal process to inactivate food bacteria has been questioned by an increasing number of investigators. For instance, we have shown that extremely pressure-resistant mutants can arise and be selected after repeated exposure of the pressure-sensitive strain *Escherichia coli* MG1655 to high pressure (Hauben et al., 1997). Very high pressure resistance was later also found among natural *E. coli* strains including pathogenic strains of the O157:H7 serotype (Alpas et al., 1999; Benito et al., 1999). Further, pressure resistance of vegetative bacteria is also largely dependent on the food matrix (Styles et al., 1991; Mackey et al., 1995; Patterson et al., 1995; Patterson and Kilpatrick, 1998). For instance, *E. coli* strain MG1655 mentioned earlier is relatively pressure-sensitive when suspended in buffer but pressure-resistant in milk at the same pH (Garcia-Graells et al., 1999). A route that is being explored to overcome these problems is to combine high-pressure treatment with another process, a preservative or any other parameter that will enhance the microbicidal efficiency. Successful combinations that have been reported include elevated temperature (Patterson and Kilpatrick, 1998; Alpas et al., 2000; Alpas and Bozoglu, 2000), reduced pH (Stewart et al., 1997; Linton et al., 1999; Jordan et al., 2001; Pagan et al., 2001), nisin or other bacteriocins, lysozyme, lactoferrin and peptide fragments derived from the latter two proteins (Kalchayanand et al., 1994; Kalchayanand et al., 1998; Garcia-Graells et al., 1999; Alpas and Bozoglu, 2000; Masschalck et al., 2001b; Masschalck and Michiels, 2001a). However, there are still problems. Kinetic experiments clearly revealed that the combined inactivation by high pressure and lysozyme or nisin is characterised by pronounced tailing, suggesting that a significant fraction of the bacterial population is resistant to the combined treatment (Masschalck et al., 2001b). Also, the cooperative effect between pressure and lysozyme or nisin appears to be limited to some bacteria. Several bacteria, including foodborne pathogens such as *Salmonella* Typhimurium, *S. enteritidis* and *Shigella flexneri* could not be sensitized to lysozyme or nisin by pressure (Masschalck et al., 2001b; Masschalck and Michiels, 2001a). Recently, we started to investigate the combination of high pressure with the lactoperoxidase (LP) system, an antimicrobial system that occurs naturally in milk and other secreta such as saliva and tears. The central component of the system is the lactoperoxidase enzyme, which catalyses the oxidation of thiocyanate (SCN^-) by hydrogen peroxide into short-lived reactive products such as the hypothiocyanite anion ($OSCN^-$) that in turn rapidly oxidises many biomolecules. Most relevant for microbial inactivation is probably the oxidation of enzymes and other proteins in the bacterial cell membrane that have exposed sulfhydryl groups (–SH), but other targets have also been suggested (Marshall and Reiter, 1976; Mickelson, 1977; Reiter, 1978). In an initial study with four strains of *Listeria monocytogenes* and four strains of *E. coli*, we found a synergistic effect of the lactoperoxidase system and high-pressure treatment against the former, but not against the latter (Garcia-Graells et al., 2000). In the present work, we show that under conditions which more closely resemble those prevailing in foods, this combined treatment also successfully inactivates *E. coli*, and we have extended the study to a wider range of relevant food bacteria, including pressure-resistant *E. coli*.

2. Materials and methods

2.1. Growth of bacteria and preparation of inocula

Bacteria and culture conditions used in this study are listed in Table 1. Permanent culture stocks were maintained at −80 °C as broth cultures containing 25% glycerol. Agar plates were streaked every 7–10 days from these stocks and stored at 4 °C. To prepare test suspensions of these bacteria, cultures were grown to stationary phase for 21 h with shaking (200 rpm) at 30 or 37 °C in the growth medium indicated in Table 1. Cells were harvested by centrifugation (3000 × *g*, 5 min) and resuspended in commercial sterilized skim milk (pH 6.7, 0.05% fat) at approximately 10^6 or 10^9 cfu/ml.

