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International Journal of Food Microbiology 89 (2003) 125–138
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Review

Combining nonthermal technologies to control foodborne microorganisms

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Received 11 January 2003; received in revised form 12 March 2003; accepted 27 March 2003

Abstract

Novel nonthermal processes, such as high hydrostatic pressure (HHP), pulsed electric fields (PEFs), ionizing radiation and ultrasonication, are able to inactivate microorganisms at ambient or sublethal temperatures. Many of these processes require very high treatment intensities, however, to achieve adequate microbial destruction in low-acid foods. Combining nonthermal processes with conventional preservation methods enhances their antimicrobial effect so that lower process intensities can be used. Combining two or more nonthermal processes can also enhance microbial inactivation and allow the use of lower individual treatment intensities. For conventional preservation treatments, optimal microbial control is achieved through the hurdle concept, with synergistic effects resulting from different components of the microbial cell being targeted simultaneously. The mechanisms of inactivation by nonthermal processes are still unclear; thus, the bases of synergistic combinations remain speculative. This paper reviews literature on the antimicrobial efficiencies of nonthermal processes combined with conventional and novel nonthermal technologies. Where possible, the proposed mechanisms of synergy is mentioned.
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Keywords: Nonthermal technology; Nonthermal processing; Combinations; Synergy; Mechanisms of inactivation; Microbial control

1. Introduction

Alternative technologies for inactivating microorganisms without relying on heat are not new concepts (Hite, 1899; Jacobs and Thornley, 1954; Sale and Hamilton, 1968), but their development for use as food preservation treatments has received considerable attention only recently, in response to consumer demands for more 'fresh' and 'natural' food products (Jeyamkondan et al., 1999). These novel nonthermal technologies have the ability to inactivate microorganisms at ambient or near-ambient temperatures, thereby avoiding the deleterious effects that heat has on the flavour, colour and nutrient value of foods (Barbosa-Canovas et al., 1999).

With the high treatment intensities required to inactivate significant numbers of microorganisms, however, certain nonthermal processes can also affect

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0168-1605/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0168-1605(03)00161-2

the sensory properties of foods. In particular, high hydrostatic pressure (HHP) can alter the structure of proteins and polysaccharides, causing changes in the texture, physical appearance and functionality of foods (Knorr, 1993; Williams, 1994). High-intensity ultrasound also can denature proteins and produce free radicals that can adversely affect the flavour of fruit-based or high-fat foods (Williams, 1994; Sala et al., 1995). Proteins, fats and carbohydrates are not notably altered by irradiation although higher doses may cause slight colour changes in beef, pork and poultry (Wood and Bruhn, 2000).

'Minimal processing' is a concept describing approaches to food safety and preservation that are designed to retain the natural and as-fresh properties of foods (Manvell, 1997). The hurdle concept (Leistner, 1992, 2000) is a minimal processing technique that exploits synergistic interactions between traditional preservation treatments. According to the hurdle concept, preservation treatments combined at lower individual intensities have additive or even synergistic antimicrobial effects, while their impact on sensory and nutritive properties of the food is minimized (Leistner, 1992).

In a similar fashion, it is possible that combining two or more novel nonthermal technologies will produce synergistic antimicrobial effects while reducing the energy input and treatment intensities required. However, the intelligent application of hurdle technology requires knowledge of the mechanisms of each hurdle applied (Leistner, 2000); it is in this area that knowledge of nonthermal technology is most lacking.

Nevertheless, combinations of nonthermal technologies and processes have been investigated in recent years, with a view to improving control over foodborne microorganisms while minimizing the intensities of the treatments applied. This paper provides an up-to-date summary of published findings, where nonthermal technologies have been combined to enhance the inactivation of foodborne microorganisms. Combinations that are covered within the review include emerging nonthermal processes with organic acids or natural antimicrobial compounds and simultaneous or consecutive application of emerging nonthermal processes. Where possible, the proposed mechanisms of synergy between combined treatments are also discussed.

