

# Relationship between Sublethal Injury and Microbial Inactivation by the Combination of High Hydrostatic Pressure and Citral or *tert*-Butyl Hydroquinone<sup>∇</sup>

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**The aim was to investigate (i) the occurrence of sublethal injury in *Listeria monocytogenes*, *Escherichia coli*, and *Saccharomyces cerevisiae* after high hydrostatic pressure (HHP) treatment as a function of the treatment medium pH and composition and (ii) the relationship between the occurrence of sublethal injury and the inactivating effect of a combination of HHP and two antimicrobial compounds, *tert*-butyl hydroquinone (TBHQ) and citral. The three microorganisms showed a high proportion of sublethally injured cells (up to 99.99% of the surviving population) after HHP. In *E. coli* and *L. monocytogenes*, the extent of inactivation and sublethal injury depended on the pH and the composition of the treatment medium, whereas in *S. cerevisiae*, inactivation and sublethal injury were independent of medium pH or composition under the conditions tested. TBHQ alone was not lethal to *E. coli* or *L. monocytogenes* but acted synergistically with HHP and 24-h refrigeration, resulting in a viability decrease of >5 log<sub>10</sub> cycles of both organisms. The antimicrobial effect of citral depended on the microorganism and the treatment medium pH. Acting alone for 24 h under refrigeration, 1,000 ppm of citral caused a reduction of 5 log<sub>10</sub> cycles of *E. coli* at pH 7.0 and almost 3 log<sub>10</sub> cycles of *L. monocytogenes* at pH 4.0. The combination of citral and HHP also showed a synergistic effect. Our results have confirmed that the detection of sublethal injury after HHP may contribute to the identification of those treatment conditions under which HHP may act synergistically with other preserving processes.**

High hydrostatic pressure (HHP) has attracted much interest as an alternative to heat for food preservative technology. This nonthermal processing technique is an effective method for increasing food safety and shelf life while preserving the organoleptic properties of food products (19, 23, 48).

Loss of cytoplasmic membrane integrity is believed to be one of the critical events leading to the death of pressure-treated microorganisms (38, 59). Several authors have inferred injury to the cytoplasmic membrane after pressure treatment based on loss of intracellular material, loss of osmotic responsiveness, or uptake of vital dyes (2, 4, 5, 38, 49, 56). However, partial loss of cytoplasmic membrane functionality or damage to the gram-negative outer membrane may not necessarily lead to cell death, but surviving cells may demonstrate enhanced sensitivity to inhibitors such as sodium chloride, bile salts, and ingredients of selective media (26). Many published reports (1, 7, 20, 33, 44, 46) have demonstrated the occurrence of sublethal injury after HHP treatment using a differential plating technique.

Because membrane damage caused by pressurization may enhance sensitivity to antimicrobial agents, it could allow the design of combined processes that increase the effectiveness of pressure processing (1, 18, 31). Published data have demonstrated that the application of specific additional hurdles such as antimicrobial peptides, the lactoperoxidase system, and phenolic compounds in combination with HHP treatments have

synergistic inactivation effects (8, 16, 17, 18, 21, 22, 30, 31, 34, 55, 58). One of the most effective phenolic compounds is *tert*-butyl hydroquinone (TBHQ), which sensitizes some barotolerant *Listeria monocytogenes* strains to HHP (8, 58).

Nowadays, people demand more healthy foods. In this vein, natural sources of antimicrobial compounds, such as plant essential oils, have been evaluated as substitutes for chemical preservatives (11). Citral is one of the essential oil compounds for which antimicrobial action against some important pathogenic microorganisms (*Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica*, and *Staphylococcus aureus*) has been demonstrated (11, 36). Citral is an acyclic unsaturated monoterpene aldehyde found naturally in the volatile oils of citrus fruits, lemongrass, and other herbs and spices. It consists of a mixture of two isomers, geranial and neral, and is used for flavoring citrus-based beverages. Its antimicrobial properties and pleasant fruity scent could make citral a suitable antimicrobial ingredient for wider use in the food industry.

The aim of this work was to investigate the occurrence of sublethal injury after HHP treatment as a function of the treatment medium pH and composition in a gram-positive (*Listeria monocytogenes*) and a gram-negative (*Escherichia coli*) bacterial species and a yeast (*Saccharomyces cerevisiae*). Also, this work investigated the relation of sublethal injury to the inactivating effect of the combination of pressure treatments and two antimicrobials, TBHQ and citral.