In experiments where survival was monitored during storage at 20 °C after pressure treatment, a

Table 1
Bacteria and culture conditions used in this study

	Growth medium	Count medium	Growth temperature (°C)
Escherichia coli MG1655	LB	TSA	37
Escherichia coli LMM1010[a]	LB	TSA	37
Listeria innocua LMG 11387[b]	TSB	TSA	37
Staphylococcus aureus LMMBM14[c]	NB	PCA	37
Enterococcus faecalis LMMB239[c]	BHI	BHI	37
Salmonella Typhimurium LMMBM01[c]	NB	PCA	37
Pseudomonas fluorescens LMMBM07[c]	NB	PCA	37
Lactobacillus plantarum LB75[c]	MRS	MRS	30

LB, Luria Bertani broth; TSB, tryptons soya broth; NB, nutrient broth; BHI, brain–heart infusion broth or agar; MRS, de Man Rogosa Sharpe broth or agar; TSA, tryptons soya agar; PCA, plate count agar.

[a] Pressure-resistant mutant derived from MG1655 (Hauben et al., 1997).

[b] Belgian Coordinated Collection of Microorganisms, Ghent, Belgium.

[c] Laboratory of Food Microbiology Collection, Leuven, Belgium.

sufficient number of replicate samples was prepared and simultaneously treated to allow destructive sampling.

2.2. Application of the lactoperoxidase system

A stock solution of 10 mg/ml of lactoperoxidase (EC 1.11.1.7; Sigma, Bornem, Belgium) was prepared in 50% glycerol and 50% phosphate buffered saline (0.1 M potassium phosphate buffer, pH 6.0, 150 mM NaCl). Aqueous 25 mM stock solutions of the substrates of the LP system, KSCN (Acros, Geel, Belgium) and H_2O_2 (Vel, Leuven, Belgium) were sterilized by passage through 0.22-μm filters and stored at 4 °C. In experiments with the LP system, the enzyme was used at 5 μg/ml together with both substrates at 0.25 mM. Two control experiments were always performed: one without addition of enzyme or substrates, and one with addition of 0.25 mM H_2O_2 only. The latter allowed us to see whether inactivation was caused by the toxic effect of H_2O_2 alone.

2.3. High-pressure treatment

Samples in heat-sealed polyethylene bags were pressurized in a small 8-ml pressure autoclave driven by a manual spindle pump and thermostatically controlled with a water jacket connected to a cryostat (Resato, Roden, The Netherlands). The pressure liquid was TP1, a mixture of glycols (Van Meeuwen, Wesp, The Netherlands). All pressure treatments were conducted at 20 °C. It should be noted, however, that sample temperatures in our experiments may reach a peak up to 30 °C during compression (Masschalck et al., 2000).

2.4. Determination of bacterial survival and reproducibility of results

Viability was determined by plating decimal dilutions on the appropriate medium for each species (Table 1) with a spiral plater (Spiral Systems, Cincinnati, OH) and incubating at 30 or 37 °C for 24–48 h depending on the species. For pressure-inactivation experiments, viability of pressure-treated samples and untreated controls was determined immediately after pressure release except when otherwise mentioned. For time-course experiments, samples were stored at room temperature and bacterial viability was determined at different times. Reduction of viable cells was expressed as the difference between the logarithms of the colony counts of the untreated and treated samples ($\log N_0 - \log N$).

Time-course experiments were done in threefold using three independent bacterial cultures, and representative results are shown. For the inactivation experiments that were only analysed immediately after pressure treatment (Figs. 1 and 2), at least two experiments at two different pressures were done in threefold for each bacterial strain, and standard deviations were calculated. Standard deviations were found to be similar for all experiments and for all bacteria and never exceeded 1 log unit. If we now assume a uniform standard deviation of 0.19 (i.e. the average of all measured standard deviations) for all experiments, a statistically significant difference ($p < 0.05$) must be at least 0.38 log units. In the discussion, only differences larger than 1.5 log units are considered relevant, and this is well above the threshold for statistical significance.