2. Nonthermal technologies and the hurdle concept

Nonthermal technologies encompass all preservation treatments that are effective at ambient or sublethal temperatures including antimicrobial additives, pH adjustment and modified atmospheres. The term 'nonthermal processing' is more apt for novel nonthermal technologies, such as high hydrostatic pressure, pulsed electric fields (PEFs), high-intensity ultrasound, ultraviolet light, pulsed light, ionizing radiation and oscillating magnetic fields, which are intended for application as microbe-inactivating processes during food manufacture. Such novel technologies have the ability to inactivate microorganisms to varying degrees (Butz and Tauscher, 2002), but the high treatment intensities required for inactivation by some processes can cause adverse changes in the sensory or functional properties of the food (Knorr, 1993; Williams, 1994; Sala et al., 1995; Wood and Bruhn, 2000). Some emerging nonthermal technologies have been deemed too energy expensive or costly to be practical for use in food processing (Raso et al., 1998a). The resilience of bacterial spores and the existence of highly resistant microbial subpopulations also currently limit the efficacies of emerging nonthermal technologies (Leistner and Gould, 2002).

The most extensively researched and promising nonthermal processes appear to be high hydrostatic pressure (HHP), pulsed electric fields (PEF) and high-intensity ultrasound combined with pressure. Gamma irradiation has high potential although its development and commercialization has been hampered in the past by unfavourable public perceptions (Resurreccion et al., 1995). Recent endorsement of food irradiation by many international food and health organizations has, however, served to increase consumer confidence and heighten interest within the food industry (Monk et al., 1995; Lacroix and Ouattara, 2000; DeRuiter and Dwyer, 2002; Ouattara et al., 2002).

The antimicrobial effect of preservation treatments can be optimized by combining them as hurdles in an overall preservation strategy. By placing a number of sublethal stresses (i.e. hurdles) on a microbial cell, the organism expends energy to overcome the hostile environment, potentially leading to metabolic exhaustion and death (Leistner, 2000). Hurdles targeting the same elements within the cell have an additive inhibitory effect only, whereas synergistic effects may

result from disturbing several functions of the cell (Leistner, 1992, 1994). According to Leistner (2000), attacking various cellular targets will have a synergistic effect by making the organism strain every possible repair mechanism simultaneously, while the activation of stress shock proteins also becomes more difficult. The multitarget approach may also help to counter stress adaptation associated with sublethal treatments (Yousef, 2001).

Two or more novel nonthermal processes and conventional preservative techniques can also be combined to enhance their lethal or inhibitory effects on microorganisms (Crawford et al., 1996; Jin et al., 1998; Pagan et al., 1999; Yousef, 2001; Leistner and Gould, 2002). Such combinations enhance food preservation at lower individual treatment intensities, and microbes that survive processes, such as irradiation and ultrasonication, generally become less resistant to other factors such as heat, pH changes and antibiotics (Williams, 1994; Barbosa-Canovas et al., 1998).

It is debatable whether the theoretical basis of the original hurdle concept can be applied to nonthermal processing combinations, however, as the modes of action of nonthermal processes are intrinsically different to those of conventional and sublethal preservation hurdles (e.g. low water activity, low-temperature storage, pH manipulation or addition of inhibitory substances). They are not a series of low-intensity stressors designed to fatigue a cell, but are relatively short-time high-intensity treatments causing irreversible damage to vital cell components. This raises questions about the mechanisms of synergy behind nonthermal processing combinations such as whether effective combinations target the same or different components within the cell. It could be argued that applying a number of treatments with a common target would improve the chances of irreversible damage being inflicted, whereas treatments targeting different sites may cause sublethal damage in several areas of the cell, but fail to inactivate the organism.