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## MATERIALS AND METHODS

**Microorganisms and growth conditions.** *E. coli* J1, an acid-resistant commensal strain, was kindly provided by Ian Booth (University of Aberdeen, Aberdeen,

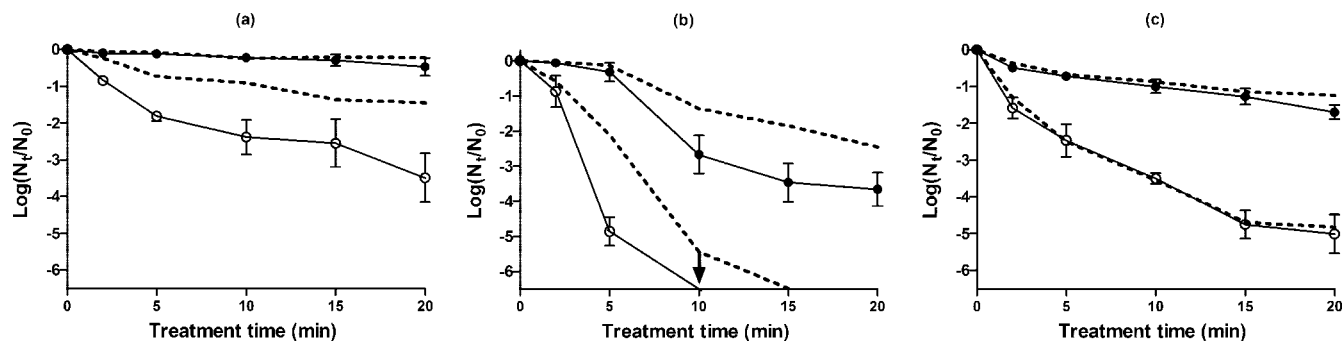


FIG. 1. Survival fractions of *E. coli* at 400 MPa (a), *L. monocytogenes* at 300 MPa (b), and *S. cerevisiae* at 200 MPa (c) treated in PBS (solid line) and in McIlvaine's buffer at pH 7.0 (dashed line). Survivors recovered on the nonselective (filled circles) and selective (open circles) media are shown. Results are means of three observations  $\pm$  standard deviations (error bars).

United Kingdom). The strains of *L. monocytogenes* NCTC 11994 and *S. cerevisiae* STCC 11034 were supplied by culture collections. The strains were maintained in frozen cultures at  $-80^{\circ}\text{C}$ . Bacterial broth subcultures were prepared by inoculating a test tube containing 10 ml of tryptone soya broth (TSB; Oxoid, Basingstoke, United Kingdom) with a single colony from a plate and incubating the resulting culture at  $37^{\circ}\text{C}$  for 6 h in a shaking incubator. For *S. cerevisiae*, Sabouraud broth (Oxoid) was used instead of TSB and tubes were incubated at  $30^{\circ}\text{C}$  for 24 h. Next, 250-ml Erlenmeyer flasks containing 50 ml of TSB or Sabouraud broth (in the case of *S. cerevisiae*) were inoculated with 0.1 ml of culture. The flasks were incubated with agitation at 130 rpm at  $37^{\circ}\text{C}$  (*E. coli* and *L. monocytogenes*) or  $30^{\circ}\text{C}$  (*S. cerevisiae*) until the stationary growth phase was reached.

**HHP treatment.** Cells were centrifuged at  $3,000 \times g$  for 20 min at  $4^{\circ}\text{C}$ , and pellets were resuspended in different treatment media, phosphate-buffered saline (PBS, pH 7.2; Oxoid) or citrate-phosphate buffer (McIlvaine's buffer), at either pH 7.0 or pH 4.0. Where indicated, 100 ppm of TBHQ or 1,000 ppm of citral was added to the buffer. These concentrations were chosen from those previously described in published literature (8, 11, 58). Cell suspensions (2 ml each) were then placed in sterile plastic pouches that were heat sealed and kept on ice before pressurization. Samples were pressure treated in a 300-ml pressure vessel (model S-FL-850-9-W; Stansted Fluid Power, Stansted, United Kingdom) at  $18^{\circ}\text{C}$ . The pressure-transmitting fluid was monopropylene glycol-water (30:70). Cells were exposed to pressures from 200 to 400 MPa for different times (0.5 to 20 min). The maximum temperature reached during pressurization was  $28^{\circ}\text{C}$ . After decompression, the pouches were removed from the unit and placed on ice until viable counts were evaluated.

**Measurement of sensitivity to subsequent holding in the HHP treatment medium.** High-pressure-treated cells were held in the treatment medium (McIlvaine's buffer at pH 7.0 and pH 4.0, with or without 100 ppm of TBHQ or 1,000 ppm of citral) at  $5^{\circ}\text{C}$  for 24 h. For a comparison, untreated native cells were also held under the same conditions. Samples were taken at preset intervals, and survivors were evaluated.

**Counts of viable cells.** Samples were adequately diluted in maximum recovery diluent (Oxoid), and 0.02 ml was spread on tryptone soya agar supplemented with 0.3% yeast extract (TSA-YE) (Oxoid) in the case of *E. coli* and *L. monocytogenes* or on potato dextrose agar (Oxoid) for *S. cerevisiae* (PDA). Both media were supplemented with 0.1% sodium pyruvate. Plates were incubated at  $37^{\circ}\text{C}$  for 24 h (*E. coli* and *L. monocytogenes*) or  $30^{\circ}\text{C}$  for 48 h (*S. cerevisiae*). Previous experiments showed that longer incubation times did not influence the amount of surviving cells. The error bars in the figures indicate the standard deviations of the means for data obtained from at least three independent experiments.