3. Results

3.1. Effect of the LP system on vegetative bacteria in milk

First, we studied the antimicrobial effect of the LP system at atmospheric pressure in sterilized skim milk. The eight bacteria of the test panel were inoculated in milk at approximately 10^6 cfu/ml. For each organism, one sample was supplemented with the complete LP system (lactoperoxidase, H_2O_2, KSCN), another sample with H_2O_2 alone and a third sample was not supplemented with any additives. The samples were incubated for 24 h at 20 °C, and viable cells counted at regular times. In the absence of additives, all the bacteria showed growth after 24h, although only very weak for *Lactobacillus plantarum* and *Pseudomonas fluorescens* (result not shown). The effect of the LP system varied with the bacterial strain from bactericidal (*P. fluorescens*) to bacteriostatic or inhibitory (*E. coli* MG1655, *L. innocua*, *Staphylococcus aureus*, *L. plantarum*, *Enterococcus faecalis*), or no effect (*S.* Typhimurium, *E. coli* LMM1010) (result not shown). The bactericidal effect on *P. fluorescens* was rapid, reaching a maximum reduction in viable count of approximately 5 log cfu/ml after 3 h, but was only temporary, since the counts increased again after 3 h at a rate similar to the growth of this strain in unsupplemented milk (result not shown). The addition of H_2O_2 alone did not affect growth of the test bacteria in milk, except for *E. coli* MG1655, for which a bacteriostatic effect was observed (result not shown).

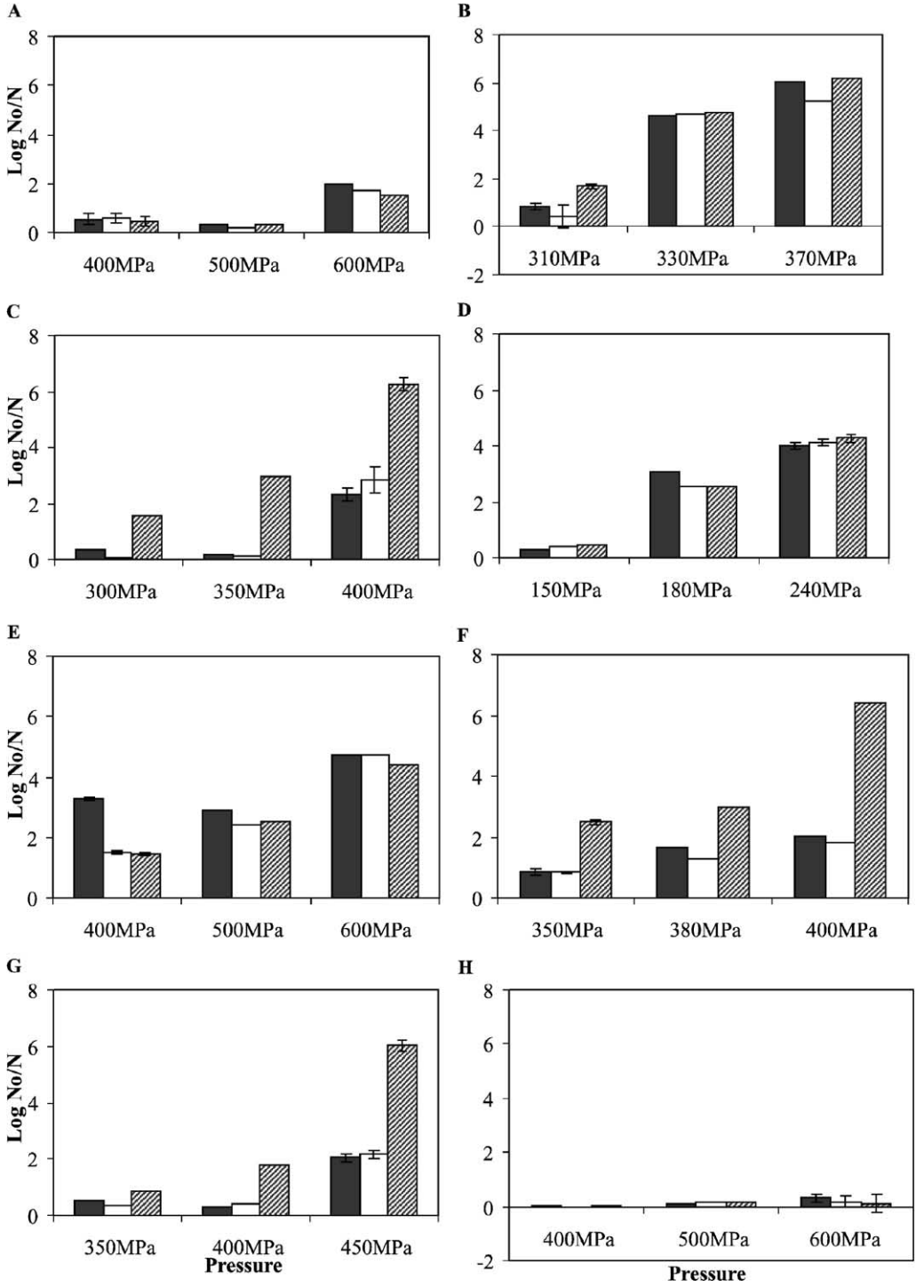
3.2. Effect of the LP system in combination with HP treatment on dense bacterial suspensions