A good understanding of the modes of action of each individual treatment is crucial to selecting effective antimicrobial combinations (Barbosa-Canovas et al., 1998; Leistner, 2000). Unfortunately, the mechanisms of microbial inactivation by nonthermal technologies are currently not well understood although theories have been developed. These proposed mechanisms are too complex to include in any detail here, but excellent reviews are available for high hydrostatic pressure (Hoover et al., 1989), pulsed electric fields (Castro et al., 1993; Barbosa-Canovas et al., 1999) and other nonthermal technologies (Pothakamury et al., 1993; Barbosa-Canovas et al., 1998; Dickson, 2001).

Despite the current gaps in understanding, combining nonthermal processes with other nonthermal technologies has been investigated to improve control over foodborne microorganisms, with promising results. A better understanding of the antimicrobial mechanisms of emerging nonthermal technologies as well as their effectiveness when combined with traditional food preservation hurdles is needed so that new food preservation strategies can be developed on a sound scientific basis (Barbosa-Canovas et al., 1998).

3. Nonthermal processing and acidification

When combined with certain nonthermal processes, acidic conditions may result in greater inactivation of vegetative bacteria (Hoover et al., 1989; Vega-Mercado et al., 1996; Barbosa-Canovas et al., 1999) and spores (Roberts and Hoover, 1996; Balasubramaniam et al., 2001) than at neutral pH. The greater inactivation of vegetative cells is presumably due to stressful changes in the cytoplasmic pH combined with loss of membrane functionality and other cell damage caused by the nonthermal processing hurdles (Vega-Mercado et al., 1996; Jeyamkondan et al., 1999). The reduction of cytoplasmic pH is more effective with acidification by organic rather than inorganic acids because organic acids are more able to permeate the cell membrane (Eklund, 1989).

3.1. High hydrostatic pressure (HHP) and lowered pH

HHP processing involves applying static pressures of 50 to 1000 MPa to solid or packaged liquid foods, with holding times varying from several seconds to minutes (Mertens and Deplace, 1993; Williams, 1994). High pressure only affects noncovalent bonds, leaving covalent bonds intact (Knorr, 1993) and, consequently, induces alterations in the structure of secondary- and tertiary-bonded molecules (Datta and Deeth, 1999).

Pressurization narrows the pH range for growth of microorganisms, having a particularly inhibitory effect on membrane ATP-ase, a very important enzyme in the acid–base physiology of cells (Hoover et al., 1989). According to Mozhaev et al. (1994), sorbic and benzoic acids enhance the effect of pressure on microorganisms, enabling lower pressures and shorter treatment times to achieve inactivation. Timson and Short (1965) observed slightly greater destruction of *Bacillus subtilis* at pH 6 than at pH 8, where pressure resistance was highest. Balasubramaniam et al. (2001) achieved an extra 2.5-log reduction of *B. subtilis* spores at 827 MPa and 75 °C, when the pH of the treatment buffer was reduced from 7.0 to 3.0. Roberts and Hoover (1996) also achieved greater inactivation of *Bacillus coagulans* spores at just 400 MPa, when the pH was lowered from neutral to pH 4.0. Other authors, however, have reported that altering pH has little effect on HHP inactivation of yeast and bacterial spores (Patterson et al., 1995; Barbosa-Canovas et al., 1998). It is worth noting that under pressurized conditions, the carboxylic acids commonly used as food preservatives show enhanced dissociation and, hence, become less effective because undissociated acid forms are believed to be the effective antimicrobial entities (Eklund, 1989; Earnshaw et al., 1995).

3.2. Pulsed electric fields (PEF) and lowered pH

PEF processing involves passing a high-voltage electric field (10–80 kV/cm) through a liquid food held between two electrodes, in very fast pulses typically of 1–100-μs duration (Zhang et al., 1994; U.S. Food and Drug Administration, 2000). Destruction of microbial cells by PEF is due to irreversible electroporation of the cell membrane, which leads to leakage of intracellular contents and eventually lysis of the cell (Qin et al., 1995; Barbosa-Canovas et al., 1999).