**Detection of sublethal injury.** In order to determine microbial cell injury, treated samples were also plated onto TSA-YE containing 2.5% NaCl (*E. coli*) or 5% NaCl (*L. monocytogenes*) or onto PDA with 7% NaCl (*S. cerevisiae*) (TSAYE-SC and PDA-SC; Fisher Scientific, Loughborough, United Kingdom). These were the maximum concentrations of sodium chloride that caused no reduction in colony counts of unstressed cells. Plates containing selective medium were incubated for 24 h more than those containing nonselective medium. Previous experiments showed that longer incubation times did not influence survival counts.

The extent of sublethal injury in a population of pressure-treated cells was expressed as the difference between the log count (CFU) on nonselective medium (TSA-YE or PDA) and the log count on selective medium (TSAYE-SC or PDA-SC). According to this representation, "2 logs of injury" means a 2-log

difference in the count on selective and nonselective media or that 99% of survivors were sublethally injured.

## RESULTS

**Effect of the treatment medium composition on microbial inactivation and on the occurrence of sublethal injury after HHP treatments.** In previous work investigating the effect of pH on sublethal injury caused by nonthermal preservation treatments, McIlvaine's citrate-phosphate buffer was used as a suspending medium because of its wide buffering range (14, 37). However, because PBS has often been used in high-pressure work (28, 38, 39), the effect of these two buffers on survival and sublethal injury was examined.

Figure 1 shows the influence of buffer composition on pressure resistance and sublethal injury in the three microorganisms. While the HHP resistance of *E. coli* and *S. cerevisiae* was unaffected ( $P > 0.05$ ) by composition of the treatment medium, the degree of inactivation of *L. monocytogenes* cells was higher in PBS than in McIlvaine's buffer ( $>1 \log_{10}$  cycle). HHP treatment in PBS caused an increase in the extent of sublethal injury in surviving populations compared with that in McIlvaine's buffer in *E. coli* and *L. monocytogenes* but not in *S. cerevisiae*. Regarding *S. cerevisiae*, there was no statistically significant difference ( $P > 0.05$ ) in cell inactivation or sublethal injury between the two buffers. Since in the case of *E. coli* and *L. monocytogenes* McIlvaine's buffer was more protective than PBS, the former was chosen for use in subsequent experiments.

**Occurrence of sublethal injury in different microorganisms after HHP treatment: effect of the pH of the treatment medium.** Figure 2 shows the surviving fractions of *E. coli*, *L. monocytogenes*, and *S. cerevisiae* cells in McIlvaine's buffer of pH 7.0 or 4.0 after HHP treatments at 400 MPa, 300 MPa, or 200 MPa, respectively, with recovery on nonselective and selective media. These treatment conditions were selected from preliminary work (data not shown) to identify pressure intensities and treatment times that would yield reasonably similar degrees of inactivation and injury in the three test organisms. The screening showed that both bacteria were more pressure resistant than *S. cerevisiae* and that *E. coli* J1 was more resistant than *L. monocytogenes* NCTC 11994.

By comparing data obtained with the nonselective medium, *E. coli* and *L. monocytogenes* were more resistant at pH 7.0 than at pH 4.0. On the contrary, in the case of *S. cerevisiae*, no