In a previous study, we observed a synergistic effect of high-pressure treatment and the LP system on inactivation of *L. innocua*, but not on *E. coli* (Garcia-Graells et al., 2000). Now, we extended this study to a number of other bacteria. Similar to this previous study, we first used dense bacterial suspensions of 10^9 cfu/ml in milk to allow measurement of inactivation over a wide range. The inoculated samples, with the LP system, with H_2O_2 or without addition, were treated at different pressures, and survivors were counted immediately after pressure treatment (Fig. 1). Pressure sensitivity varied largely between the different bacteria tested. Most sensitive was *P. fluorescens*, with a reduction of 4 log cfu/ml at 240 MPa. Most resistant were both *E. coli* strains, with reductions at 600 MPa of 2 log cfu/ml for MG1655, and only 0.3 log cfu/ml for the pressure-resistant strain LMM1010. None of the bacteria tested were more sensitive to HP in the presence of H_2O_2 than in its absence. However, in the presence of the LP system, high-pressure sensitivity was affected depending on the bacterial species. Enhanced inactivation was observed for *L. innocua*, *E. faecalis* and *L. plantarum* in the presence of the LP system and this effect increased at higher pressures. As a result of the addition of the LP system, these bacteria were efficiently inactivated (≥ 6 log cfu/ml) at relatively mild pressures (400–450 MPa), whereas in the absence of the LP system, the same pressure caused only about 2 log cfu/ml reduction. For the other bacteria tested, *E. coli*, *S.* Typhimurium, *S. aureus* and *P. fluorescens*, no such synergy occurred, even when HP inactivation in the absence of the LP system was up to 4 log cfu/ml. Increasing pressure decreased the number of surviving bacteria, but did not make the survivors sensitive to the LP system.

3.3. Effect of the LP system in combination with HP treatment on dilute bacterial suspensions

To investigate whether the initial cell concentration of the bacterial suspensions would affect the efficiency of the combined treatment, we inoculated the test bacteria at 10^6 cfu/ml instead of 10^9 cfu/ml (Fig. 2). Comparison of the results at low and high cell density leads to a number of observations. First, lower cell densities do not seem to result in higher inacti-