The PEF inactivation of *Escherichia coli* O157:H7 in a 10% glycerol solution was enhanced synergistically by lowering the pH from 6.4 to 3.4 using benzoic or sorbic acid (Liu et al., 1997). Similarly, adjusting the pH of skim milk and liquid eggs with organic acids has shown additive and synergistic inactivation of bacteria when combined with PEF treatment (Gongora-Nieto et al., 1999; Fernandez-Molina et al., 2001a,b). PEF plus acidification with hydrochloric acid, on the other hand, resulted in no extra inactivation of raw milk's microflora when compared with PEF alone (Smith et al., 2002). The synergism between acetic acid and PEF might be due to the fact that both treatments target the cell membrane (Fernandez-Molina et al., 2001a). In this way, PEF could increase the permeability of the cell wall and membrane, enhancing the entry of undissociated acids into the bacterial cell.

It should be noted that lowered pH as a sublethal hurdle may also jeopardize the efficacy of other antimicrobial processes. For example, Evrendilek and Zhang (2001) found that exposing *E. coli* O157:H7 to pH 3.6 before PEF treatment resulted in lower inactivation than exposure to pH 5.2 or 7.0. It was concluded that adaptation of *E. coli* to the acid stress resulted in greater survivability during PEF treatment.

Emerging nonthermal processes, such as HHP and PEF, are gaining commercial application most rapidly with juices and other fruit-derived products (Mermelstein, 1997; Qiu et al., 1998; Jia et al., 1999; Leistner and Gould, 2002), as the hurdle of low pH exists naturally in the raw material. Much more research is needed before these technologies can be adapted for the production of shelf-stable low acid foods (Yousef, 2001).

3.3. Ultrasonication and lowered pH

High-frequency alternating electric currents can be converted into ultrasonic waves via an ultrasonic transducer (Mason, 1998). These ultrasonic waves can be amplified and applied to liquid media by an ultrasonic probe, which is immersed in the liquid to be treated, or an ultrasound bath, which is filled with the treatment liquid.

The antimicrobial effect of ultrasonication is due to cavitation, i.e. the extremely rapid creation and collapse of bubbles formed by ultrasonic waves in a medium (Earnshaw, 1998). Cavitation produces intense localized changes in pressure and temperature, causing shear-induced breakdown of cell walls, disruption and thinning of cell membranes, and DNA damage via free radical production (Lillard, 1994; Earnshaw et al., 1995; Sala et al., 1995; Manvell, 1997).

Pagan et al. (1999) found that the effect of ultrasonic treatment (20 kHz, 117 μm) of *Listeria monocytogenes* under sublethal pressure (200 kPa) was not greatly influenced by a pH reduction from 7.0 to 4.0. The acidic conditions had a much greater effect on the organism's resistance to heat than its sensitivity to ultrasonication. Similar findings were reported much earlier by Kinsloe et al. (1954), who exposed bacterial and yeast cells to a sonic field in saline suspensions of varying pH. Lowering the pH from neutral to 4.0 did not alter the death rates of *Pseudomonas aeruginosa* or *Saccharomyces cerevisiae* under sonication. For *E. coli*, *Serratia marcescens* and *Micrococcus varians*, higher death rates were only observed when a higher treatment temperature (45 °C) was combined with both sonication and lowered pH (Kinsloe et al., 1954).

3.4. Irradiation and lowered pH

Irradiation of bulk or prepackaged foods is achieved by exposing the product to a source of ionizing energy, typically Cobalt-60 (Wood and Bruhn, 2000). The product to be treated is conveyed through a shielded chamber containing the radiation source, and irradiation dosage is controlled by the speed of the conveyor. Inactivation of organisms by ionizing radiation is primarily due to DNA damage, which destroys the reproductive capabilities and other functions of the cell (Dickson, 2001; DeRuiter and Dwyer, 2002).

