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Inactivation of Barotolerant Strains of *Listeria monocytogenes* and *Escherichia coli* O157:H7 by Ultra High Pressure and *tert*-Butylhydroquinone Combination

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Antimicrobial efficacy of ultra-high-pressure (UHP) can be enhanced by application of additional hurdles. The objective of this study was to systematically assess the enhancement in pressure lethality by TBHQ treatment, against barotolerant strains of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. Two *L. monocytogenes* Scott A and the barotolerant OSY-328 strain, and two *E. coli* O157:H7 strains, EDL-933 and its barotolerant mutant, OSY-ASM, were tested. Cell suspensions containing TBHQ (50 ppm, dissolved in dimethyl sulfoxide) were pressurized at 200 to 500 MPa (23±2°C) for 1 min, plated on tryptose agar and enumerated the survivors. The TBHQ-UHP combination resulted in synergistic inactivation of both pathogens, with different degrees of lethality among strains. The pressure lethality threshold, for the combination treatment, was lower for *E. coli* O157:H7 (≥ 200 MPa) than for *L. monocytogenes* (> 300 MPa). *E. coli* O157:H7 strains were extremely sensitive to the TBHQ-UHP treatment, compared to *Listeria* strains. Interestingly, a control treatment involving DMSO-UHP combination consistently resulted in higher inactivation than that achieved by UHP alone, against all strains tested. However, sensitization of the pathogens to UHP by the additives (TBHQ in DMSO) was prominently greater for UHP than DMSO. Differences in sensitivities to the treatment between these two pathogens may be attributed to discrepancies in cellular structure or physiological functions.

***Keywords*:** *Listeria monocytogenes*, *Escherichia coli* O157:H7, ultra-high-pressure, *tert*-butylhydroquinone, dimethylsulfoxide

High pressure processing is a novel food processing method where food is subjected to elevated pressures (up to 87,000 psi, 600 MPa or approximately 6,000 atm) to achieve microbial inactivation. This process is also known as high hydrostatic pressure processing and ultra-high-pressure processing. Ultra-high-pressure (UHP) processing is a promising heat-alternative treatment which has the potential to meet the demands for high quality foods that are microbiologically safe. Pressure is distributed instantly and uniformly throughout the foods so that the processing is not restricted by the size and the geometry of the products. Therefore, it offers a significant advantage over conventional thermal processes, where the heat transfer is limited by the intrinsic properties of foods and thus frequently leads to the size reduction of the products (San Martin *et al*., 2002). In addition, high pressure can be applied at ambient temperature, leading to quality enhancements and energy efficiency.

In general, pressures ranging from 300 to 600 MPa can inactivate most pathogenic and spoilage vegetative cells, yeasts, and molds (Smelt, 1998). However, the inactivation curves induced by pressure treatment rarely obey the first-order kinetics, and often results in the inactivation pattern that leaves a small fraction of the microbial population viable. These 'survivor tails' raise practical problems if manufacturers are considering the development of processes to sterilize food or eliminate pathogens. Microbial efficacy of UHP can be increased by the application of additional hurdles such as mild heat and various biopreservatives (Kalchayanand *et al*., 1998; Masschalck *et al*., 2000, 2001a, 2001b). Certain combinations of hurdles, superior to others, inactivate barotolerant foodborne pathogens. According to the studies in this laboratory, addition of *tert*-butylhydroquinone (TBHQ) to cells, in suspension or inoculated in food, increased the lethality of UHP against *Listeria monocytogenes* (Chung *et al*., 2005; Vurma *et al*., 2006) and *Escherichia coli* O157:H7 (Malone *et al*., unpublished data) strains. However, these results were obtained from the independent experiments under different environments. For feasibility of using this combination in food, synergy between UHP and TBHQ should be demonstrated against barotolerant Gram-positive and Gram-negative pathogens. The direct comparison of synergistic effect against these bacterial species under the same treatment conditions may aid the elucidation of microbial inactivation mechanism of this combination treatment.