Fig. 1. High-pressure inactivation of high-density cell suspensions (10^9 cfu/ml) of *E. coli* MG1655 (A), *S.* Typhimurium (B), *L. innocua* (C), *P. fluorescens* (D), *S. aureus* (E), *L. plantarum* (F), *E. faecalis* (G) and *E. coli* LMM1010 (H) in the absence of additives (black), presence of 0.25 mM H_2O_2 (white) and presence of the full LP system (0.25 mM H_2O_2, 0.25 mM KSCN and 5 μg/ml LP) (grey). Error bars represent standard deviations (N_0 = initial viable cell numbers; N = viable cell numbers after treatment).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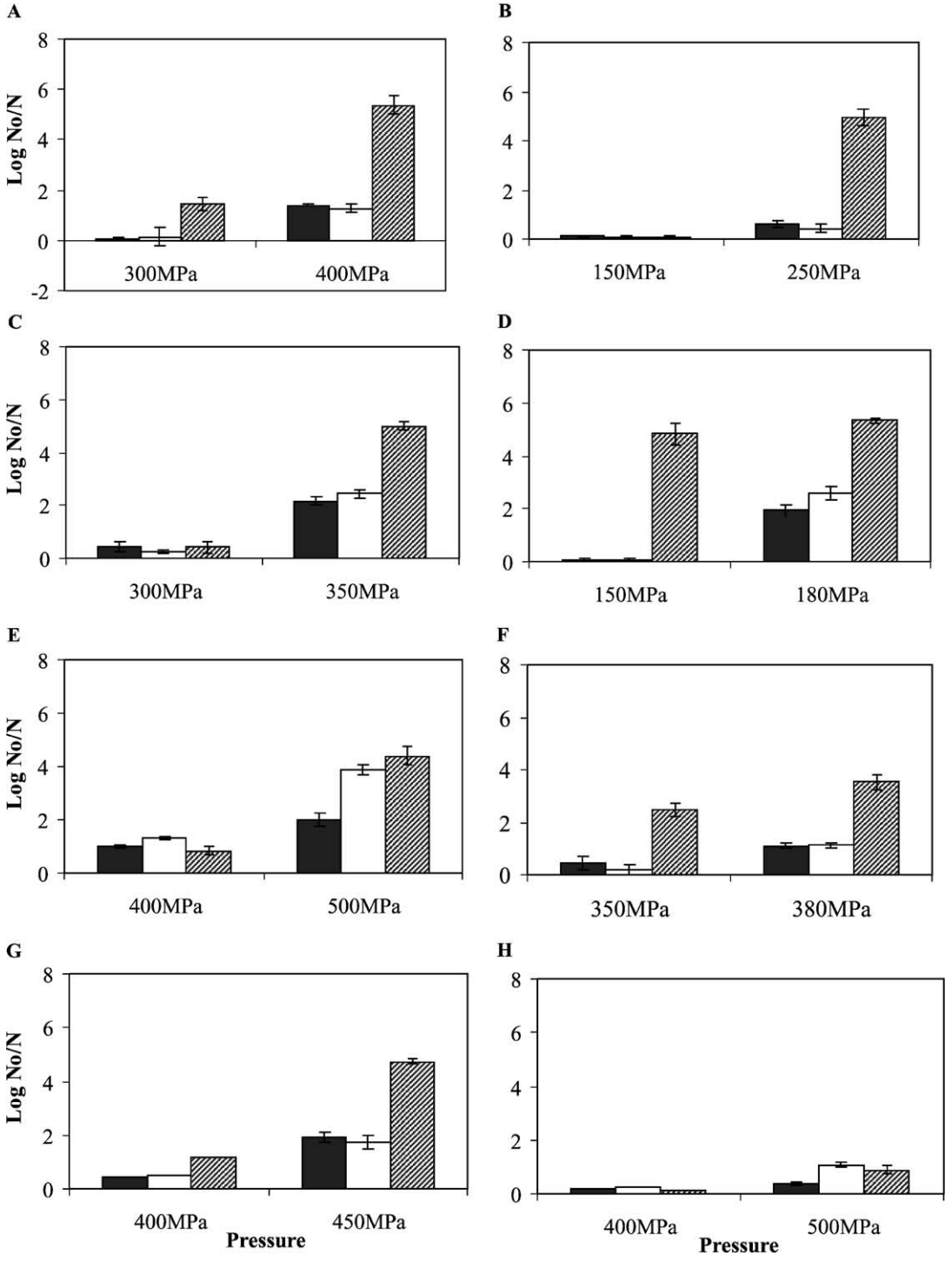
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vation levels by high pressure alone, except perhaps for *L. innocua*, which at 350 MPa showed 2 log cfu/ml higher inactivation at low cell density. Second and more important, some of the bacteria that were insensitive to the LP system under pressure at high cell density (*E. coli* MG1655, *S.* Typhimurium, *S. aureus* and *P. fluorescens*) became highly sensitive at low cell density. For instance, inactivation by the combined treatment of *E. coli* MG1655 was 5 log cfu/ml at 400 MPa when treated at low cell density, compared to only 2 log cfu/ml at 600 MPa at high cell density. For *P. fluorescens* and *S.* Typhimurium, a 5 log cfu/ml reduction was achieved by the combined treatment at, respectively, 150 and 250 MPa at low cell density, while at least 240 and 330 MPa were required for the same effect at high cell density. For *S. aureus*, the pressure required for a reduction of somewhat less than 5 log cfu/ml in the presence of the LP system was reduced from 600 to 500 MPa at low cell density. Remarkably, low density cell suspensions of *S. aureus* were also sensitized to H_2O_2. This was not the case for any of the other bacteria. Third, the pressure-resistant *E. coli* LMM1010 seems not to be sensitized to the LP system under pressure, even at low cell density. The level of inactivation achieved for this strain remains below 1 log cfu/ml, even at 500 MPa.