The lethal effect of ionizing radiation is not greatly enhanced by mild acidification. For example, the destruction of bacteria in and the shelf life of irradiated chicken and ground beef were not improved by low concentrations of infused or added acetic acid (Hausam et al., 2002; Ouattara et al., 2002). In another study, the differences in pH of five commercial orange juice formulations (from pH 3.87 to 4.13) had no influence on the radiation dose required for 90% inactivation of a *Salmonella* Enteritidis strain isolated from a citrus juice associated with an outbreak of salmonellosis (Niemira, 2001).

Farkas and Andrassy (1993) investigated irradiation combined with pH reduction (with different acidulants) for their effects on the shelf life of a chilled and temperature abused beef/pork product. Bacteria of the Enterobacteriaceae family were both destroyed and injured by irradiation, and injured cells were unable to grow at the acidic pH even in temperature abused product. Radiation-resistant lactic acid bacteria subsequently became the dominant microflora, suggesting that the selection for lactic acid bacteria in irradiated foods might serve as an intrinsic factor for control of the growth of certain pathogens (Farkas and Andrassy, 1993).

4. Nonthermal processing and antimicrobial agents

The addition of naturally occurring antimicrobials has proven to be an effective hurdle when combined with nonthermal processing techniques. Bacteriocins, such as those produced by lactic acid bacteria, are legally considered natural if used in concentrations equal to or below what are found in foods naturally fermented with the bacteriocin-producing starter culture (Cleveland et al., 2001). Nisin is currently the only bacteriocin approved for use in food by the World Health Organization although many lactic acid bacteria-produced bacteriocins have potential applications (Pol et al., 2000; Cleveland et al., 2001).

Aside from regulatory control, the use of natural antimicrobials in commercial food supplies is also restricted by high costs and inhibition of their bactericidal activity in complex food substrates (Garcia-Graells et al., 1999; Terebiznik et al., 2000). According to Ganzle et al. (1999), bacteriocin activity may be inhibited in food matrices due to changes in solubility and charge of the bacteriocins, binding of bacteriocins to food components and inactivation of bacteriocins by proteases.

Two extensively studied natural antimicrobial compounds with potential to control foodborne microorganisms are nisin and lysozyme. Nisin is a peptide that causes ion-permeable pore formation in the cytoplasmic membrane of cells, while the enzyme lysozyme weakens cell peptidoglycan layers by hydrolyzing glycosidic linkages in the peptidoglycan (Chung and Hancock, 2000; Sablon et al., 2000). Nisin and lysozyme are normally only effective against Gram-positive organisms, as the outer lipopolysaccharide component of the membrane of Gram-negative bacteria prevents such antimicrobial compounds accessing the cytoplasmic membrane or peptidoglycan layer (Ibrahim et al., 1992; Hauben et

al., 1996). However, lysozyme, nisin and other bacteriocins have been shown to act on several species of Gram-negative bacteria, provided that the barrier properties of the outer membrane are first disrupted (Kalchayanand et al., 1992; Ray, 1993; Hauben et al., 1996; Masschalck et al., 2001a; Smith et al., 2002).

4.1. High hydrostatic pressure (HHP) and antimicrobial agents

Nisin, lysozyme, pediocin AcH, lacticin 3147, bovine lactoferrin and lactoferricin have significantly enhanced the destruction of both Gram-positive and Gram-negative bacteria in skim milk, whey and buffers under HHP (Kalchayanand et al., 1994; Hauben et al., 1996; Roberts and Hoover, 1996; Kalchayanand et al., 1998; Garcia-Graells et al., 1999; Morgan et al., 2000; Masschalck et al., 2001a,b; Ray, 2001). The primary cause of vegetative cell damage by HHP is permeabilization of the cell membrane due to irreversible changes in the structure of membrane macromolecules such as proteins (Hoover et al., 1989; Balny and Masson, 1993). Permeabilization of the membrane is also affected by compression of the membrane's bilayer and reduction in the cross-sectional area per phospholipid molecule (Sale et al., 1970; Hoover et al., 1989; Datta and Deeth, 1999).