The mechanism of microbial inactivation by UHP is still not fully understood, but studies suggest that UHP inactivation involves multi-targets in the cell. Currently, the membrane damage is believed to be the basis of bacterial inactivation by pressure in several works (Pagán and Mackey, 2000; Ganzle and Vogel, 2001; Ritz *et al*., 2002; Russell,

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2002; Mañas and Mackey, 2004). Using UHP in the combination of chemical compound such as TBHQ, the fundamental insight in the perception of inactivation mechanism in microbial cells may be complicated. The objectives of this study were to assess the enhancement in pressure lethality by TBHQ treatment, against *E. coli* O157:H7 and *L. monocytogenes* strains, and to compare the measurement of the synergistic effect between these bacterial species.

Materials and Methods

Strains

Two *L. monocytogenes* strains, Scott A and barotolerant OSY-328 strain, and two *E. coli* O157:H7 strains, EDL-933 and its barotolerant mutant OSY-ASM, were obtained from the culture collection of the Food Safety Laboratory at The Ohio State University (USA) and tested in this study. All strains were grown from a 0.1% inoculum in Tryptose Broth (TB; Becton, Dickinson and Co., USA) at 35°C for 18 h. All cultures were transferred at least twice before experimentation.

Tert-butylhydroquinone preparation

Tert-butylhydroquinone (TBHQ) was obtained from Sigma Chemical Co. (USA). Solutions were prepared by dissolving TBHQ in dimethyl sulfoxide (DMSO; 99.9% spectrophotometric grade, Sigma), and 200 μl of this stock solution was added to 1,800 μl of cell suspension (i.e., 10%, v/v) to achieve a final concentration of 50 ppm.

Pressure treatment

All strains were grown to the stationary phase (~1.0×10^9 CFU/ml) in 10 ml TB. Portions of the culture (1,800 μl) and TBHQ solution (200 μl) were aseptically transferred to a sterile stomacher bag (Fisher Scientific, USA) and the bags were sealed using a vacuum sealer (VacMaster, USA). Control treatments included cultures that were not treated, or treated with DMSO, TBHQ in DMSO, or DMSO in combination with UHP. Samples were pressurized at ranges from 200 to 500 MPa (23±2°C holding temperature) for 1 min in a high-pressure processor (Quintus® QFP-6, Flow Pressure Systems, USA). The initial temperature of the pressure transmitting fluid was controlled to account for the compression heating (3 to 4°C/100 MPa). An untreated control was held at 25°C and atmospheric pressure (0.1 MPa) while each treated sample was being pressure-treated. After pressure treatments, samples were serially-diluted using 0.1% peptone water, plated on Tryptose Agar (TA; Becton, Dickinson and Co., USA), and incubated at 35°C for 48 h to enumerate the survivors.

Results

Direct comparison of UHP-sensitivity of two pathogens, *L. monocytogenes* Scott A and *E. coli* O157:H7 EDL-933, was examined (Fig. 1). To compare the differences in UHP-sensitivity as straight as possible, these cells were grown to the stationery phase under same conditions, resuspended in phosphate buffer, and the initial population before UHP for each strain was 10^9 CFU/ml. Pressure lethality threshold was lower for *E. coli* O157:H7 EDL-933 than *L. monocytogenes* Scott A (Fig. 1). Slight inactivation of *E. coli* O157:H7 EDL-933 was noticed at 200 MPa and 23°C for 1 min treatment, while no lethality was observed at 300 MPa and 23°C for 1 min treatment for *L. monocytogenes* Scott A. However, at a higher pressure level (500 MPa) EDL-933 strain was more resistant than Scott A.

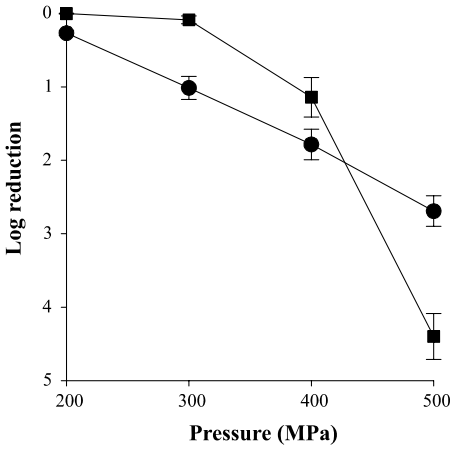

Fig. 1. Inactivation of *Listeria monocytogenes* Scott A (■) and *Escherichia coli* O157:H7 EDL-933 (●) by high pressure treated at 200 to 500 MPa and 23°C for 1 min. Error bars represent the standard error of 2~6 experiments.