3.4. Bacterial sensitivity to the LP system after pressure treatment

A final issue concerns the fate of the surviving bacteria after combined treatment with high pressure and the LP system. Since the LP system retains full activity after pressure treatments up to 600 MPa at room temperature in milk (Garcia-Graells et al., 2000), it was of interest to determine whether the number of surviving bacteria will remain stable during an extended period of time after pressure treatment or will further decline. The former would be expected if the bacteria surviving the pressure treatment behave as a fresh inoculum of intact cells, the latter would be an indication of some form of sublethal injury caused by high pressure and causing sensitivity to the LP system. Because the previous experiments had failed to show any synergistic effect of high pressure and the LP system on *E. coli* LMM1010, we used only this strain and its parental strain MG1655 in the current experiment to investigate the existence of such a postprocess effect. Both strains at an inoculum density of 10^6 cfu/ml were subjected to pressure treatments of 300, 400 and 500 MPa, and the evolution of the bacterial counts during subsequent storage of the samples at room temperature was determined up to 24 h (Fig. 3). For MG1655, treatment of the samples supplemented with the LP system at 400 and 500 MPa resulted in almost complete inactivation, in agreement with the previous results in Fig. 2 (data not shown). Upon treatment at 300 MPa, the immediate inactivation was far less than 1 log cfu/ml. However, in the presence of the LP system, the bacterial counts decreased to below detection limit (20 cfu/ml) within 6 h after pressure treatment, and remained at that level after 24 h (Fig. 3A). For the pressure-resistant strain LMM1010, treatment at 300 MPa did not cause any immediate inactivation irrespective of the presence of the LP system, and also did not cause any postprocess inactivation. Nevertheless, there was some indication of sensitization because no growth of LMM1010 occurred within 24 h in this experiment, while without pressure treatment, LMM1010 can grow in the presence of the LP system (results not shown). At 400 MPa, although there was still no immediate inactivation, LMM1010 was more strongly sensitized and underwent complete inactivation within 24 h (Fig. 3C). At 500 MPa, some immediate inactivation could be detected, and subsequent inactivation was complete in 6 h, without any sign of regrowth within the 24-h measurement period. Also included in this experiment were bacterial samples containing only H_2O_2 and, although H_2O_2 is clearly less effective than the complete LP system, the results suggest that sufficiently high pressures can also sensitize strain LMM1010 to H_2O_2. Upon 500-MPa treatment, the

Fig. 2. High-pressure inactivation of moderate-density cell suspensions (10^6 cfu/ml) of *E. coli* MG1655 (A), *S.* Typhimurium (B), *L. innocua* (C), *P. fluorescens* (D), *S. aureus* (E), *L. plantarum* (F), *E. faecalis* (G) and *E. coli* LMM1010 (H) in the absence of additives (black), presence of 0.25 mM H_2O_2 (white) and presence of the full LP system (0.25 mM H_2O_2, 0.25 mM KSCN and 5 μg/ml LP) (grey). Error bars represent standard deviations.

strain seems to have lost its capacity to grow in the presence of H_2O_2 under the conditions of the experiment.