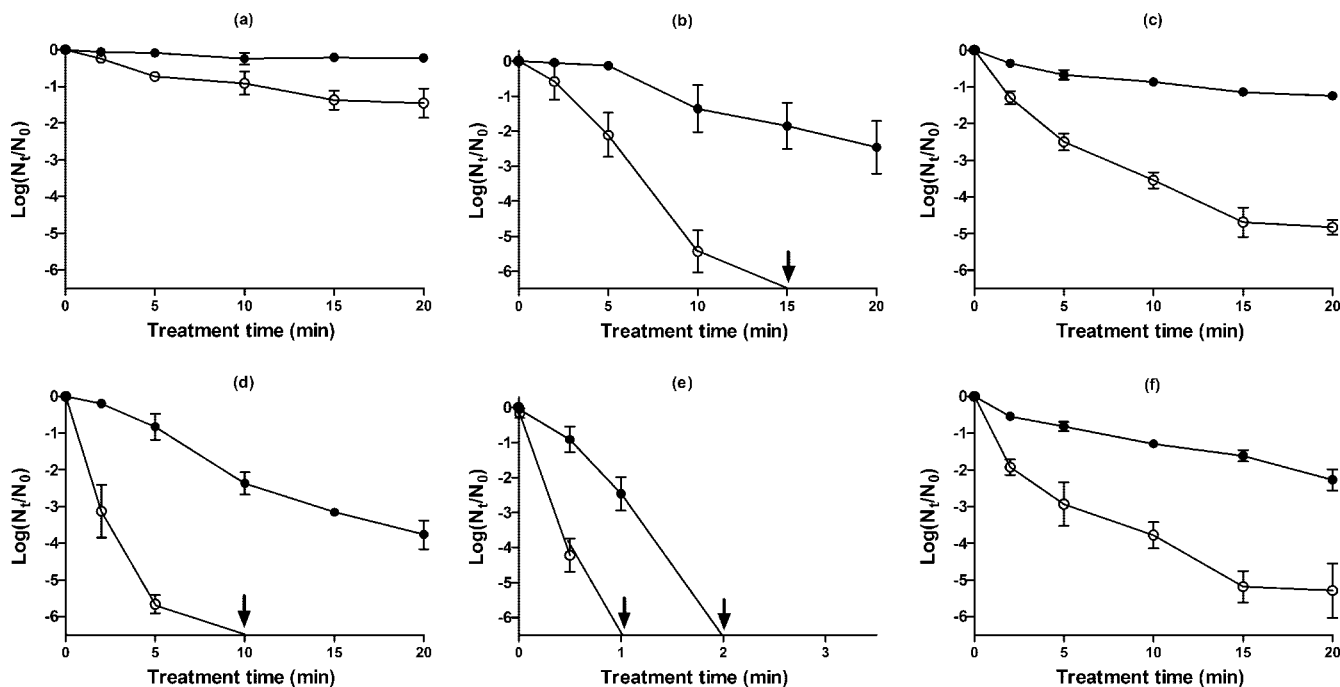


FIG. 2. Shown are survival fractions of *E. coli* at 400 MPa (a and d), *L. monocytogenes* at 300 MPa (b and e), and *S. cerevisiae* at 200 MPa (c and f) treated in McIlvaine's buffer at pH 7.0 (a, b, and c) and pH 4.0 (d, e, and f). Survivors recovered on the nonselective (filled circles) and the selective (open circles) media are shown. Results are means of three observations  $\pm$  standard deviations (error bars). Arrows indicate that the viable count was below the limit of detection (500 CFU/ml).

statistically significant difference ( $P < 0.05$ ) was observed between survival curves at either pH value. *Listeria monocytogenes* showed the greatest extent of sensitization at pH 4.0, where  $>6 \log_{10}$  cycles of inactivation were achieved after 3 min at 300 MPa compared with  $<3 \log_{10}$  cycles when the bacterium was pressurized at pH 7.0 for 20 min.

A high proportion of sublethally injured cells was seen after all treatments except with *E. coli* at pH 7.0. The maximum proportion of sublethally injured cells ( $>99.99\%$  of the survivors, equivalent to a 4-log difference in count on selective and nonselective media) was observed when *L. monocytogenes* was pressurized at pH 7.0 for 15 min at 300 MPa or when *E. coli* and *L. monocytogenes* were pressurized at pH 4.0 for 5 and 0.5 min at 400 and 300 MPa, respectively. In general, the extent of injury remained constant or slightly decreased during longer treatment times. In the case of *S. cerevisiae*, the extent of injury did not vary as a function of the treatment medium pH. The effect of pressure combined with TBHQ or citral at different pH values was investigated for the two bacterial species, which were more pressure resistant than the yeast.

**Microbial inactivation by TBHQ or citral.** Native cells of *E. coli* and *L. monocytogenes* at a concentration of  $10^9$  CFU/ml were insensitive to incubation in McIlvaine's buffer at pH 7.0 or pH 4.0 for 24 h at 5°C (Tables 1 and 2). The populations of both organisms remained constant ( $P > 0.05$ ) on both nonselective and selective recovery media. The addition of 100 ppm TBHQ did not cause inactivation or injury to *E. coli* cells at either pH (Table 1). Regarding *L. monocytogenes*, the presence of 100 ppm TBHQ also caused no inactivation but resulted in  $>1 \log_{10}$  cycle of injury when suspended at a pH of 7.0 and

approximately 0.4  $\log_{10}$  cycles of injury when suspended at a pH of 4.0.

During refrigerated holding in the presence of 1,000 ppm citral, no inactivation or sublethal injury was detected in *E. coli* at pH 4.0, but viable numbers of bacteria that were decreased by almost 5  $\log_{10}$  cell cycles and more than one extra  $\log_{10}$  cycle were injured at pH 7.0. Under the same experimental conditions, the behavior of native cells of *L. monocytogenes* was the opposite, with inactivation and sublethal injury being greater at pH 4.0 than at pH 7.0.

**Microbial inactivation by combining TBHQ or citral and HHP.** Cells were pressure treated in McIlvaine's buffer at pH 4.0 or pH 7.0, either alone or in combination with 100 ppm of TBHQ or 1,000 ppm of citral. *Escherichia coli* cells were treated at 400 MPa for 5 min (pH 4.0) or 10 min (pH 7.0). *Listeria monocytogenes* cells were treated at 300 MPa for 0.5 min (pH 4.0) or 10 min (pH 7.0). These pressure treatment conditions were chosen from the preliminary studies because of their capacity to cause the maximum proportion of sublethally injured cells among survivors.