Two different strains, Scott A and OSY-328, of *L. monocytogenes* showed different UHP-sensitivity at pressures ≥400 MPa (Fig. 2). Similarly, two strains, EDL-933 and OSY-ASM, of *E. coli* O157:H7 exhibited different sensitivities to the pressure treatment (Fig. 3). Generally, the increment in log reduction by UHP against *L. monocytogenes* strains was more, as pressure levels increase, than against *E. coli* O157:H7 strains (Fig. 1~3).

Dimethyl sulfoxide is an excellent solvent for a hydrophobic compound such as TBHQ and the resulting solution is conveniently miscible with aqueous media of cell suspensions. As a control treatment, inactivation of the strains by DMSO-UHP combination was compared to that of UHP alone and the TBHQ-UHP combination. However, this solvent seems to take part in cellular reactions induced by pressure. Although the contribution of DMSO to the lethality of UHP was prominently less than that of TBHQ in combination treatment, DMSO-UHP combination consistently results in higher inactivation than UHP alone against all strains tested (Fig. 2 and 3). This treatment, DMSO-UHP, however, seems to inactivate more against *E. coli* O157:H7 strains than *L. monocytogenes* strains.

The combination treatment of TBHQ (50 ppm, dissolved in DMSO) and UHP resulted in synergistic inactivation of both pathogens, with different degree of lethality among strains (Fig. 2 and 3). It appears that a threshold pressure should be achieved before bacteria are sensitized by TBHQ. When TBHQ was combined with pressures ranging from 100 to 200 MPa, for 5~15 min (23°C), no synergistic lethality between pressure and TBHQ, against *E. coli* O157:H7, was observed (data not shown). Similarly, TBHQ-UHP in-

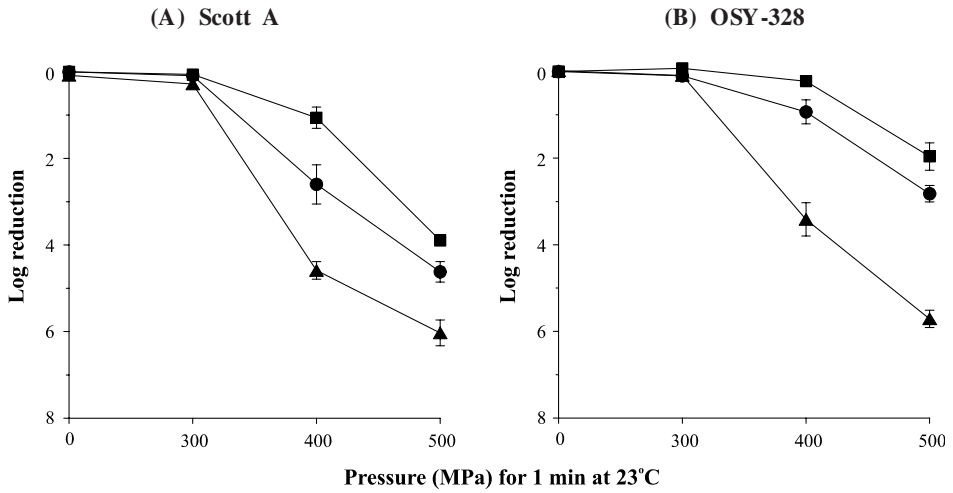

Fig. 2. Inactivation of *Listeria monocytogenes* (A) Scott A and (B) OSY-328 by UHP alone (■), DMSO-UHP (●), or TBHQ-UHP (▲). Samples were treated with pressure at 300 to 500 MPa and 23°C for 1 min, in the absence or presence of DMSO (10%, v/v) or TBHQ (50 ppm, dissolved in DMSO). Error bars represent the standard error of three experiments.