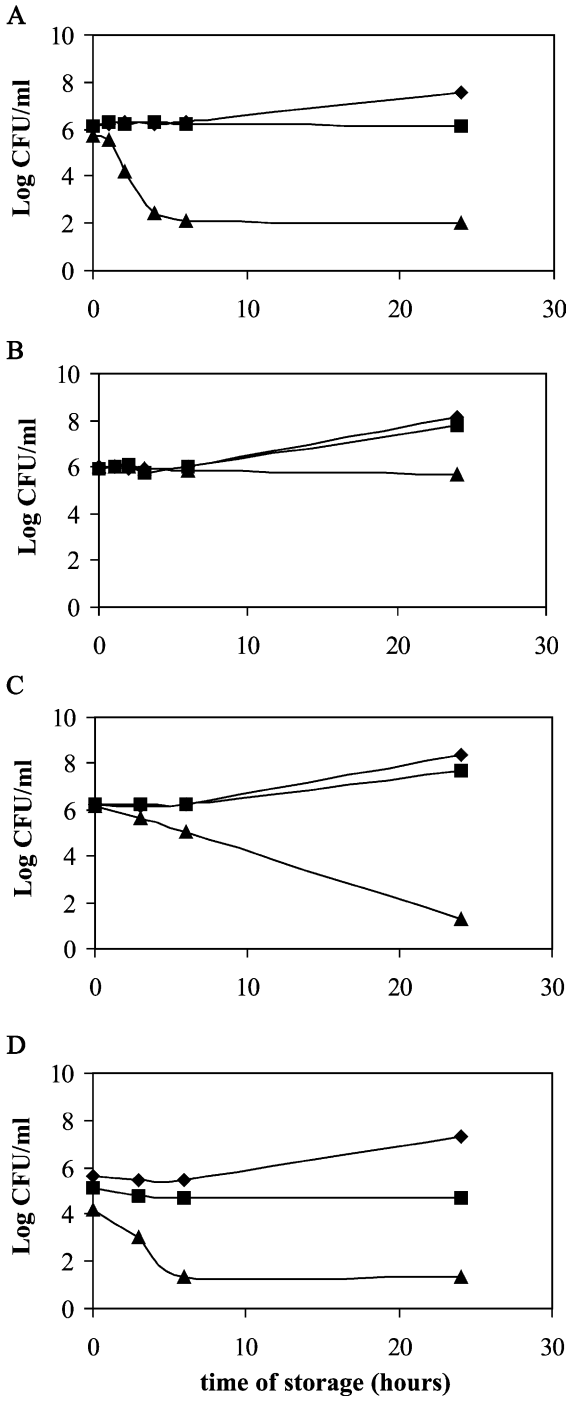

Fig. 3. Evolution of viable counts during storage at 20 °C after pressure treatment of *E. coli* wild-type MG1655 at 300 MPa (A), and the pressure-resistant strain LMM1010 at 300 (B), 400 (C) and 500 MPa (D) in milk without any additives (◆), supplemented with H_2O_2 (■) or supplemented with the complete LP system (▲).

4. Discussion

We have demonstrated in this work a strong cooperative effect of high-pressure treatment and the LP system on bacterial inactivation. This combined treatment has a high potential to be developed into a successful cold pasteurisation technique because it allows efficient inactivation of a wide range (thus far without limitation) of vegetative bacteria in a complex food matrix under mild process conditions. These features make this combination superior to high-pressure treatment alone and to other combinations of high pressure with antimicrobials that have been previously described.

In a previous study on the inactivation of *E. coli* and *L. innocua* by combined treatment with HP and the lactoperoxidase (LP) system, we found a synergistic effect only for the latter species but not for *E. coli* (Garcia-Graells et al., 2000). However, in that study, synergy was only evaluated at high cell densities (10^9 cfu/ml). Such high cell densities are commonly used in bacterial inactivation studies to allow for determination of up to seven decimal reductions, but normally do not occur in food products to be subjected to a preservation process. In the current work, we extended our observations to a variety of other food related bacteria and we demonstrated that the combined treatment effectively inactivated all the species tested, including *E. coli*, at moderate cell concentration (10^6 cfu/ml), more likely to be encountered in foods to be treated.

In a first experiment, we compared the sensitivity of the bacteria in our test panel to the LP system in absence of a pressure treatment. We observed wide differences in LP sensitivity among the test bacteria but, contrary to some reports in the literature (Björck et al., 1975; Marshall and Reiter, 1980; Reiter et al., 1976; Thomas et al., 1983; Siragusa and Johnson, 1989), we did not observe a systematic difference in LP sensitivity between Gram-negative and Gram-positive bacteria. Both the most sensitive (*P. fluorescens*) and the most resistant organisms (*S.* Typhimu-

rium and *E. coli* LMM1010) in our experiment are Gram-negative. An interesting observation is the increased resistance of *E. coli* LMM1010 compared to its parent strain MG1655. LMM1010 was isolated as a pressure-resistant derivative of MG1655 (Hauben et al., 1997) but was subsequently shown to have also increased resistance to acid and oxidative stress, and to have derepressed synthesis of a number of stress related proteins (Hauben et al., 1999). The current finding that LMM1010 is more resistant to hydrogen peroxide and to the LP system is in line with these previous findings.