Figure 3 shows the number of  $\log_{10}$  cycles of inactivation of *E. coli* cells suspended in McIlvaine's buffer at pH 4.0, or in buffer containing TBHQ or citral, after HHP and recovery on nonselective and selective media. Survivors were evaluated immediately after pressure treatment (Fig. 3a) and after 24-h storage at 5°C (Fig. 3b). Treatment in buffer alone resulted in a reduction in viable numbers of slightly  $<1 \log_{10}$  cell cycle and a further 4  $\log_{10}$  cell cycles of sublethally injured cells. The presence of TBHQ or citral during the treatment caused a further decrease in viability of between 0.25 and 1  $\log$  cycle,

TABLE 1. Log<sub>10</sub> cycles of inactivation of *E. coli* after HHP treatment at 400 MPa for 5 min (at pH 4.0) and for 10 min (at pH 7.0) at time zero and after a storage of 24 h at refrigeration temperatures<sup>a</sup>

Type of cell	pH	Medium	Mean (± SD) at:					
			0 h			24 h		
			McIlvaine's buffer	TBHQ 100 ppm	Citral 1,000 ppm	McIlvaine's buffer	TBHQ 100 ppm	Citral 1,000 ppm
Native	7.0	NS	NE	NE	NE	NE	NE	4.91 (0.18)
		S	NE	NE	NE	NE	NE	>6.3
	4.0	NS	NE	NE	NE	NE	NE	NE
		S	NE	NE	NE	NE	NE	NE
Pressurized	7.0	NS	0.08 (0.06)	1.15 (0.26)	0.30 (0.26)	0.01 (0.09)	>6.3	6.06 (0.71)
		S	0.96 (0.37)	2.58 (0.31)	4.17 (0.43)	0.55 (0.35)	>6.3	>6.3
	4.0	NS	0.75 (0.15)	0.96 (0.10)	1.60 (0.16)	0.79 (0.08)	>6.3	5.47 (0.66)
		S	4.93 (0.28)	>6.3	>6.3	5.93 (0.17)	>6.3	>6.3

<sup>a</sup> NS, nonselective medium; S, selective medium; NE, no effect was found at this condition.

respectively, while counts on the selective medium were below the detection limit, indicating >4 to 5 logs of injury.

During subsequent holding of pressure-treated *E. coli* cells in McIlvaine's buffer at pH 4.0, there was no reduction in viable numbers, but the number of injured cells increased by 1 log<sub>10</sub> cycle. However, holding for 24 h in the presence of TBHQ or citral caused the inactivation of >5 log<sub>10</sub> cycles. The survival counts obtained on selective and nonselective recovery media were very similar, indicating that most HHP-damaged cells were sensitive to the presence of TBHQ or citral. The final degree of inactivation of *E. coli* cells due to the combined process (approximately 6 log<sub>10</sub> cycles) was much higher than that obtained by adding the effects of HHP treatment and incubation with TBHQ or citral acting separately (<1 log<sub>10</sub> cycle). Therefore, at pH 4.0, treatment with citral or TBHQ and HHP was synergistic when these antimicrobials were simultaneously applied.

At pH 7.0, *E. coli* cells showed considerable HHP resistance when suspended in McIlvaine's buffer without any antimicro-

bial added (Table 1). The presence of TBHQ or citral increased both inactivation and injury when assessed immediately after HHP treatment, particularly with citral, which resulted in 4 log<sub>10</sub> cycles of injury. Again, the final degree of inactivation of *E. coli* cells at a pH of 7.0, due to the combined process of pressure plus TBHQ (6 log<sub>10</sub> cycles), was much higher after holding for 24 h than that obtained by adding together the separate effects of HHP treatment and incubation with TBHQ (<1 log<sub>10</sub> cycle), showing a synergistic effect. The combined pressure treatment with citral did not exert a significant synergistic effect, because citral acting alone affected the whole population of native cells after 24 h of refrigeration (Table 1).