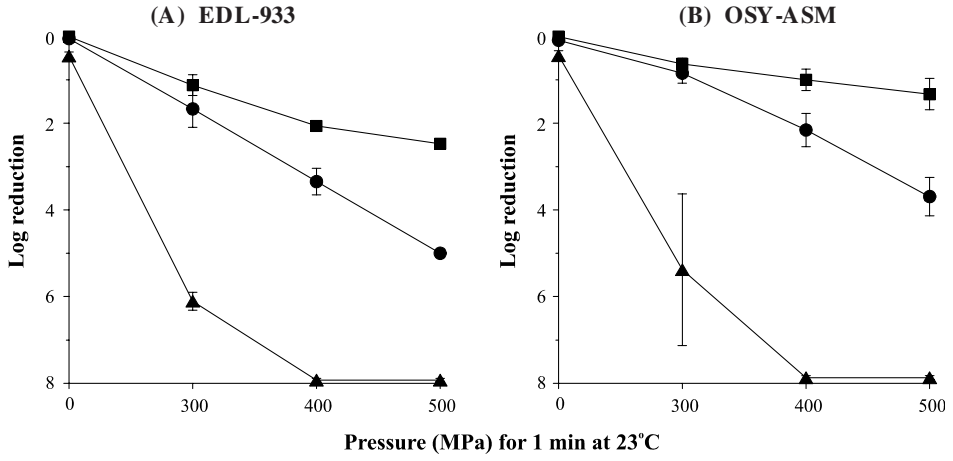

Fig. 3. Inactivation of *Escherichia coli* O157:H7 (A) EDL-933 and (B) OSY-ASM by UHP alone (■), DMSO-UHP (●), or TBHQ-UHP (▲). Samples were treated with pressure at 300 to 500 MPa and 23°C for 1 min, in the absence or presence of DMSO (10%, v/v) or TBHQ (50 ppm, dissolved in DMSO). Error bars represent the standard error of three experiments.

duced lethality was not obtained at 300 MPa (at 23°C for 1 min) for *L. monocytogenes* strains. Most noticeably, *E. coli* O157:H7 strains were extremely sensitive to the TBHQ-UHP combination treatment, compared to *L. monocytogenes*. No survivors of *E. coli* O157:H7 were detected by the plate counting method on nonselective agar when treated at pressures ≥400 MPa in the presence of TBHQ (Fig. 3).

Discussion

Variation in strain sensitivity to a certain process is an important consideration when designing processing technologies. These technologies should be effective against the most resistant strain of the microorganism of concern. Two strains, one is barosensitive and the other is barotolerant, for each *L. monocytogenes* and *E. coli* O157:H7 were selected based on our previous results (Chung *et al*., 2005; Vurma *et al*., 2006; Malone *et al*., unpublished data). Different lethality due to UHP alone was confirmed between the sensitive and the resistant strains of each pathogen. The variability in pressure resistance was also commonly observed from other studies for both Gram-positive and Gram-negative pathogens including *L. monocytogenes* (Alpas *et al*., 1999; Tay *et al*., 2003), *Staphylococcus aureus* (Alpas *et al*., 1999), *Salmonella* spp. (Alpas *et al*., 1999), and *E. coli* O157:H7 (Alpas *et al*., 1999; Benito *et al*., 1999; Malone *et al*., 2006).

Currently, the membrane damage is believed to be the basis of bacterial inactivation by pressure in several works (Pagán and Mackey, 2000; Ganzle and Vogel, 2001; Ritz *et al*., 2002; Russell, 2002; Mañas and Mackey, 2004). Biomembranes are dynamic structures, which liquid crystalline lamellar phospholipids phases (L_α) serve as a basic structural element. Phospholipids bilayers exhibit a structural polymorphism, depending on their molecular structure and environmental

conditions, such as the water content, pH, ionic strength, temperature and pressure (Gruner, 1985). In the liquid crystalline phase, the acyl chains of the lipid bilayers are conformationally disordered, whereas in the gel phases (L_β), the chains are more extended and ordered. Phase transition, from liquid crystalline to gel, which is observed in the case of temperature downshift, of several single-component phospholipids bilayers, was reported after pressure treatment (Chong and Cossins, 1983; MacDonald, 1993; Winter, 2001). Kato and Hayashi (1999) also reported that high pressure induces the phase transition of natural membranes, which leads to decrease in membrane fluidity, and this may result in breakage of the membrane. Although phase transition of membrane lipids is not necessarily lethal to bacteria, it has been demonstrated that membrane fluidity is linked to the increased pressure resistance (Casadei *et al*., 2002). Similarly, Braganza and Worcestor (1986) indicated that pressure increases the packing density of membrane lipids and induces phase separations due to differences in compressibility between lipids and proteins in cell membrane. Therefore, different membrane composition may be one of the reasons to induce different pressure sensitivity among different strains. Successful treatment of pathogens, in aqueous suspensions, with the hydrophobic TBHQ depended largely on the use of DMSO as a solvent. The dipolar S=O group of DMSO can interact with each other to create chain-like polymeric association (dipole-dipole interactions) and also forms hydrogen bonds with two molecules of water. This amphiphilic property of DMSO may explain why it is an efficient solvent for both polar and nonpolar molecules (Yu and Quinn, 1994). According to our observation, DMSO exhibited some lethal effect against both *L. monocytogenes* and *E. coli* O157:H7 strains, indicating it served beyond its role as a solvent, when it is used under pressure treatment. The most probable action site of DMSO seems to be at the level of cell membranes (Yu and Quinn, 1998).