The high levels of inactivation seen with antimicrobial agents and HHP hurdles are believed to be due to the combined factor destabilization of membrane structure or function although their specific modes of action are different (Kalchayanand et al., 1994; Masschalck et al., 2001a; Ray, 2001). High-pressure membrane damage can increase the cell penetrability and activity of antimicrobial agents, while, conversely, treatment with cell wall weakening agents sensitizes pressure-resistant bacteria to HHP (Earnshaw et al., 1995). In addition, residual bacteriocins in the food matrix would continue to exert their inhibitory effect after treatment with the nonthermal processing hurdle, thereby inactivating and preventing the recovery of any sublethally injured cells (Morgan et al., 2000).

Timing of antimicrobial addition is important for additive or synergistic effects to occur with HHP. For example, Hauben et al. (1996) found that *E. coli* was not sensitive to nisin or lysozyme after HHP treatment, despite a significant degree of sublethal injury. Addition of lysozyme and nisin during the pressurization treatment, however, increased lethality in an additive manner. Masschalck et al. (2001a) observed the same phenomenon when using nisin on one Gram-positive and seven Gram-negative bacterial strains exposed to HHP. It was concluded in both studies that reversible permeabilization of the outer cell membrane was induced under pressure, enabling temporary access of nisin to the cytoplasmic membrane, or lysozyme to the peptidoglycan layer surrounding the cell.

The method of application of an HHP treatment can also influence the bactericidal efficiency of added antimicrobials compounds. For example, Garcia-Graells et al. (1999) found that pulsed HHP application (i.e. 10-min HHP treatments interrupted by brief decompressions) significantly increased the sensitivity of four *E. coli* strains to both nisin and lysozyme in milk, as compared with exposure to the antimicrobials during a single, continuous HHP application.

Morgan et al. (2000) observed a synergistic killing effect of HHP in the presence of the bacteriocin lacticin 3147. HHP treatment at 250 MPa caused a 2.2-log reduction of *Staphylococcus aureus* in reconstituted skim milk, while 10,000 AU/ml lacticin 3147 alone caused a 1-log cycle reduction. Both treatments combined, however, resulted in destruction of more than 6-log cycles of the pathogen. Lacticin 3147 exerts a bactericidal effect by disturbing the selective permeability of the membrane of sensitive cells, and the authors found that as pressure increased, the effect of lacticin 3147 became more pronounced (Morgan et al., 2000).

HHP destruction of bacterial spores is enhanced in the presence of antimicrobial compounds such as nisin and the emulsifier sucrose laurate (Roberts and Hoover, 1996; Shearer et al., 2000). In a study using food-approved emulsifiers, the researchers noted that sucrose laurate seemed to be inhibitory rather than lethal to spores, as its effect was most pronounced when added to both the HHP-treated food and the enumeration media following treatment (Shearer et al., 2000).

Popper and Knorr (1990) found no synergistic effects when pretreating microbial suspensions with lysozyme or chitosan before to high-pressure homogenization. It was suggested that the lysozyme caused

loosening of the cell wall structure, thereby changing its elasticity and susceptibility to mechanical stress, while the chitosan only affected transport mechanisms through cell walls and not necessarily the wall's integrity. It was also speculated that synergistic inactivation might only have become apparent at pressures greater than those applied.

4.2. Pressure and carbon dioxide

Several reports have been published on the high rates of microbial inactivation achieved using carbon dioxide (CO_2) under pressure (Haas et al., 1989; Heinz and Knorr, 1995; Balaban et al., 2001; Erkman, 2001). Even supercritical CO_2 (at 20 MPa and 35 °C) has been shown to cause up to 7-log cycle reductions of bacteria and yeast due presumably to the low-pH inactivation of metabolic enzymes and/or the extraction of cellular substances such as phospholipids (Kamihara et al., 1987). Exposure to supercritical CO_2 has also been used to reduce both viability and pressure resistance of *Bacillus* spores (Kamihara et al., 1987; Heinz and Knorr, 1995).