When *L. monocytogenes* was pressure treated at a pH of 4.0, the extent of sublethal injury assessed immediately after HHP treatment was increased by the presence of TBHQ or citral (Table 2; Fig. 4a). Pressure-treated cells of *L. monocytogenes* were sensitive to subsequent incubation for 24 h, even in the absence of added antimicrobials: a further reduction of 2 log<sub>10</sub>

TABLE 2. Log<sub>10</sub> cycles of inactivation of *L. monocytogenes* after an HHP treatment of 300 MPa for 0.5 min (at pH 4.0) and for 10 min (at pH 7.0) at time zero and after a storage of 24 h at refrigeration temperatures<sup>a</sup>

Type of cell	pH	Medium	Mean (±SD) at:					
			0 h			24 h		
			McIlvaine's buffer	TBHQ (100 ppm)	Citral (1,000 ppm)	McIlvaine's buffer	TBHQ (100 ppm)	Citral (1,000 ppm)
Native	7.0	NS	NE	NE	NE	NE	NE	0.74 (1.04)
		S	NE	NE	NE	NE	1.19 (0.88)	0.95 (0.26)
	4.0	NS	NE	NE	NE	NE	NE	2.67 (0.88)
		S	NE	NE	NE	NE	0.41 (0.30)	4.53 (0.80)
Pressurized	7.0	NS	1.03 (0.38)	>6.3	1.75 (0.67)	0.80 (0.19)	>6.3	3.31 (0.23)
		S	6.26 (0.74)	>6.3	>6.3	4.43 (0.58)	>6.3	>6.3
	4.0	NS	0.56 (0.16)	1.11 (0.40)	1.15 (0.49)	3.46 (1.01)	>6.3	4.92 (0.51)
		S	3.01 (0.60)	>6.3	4.89 (0.62)	>6.3	>6.3	>6.3

<sup>a</sup> NS, nonselective medium; S, selective medium; NE, no effect was found at this condition.



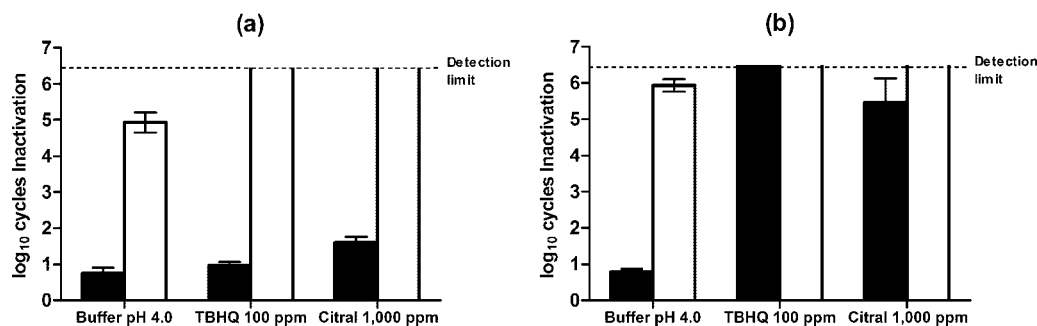


FIG. 3. Log<sub>10</sub> of cycles of inactivation of *E. coli* after a HHP treatment at 400 MPa for 5 min in McIlvaine's buffer at pH 4.0 or in the same buffer with 100 ppm of TBHQ or with 1,000 ppm of citral added at time zero (a) and after a storage of 24 h (b) at refrigeration temperatures. Survivors recovered on the nonselective (black bars) and selective (white bars) media are shown. Results are means of three observations  $\pm$  standard deviations (error bars).

cell cycles occurred during holding at a pH of 4.0 for 24 h with three extra log<sub>10</sub> cell cycles of injury. Incubation of HHP-treated cells for 24 h in the presence of TBHQ caused the inactivation of most sublethally HHP-injured cells, achieving >6 log<sub>10</sub> cycles of inactivation (Fig. 4b). Therefore, at a pH of 4.0, the combination of TBHQ and HHP treatment also acted synergistically when simultaneously applied to *L. monocytogenes* cells suspended at a pH of 4.0. Sublethally injured cells also died in the presence of citral during the 24-h incubation at 5°C, achieving approximately 5 log<sub>10</sub> cycles of inactivation. Nevertheless, the final degree of inactivation was similar to that obtained by the incubation for 24 h in the presence of citral and the HHP treatment acting separately, and thus, the effect was additive rather than synergistic.

Inactivation of *L. monocytogenes* cells at pH 7.0 showed different behavior (Table 2). The combination of HHP and TBHQ showed a very large lethal effect such that the whole cell population was inactivated. A comparison of survival after combined and individual treatments showed this to be a synergistic effect. In contrast, the presence of citral scarcely increased the efficacy of the HHP treatment. After 24 h of incubation at pH 7.0 in the absence of additives, pressure-treated cells showed no decrease in viability, while some repair of injury occurred as shown by a 2-log decrease in the difference between counts on selective and nonselective media. Since the whole cell population had been inactivated by the presence of TBHQ during the HHP treatment, it was not

possible to assess whether the subsequent incubation with TBHQ caused any extra inactivation. For cells pressure treated with citral, incubation for 24 h at pH 7.0 caused only a small further reduction in viability of about 3 log<sub>10</sub> cycles, but the extent of injury remained large.