The properties of cell membranes depend greatly on the solvation effects of water, and the biological actions of DMSO may be based on modulating these solvent effects. Studies suggested that DMSO affects the hydration forces at the phospholipids-solvent interface of the membrane surface by displacing water. Therefore, the membrane surfaces move closer to each other, due to the changes in hydrogen bonding network of water by DMSO (Gordeliy *et al*., 1998; Smondyrev and Berkowitz, 1999). These interactions of DMSO and membrane surface also enhanced the association between the head groups and the ordering of head groups, thus affected the close packing of hydrocarbon chains of lipid bilayers. Consequently, this leads to increase in membrane rigidity and the phase transition temperatures of membrane (Gordeliy *et al*., 1998; Tristram-Nagle *et al*., 1998; Yu and Quinn, 1998; Smondyrev and Berkowitz, 1999). The effect of DMSO on the increase in phase transition temperature of phospholipids bilayers, namely DMSO favors the gel phases of membrane bilayers, was similar to that of UHP on the phase transition of membrane phospholipids (Chong and Cossins, 1983; MacDonald, 1993; Kato and Hayashi, 1999; Winter, 2001), as mentioned above. Therefore, the enhanced lethality due to DMSO and UHP combination, obtained from the current study, may have resulted from the effect on gel phase stabilization of these treatments (Fig. 4).

Other studies also demonstrated that DMSO induced to

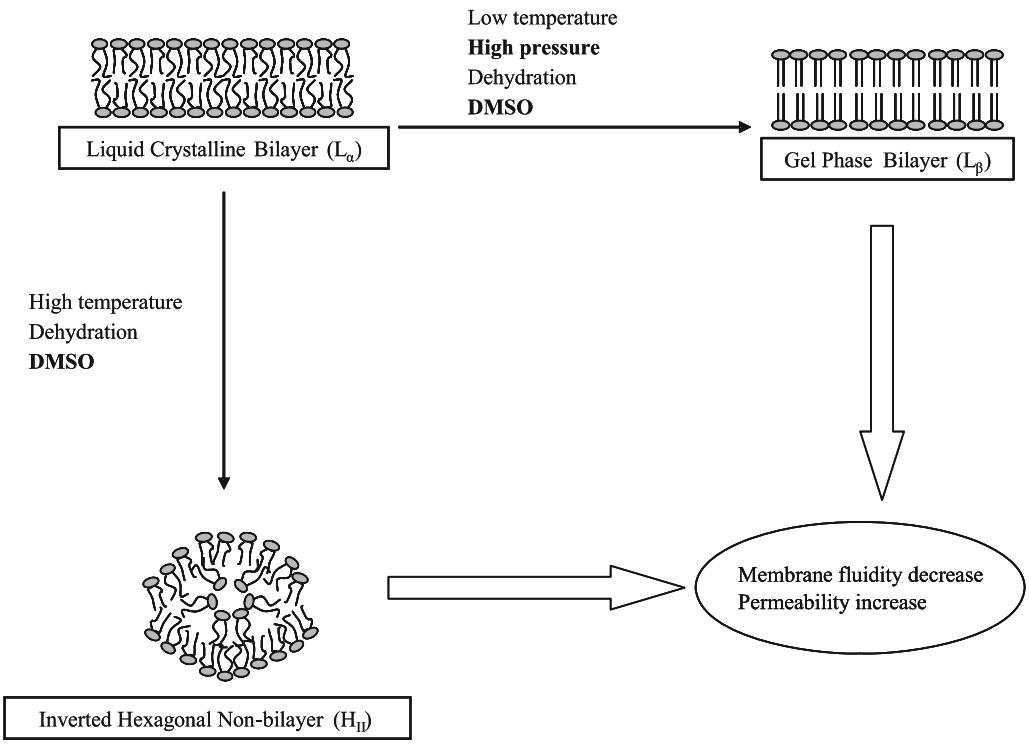

Fig. 4. Factors affecting the phase transition of membrane lipids (Adapted from Yu and Quinn, 1994).