In the next set of experiments, we compared inactivation of the test bacteria by high pressure in the presence of the LP system, both at high (10^9 cfu/ml) and at low (10^6 cfu/ml) cell density. Some studies have indeed indicated that the LP system is more efficient against cells at low than at high concentrations (Thomas et al., 1983; Farrag et al., 1992; Wolfson and Sumner, 1994), indicating that it is not the concentration of hypothiocyanite produced by the LP system per se that determines effectiveness, but the concentration of hypothiocyanite per target cell. On the other hand, the situation in the current study is different because the target cells are also treated with high pressure, a treatment that may increase their sensitivity for the LP system. Therefore, we considered it interesting to use both high and low cell concentrations in these experiments. The results (Figs. 2 and 3) show that sensitization to the LP system can indeed be achieved for all except one of the test bacteria at low cell concentration, and for three of the test bacteria also at high cell concentration. Remarkably, *P. fluorescens*, the most LP-sensitive organism in absence of a pressure treatment, could only be sensitized at low cell concentration. The only organism that was not sensitized even at low cell concentration is *E. coli* LMM1010, a finding that further illustrates the multiple stress-resistant phenotype of this strain. Another observation relates to the threshold pressure that is required for sensitization to the LP system. Although our data do not allow precise estimation of the threshold level for all bacteria, it is clear that this threshold pressure is independent of a certain inactivation threshold by high pressure alone. For instance, in Fig. 3, *S. aureus* at 400 MPa was reduced 1 log cfu/ml but was not sensitized to the LP system, while *P. fluorescens* at 150 MPa was strongly sensitized to the LP system but was virtually not inactivated by pressure alone.

Once we had demonstrated that high pressure can sensitize bacteria for the LP system when both stressors are applied simultaneously, we addressed the question of whether cells remain sensitive for the LP system after pressure treatment. In a previous work, we have demonstrated that high-pressure sensitization of bacteria for lysozyme and nisin is transient, i.e. that the cells regain their natural resistance level immediately after pressure release (Hauben et al., 1996; Garcia-Graells et al., 1999; Masschalck et al., 2000). To investigate whether sensitization for the LP system, contrary to sensitization for lysozyme and nisin, would sustain for a certain time after pressure treatment, we chose *E. coli* strains MG1655 and LMM1010. For the latter strain in particular, this experiment was highly relevant because this was the only strain for which we failed to demonstrate sensitization during or immediately after pressure treatment. The results show that sensitization for the LP system of strain LMM1010 does take place but, depending on the pressure level that is applied, becomes only detectable a few hours after pressure treatment. The levels of inactivation that are reached after 24 h, however, are considerable (>5 log units). It can be seen that LMM1010 requires a higher pressure than MG1655 to become sensitized for the LP system. Together with the finding that LMM1010 is more resistant to the LP system than MG1655 (Fig. 1), these data suggest that pressure resistance is somehow linked to LP resistance, or maybe more generally, to oxidative stress resistance.

In conclusion, high-pressure treatment and the LP system cause a strongly synergistic inactivation of a wide range of Gram-negative and Gram-positive bacteria, and the combined application of both treatments, therefore, provides an attractive basis for the development of microbial inactivation treatments such as food pasteurisation. Compared to previously reported combinations with lysozyme, nisin and other bacteriocins, the combination with the LP system is more effective, allowing up to at least 5 log units extra reduction compared to pressure treatment alone, and is effective on a wider range of bacteria, in fact on all bacteria tested so far. Although more extensive validation is necessary, the combined pressure–LP system treatment promises to be effective against pressure-resist-

ant bacteria and in the complex environment of real foods, and thereby overcomes some of the major limitations of the use of high pressure alone. The combination of pressure with the LP system is also economically attractive because it allows the use of lower pressures.

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