## DISCUSSION

*Saccharomyces cerevisiae* was less pressure resistant than either *E. coli* or *L. monocytogenes*, in line with the general observation that vegetative eukaryotic cells are less resistant to HHP treatments than prokaryotic microorganisms (19, 51). Both bacterial species were more sensitive to pressure at acidic pHs, as expected, but there was no difference in the resistance of *S. cerevisiae* when the yeast was pressurized at pH 7.0 or pH 4.0. This might be regarded as unexpected, though survival of *S. cerevisiae* during pulsed electric field (PEF) treatment was similarly unaffected by pH (54). The extent of sublethal injury after HHP treatment was also greater at pH 4.0 than at pH 7.0 in *E. coli* and *L. monocytogenes*, whereas in *S. cerevisiae*, injury did not depend on the treatment medium pH. As with pressure, the occurrence of sublethal injury in yeast after PEF has been shown to be independent of the treatment medium pH (54). The similarity in the effect of pH on inactivation and injury by HHP and PEF may perhaps be related to the fact that the cytoplasmic membrane is a major target for damage in both processes (40).

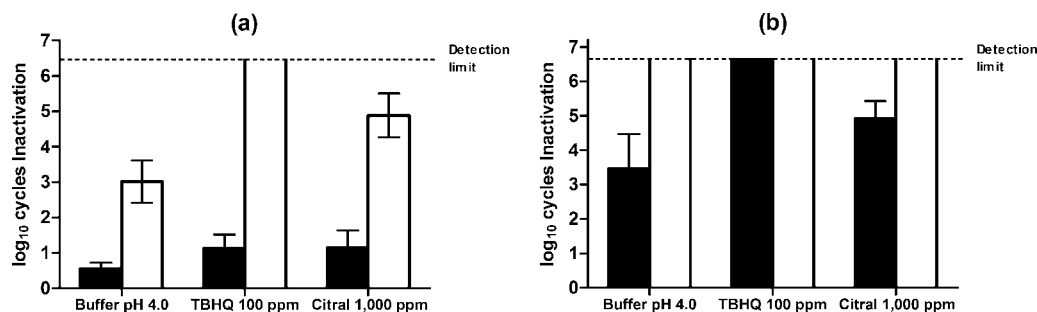


FIG. 4. Log<sub>10</sub> cycles of inactivation of *L. monocytogenes* after an HHP treatment at 300 MPa for 0.5 min in McIlvaine's buffer at pH 4.0 or in the same buffer with 100 ppm of TBHQ or 1,000 ppm of citral added at time zero (a) and after a storage of 24 h (b) at refrigeration temperatures. Survivors recovered on the nonselective (black bars) and selective (white bars) media are shown. Results are means of three observations  $\pm$  standard deviations (error bars).

Many others (1, 7, 20, 33, 44, 46) have demonstrated the occurrence of sublethally injured bacterial cells after HHP treatment using a selective medium plating technique, but to the best of our knowledge, only Pandya et al. (42) have demonstrated a kind of pressure-induced injury of yeast by plate count differential between PDA and PDA supplemented with glucose. Sublethal injury measured in this study by using NaCl as a selective compound in the recovery medium is presumably a consequence of the loss of osmoregulatory functions associated with the cytoplasmic membrane (35). The detection of sublethally injured cells after HHP treatment confirms that microbial inactivation by HHP is not an all-or-nothing event and that restoration of membrane function is an important event either in bacterial or yeast recovery after HHP.

The two bacterial species showed a higher HHP resistance and less sublethal injury in McIlvaine's buffer than in PBS, whereas *S. cerevisiae* cells showed the same pressure resistance in both. This suggests that the critical targets for pressure inactivation in bacteria and yeasts are affected differently by environmental factors (such as medium composition and pH). The reason for the greater bacterial sensitivity in PBS is not known but might be related to the presence of NaCl in the PBS but not in McIlvaine's buffer. The concentration in PBS (8.0 g/liter), though not high, might be sufficient to sensitize cells if present during the pressure treatment (20). Alternatively, the higher concentration of phosphate in McIlvaine's buffer (0.18 M) than in PBS buffer (0.01 M) might have a protective effect. It is well known that pressure induces changes in the pH of some buffers, but the effect of this on survival and the specific effects of buffer composition on survival have seldom been studied (32).

As noted by Hauben et al. (18), the detection of sublethal injury may help clarify the environmental circumstances under which other processes for food preservation in combination with HHP treatments may increase their bactericidal efficiency. The effect of combined treatments was studied further in the two bacterial species, which were more resistant to HHP than yeast.

Synergistic inactivation effects have been described when HHP treatments were combined with low pHs (15, 25) or with different antimicrobial substances, such as lysozyme (16, 18, 30), pediocin (17, 21, 22), nisin (8, 17, 18, 21, 30, 55), and lactacin (34). Several workers have reported the lethal effect of HHP in combination with phenolic antioxidants, such as TBHQ or butylated hydroxyanisole (8, 27, 58), or with plant essential oils, such as citral (41).