form a spontaneous curvature (i.e., nonlamellar) structure of the membrane to reduce the interfacial surface area (Yu *et al*., 1996; Kinoshita *et al*., 2001). These effects of DMSO on phase transition relate the decreased membrane fluidity and increased membrane permeability (Fig. 4). It was reported that DMSO does not penetrate extensively in the hydrophobic alkyl chain region of the lipid bilayer (Smondyrev and Berkowitz, 1999) and this may be related the low (0.0030) oil-in-water partition coefficient of DMSO (Bunch and Edwards, 1969). Therefore, these effects of DMSO on the phase stability of membranes may be intensified under UHP, which support our results. In addition, DMSO seems to affect mostly on physical properties of membranes, while TBHQ seems to affect largely on the physiology of the bacterial cells, which will be discussed next.

According to the recent reports in our laboratory, TBHQ sensitized different *L. monocytogenes* (Scott A, OSY-8578, OSY-328) (Chung *et al*., 2005; Vurma *et al*., 2006) and *E. coli* O157:H7 (EDL-933, OSY-ASM) (Malone *et al*., unpublished data) strains to UHP treatment. However, these results were obtained from the independent experiments under different environments. In this study using the same treatment conditions, *Listeria* strains were more resistant to TBHQ-UHP combination than were *E. coli* O157:H7 strains (Fig. 2 and 3). This different sensitivity between these two pathogens may be attributed to discrepancies in cellular structure or physiological functions. Sensitization of several antimicrobial peptides and proteins (i.e., bacteriocins, lactoferrin, lactoferricin, and lysozyme) of Gram-negative bacteria under pressure has been ascribed to the permeabilization of the outer membrane (Kalchayanand *et al*., 1994; Hauben *et al*., 1996; Kalchayanand *et al*., 1998; Masschalck *et al*., 2000, 2001a, 2001b). Masschalck *et al*. (2003), however, indicated that pressure-induced outer membrane permeabilization is dependent on the organism, outer membrane properties, and the nature of antimicrobial compounds, and thus this observation cannot be explained by a unifying mechanism. The current study also indicates that UHP was necessary to obtain the sensitization effect of TBHQ against both Gram-positive *Listeria* and Gram-negative *Escherichia* strains. In case of *E. coli* O157:H7, TBHQ may access more easily to the cell target than *Listeria*, since Gram-negative bacteria have thinner peptidoglycan layer than Gram-positives. This can be one of the possible explanations for high sensitivity of *E. coli* O157:H7 to the combination treatment.

On the other hand, TBHQ treatment without pressure actually reduced the population of both *E. coli* O157:H7 strains by less than 0.5 log (Fig. 3), but not *L. monocytogenes* (Fig. 2). Therefore, some physiological differences between *L. monocytogenes* and *E. coli* O157:H7 may also be involved, in addition to the structural differences, in their dissimilar sensitivities to this TBHQ-UHP combination treatment. Considering the antioxidant properties of TBHQ, presence of this compound during pressure treatment may have altered the cell metabolic pathways governing redox homeostasis. Malone *et al*. (unpublished data) investigated the synergistic mechanism between TBHQ and UHP by using *E. coli* K12 mutants, that are defective in genes involved in maintaining redox homeostasis and anaerobic metabolism. Especially, iron-sulfur cluster proteins, which have essential functions in electron transfer and catalysis of oxidoreductase/hydrolase in bacterial cells, were critically related to the TBHQ-UHP treatment in *E. coli* (Malone *et al*., unpublished data). These authors stated that the redox cycling activity of iron-sulfur proteins may bioactivate TBHQ, which potentiates the generation of reactive oxygen species. Further investigation of physiological differences between *L. monocytogenes* and *E. coli* O157:H7 strains in response to TBHQ-UHP treatment may help to elucidate the synergistic mechanism of this combination.

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