Many food-grade phenolic antioxidants, including TBHQ, have antimicrobial properties (13, 45, 60). TBHQ and HHP treatment also act synergistically when simultaneously applied to *L. monocytogenes* at pH 7.0 (8, 58). We found that 100 ppm of TBHQ acting alone at pH 7.0 did not affect the survival of *L. monocytogenes*, in agreement with Vurma et al. (58), but caused extensive sublethal injury following refrigerated storage for 24 h. This sensitizing effect might explain the efficacy of the combined treatment of HHP and TBHQ in *L. monocytogenes*, which allowed the inactivation of  $>6 \log_{10}$  cycles at pH 7.0. Conversely, *E. coli* cells were not sensitized by the presence of TBHQ alone, but a synergistic killing effect of HHP and TBHQ was observed after incubation for 24 h at refrigeration temperatures, also allowing the inactivation of  $>6 \log_{10}$  cycles

of *E. coli* cells. To the best of our knowledge, the combined effect of HHP and TBHQ on a gram-negative bacterial cell has not previously been investigated. Several authors have suggested that phenolic compounds might affect the cell envelopes (50, 57, 58). The synergistic effect might result from the same component of the microbial cell, the cytoplasmic membrane, being targeted simultaneously by both HHP and TBHQ agents.

The demand by consumers for natural, healthy foods has stimulated the use of plant essential oils in the development of new combined processes. Many plant essential oil compounds have antimicrobial action (9, 24, 29, 52). Moreover, a majority of essential oils are classified as being generally recognized as safe. Citral is the major constituent of the essential oil fraction of lemongrass (43) and is one of the most commonly used flavor compounds worldwide (53). Combining citral with pressurization resulted in a significantly greater reduction in viable counts of *Colletotrichum gloeosporioides* in papaya than that caused by HHP alone (41). To the best of our knowledge, there have been no previous attempts to evaluate the combined effect of HHP and citral against bacteria.

Exposure to citral alone at refrigeration temperatures caused inactivation and sublethal injury in both *L. monocytogenes* and *E. coli*, whereas in *L. monocytogenes*, the effect was greater at pH 4.0 than at pH 7.0, and the opposite was true for *E. coli*. Friedman et al. (11) observed the bactericidal effect of citral by using a microplate assay, but only at pH 7.0. Our results confirm its bactericidal effect also at pH 4.0 against the gram-positive bacterium investigated. The mechanism of action of citral is unknown, but the enhanced sensitivity to salt suggests impairment of osmotic homeostasis, possibly involving membrane perturbations caused by the lipophilic citral. The different responses to citral in *L. monocytogenes* and *E. coli* could thus arise from differences in envelope composition, but this would require further investigation.

The effect of citral combined with HHP also varied depending on the treatment medium pH and the microorganism investigated. The combined treatment showed a synergistic effect at pH 7.0 in both microorganisms. However, at pH 4.0, the effect was synergistic in *E. coli* but only additive in *L. monocytogenes*. Most sublethally injured cells detected immediately after HHP in the presence of citral were inactivated following holding for 24 h in the same treatment medium under refrigeration conditions. With the exception of *L. monocytogenes* suspended in McIlvaine's buffer at pH 7.0, the combined treatment caused a reduction in viability of at least 5  $\log_{10}$  cycles.

Citral is used as a flavoring in a variety of foods, including juices, beverages, certain baked goods, confectionary foods, and ice cream. It is added as an essential oil concentrate at concentrations of up to 800 ppm or as a pure compound at concentrations of up to 170 ppm (3, 47). The average daily intake of citral in humans is estimated to be 5 mg/kg (Council of Europe). Because of its fruity odor, citral is used mainly with products where a "green" or fruit flavor is required. It has been particularly recommended as an antimicrobial additive for soft drinks, orange juice, and apple juice (3, 10, 12). In these fruit-flavored products, the use of citral at relatively high concentrations where its odor is obvious is likely to be acceptable.

In summary, we have demonstrated that the extent of sublethal injury after pressurization treatments depended on the

type of microorganism, the pH, and the composition of the treatment medium. It is generally accepted that essential oils are most effective at acidic pHs (6), but as shown here, this did not apply to citral. The generalization that gram-positive organisms are more sensitive to essential oils than gram-negative ones was also shown to depend on pH. Despite the differences observed between microorganisms and the effects of treatment medium pH, the use of TBHQ in combination with HHP showed a synergistic effect, guaranteeing a 5-log reduction of *L. monocytogenes* and *E. coli* under any treatment condition investigated. The synergistic effect was sometimes observed immediately after the HHP treatment, as occurred when *L. monocytogenes* was treated at pH 7.0, but otherwise the effect was observed after a 24-h incubation under refrigeration conditions. The combination of citral and HHP also showed a synergistic effect, allowing the achievement of either a higher degree of inactivation or a higher proportion of sublethally injured cells. Our results have also confirmed that the detection of sublethal injury by the selective medium plating technique after HHP treatments may contribute to the identification of those treatment conditions under which HHP may act synergistically with other preserving processes.

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