Balaban et al. (2001) designed a system to continuously mix fruit juice with liquid CO_2, pressurized to the desired level and then depressurized to remove CO_2 from the juice. Using this system with a pressure application of just 35 MPa, *E. coli* was reduced by 4-log cycles, while *Salmonella* and *Listeria* both showed a 6-log cycle reduction. Haas et al. (1989) reported 5- to 8-log cycle reductions in microbial numbers using just 55 MPa in the presence of CO_2, while Erkman (2001) observed more than 5-log reductions of *Yersinia enterocolitica* with carbon dioxide at 6 MPa.

It has been suggested (Haas et al., 1989) that pressurized CO_2 penetrates cells relatively easily, causing a greater intracellular pH change than other acids. Others have suggested that the CO_2 dissolves in the aqueous phase as carbonic acid, then expands upon sudden pressure release to cause damage to the cell (Patterson et al., 1995).

4.3. Pulsed electric fields (PEF) and antimicrobial agents

Nisin combined with PEF treatment has caused additive inactivation of bacteria in liquid foods, whether nisin was added to the sample prior to PEF exposure or added to an agar growth medium before plating of microorganisms subjected to PEF (Calderon-Miranda et al., 1999a; Terebiznik et al., 2000). Under conditions of low treatment intensity, PEF application followed by nisin exposure caused additive inactivation of *Listeria innocua* in liquid egg, but a synergistic interaction was observed as the intensity of PEF or concentration of nisin was increased (Calderon-Miranda et al., 1999b).

Numerous other researchers (Iu et al., 2001; Jagus et al., 1999; Liang et al., 2002; Pol et al., 2000; Smith et al., 2002) have also observed synergistic inactivation of Gram-positive and Gram-negative bacteria when PEF treatments are combined with nisin and/or lysozyme addition. While the mechanism of synergy is not yet fully understood, the additional stress of PEF probably facilitates the antimicrobial agent's access to the cytoplasmic membrane. Both treatments may inflict damage, resulting in more or larger pores, or pores with greater stability (Pol et al., 2000; Smith et al., 2002). The bactericidal effects of nisin/lysozyme mixtures on PEF-treated cells were more pronounced than addition of either nisin or lysozyme alone (Liang et al., 2002).

PEF alone appears to have very little effect on bacterial spores (Knorr et al., 1994; Sitzmann, 1995; Grahl and Markl, 1996; Pagan et al., 1998) although some studies have reported successful destruction in saline solutions (Marquez et al., 1997; Dantzer et al., 1999). PEF does not induce germination of spores (Knorr et al., 1994; Marquez et al., 1997), but if germination is initiated by other methods, the resulting vegetative cells will become sensitive to an electric field (Knorr et al., 1994; Barbosa-Canovas et al., 1998). Su et al. (1996) used this approach to inactivate more than 95% of *B. subtilis* spores when PEF was applied to a saline spore suspension containing 0.01% of the germinating agent L-alanine. Lysozyme combined with PEF has shown mixed abilities to inactivate spores (Barbosa-Canovas et al., 1998; Pagan et al., 1998). Where significant spore inactivation was achieved, it was possible that the lysozyme dissolved the spore coat, thus rendering the cell susceptible to an electric field (Barbosa-Canovas et al., 1998).

Similar increases in microbial inactivation under PEF and HHP have been observed with other anti-

microbial agents. Ethylenediaminetetraacetic acid (EDTA) enhanced the lethal effect of pressure against *E. coli* in buffer (Hauben et al., 1996) and nonspore-forming bacteria in milk (Timson and Short, 1965). EDTA binds divalent cations, such as Ca^{++} and Mg^{++}, which are essential for maintaining structural integrity of Gram-negative outer membranes (Wilkins and Board, 1989). The presence of EDTA and other chelating agents also enhances the action of nisin against Gram-negative bacteria (Stevens et al., 1992). The EDTA disrupts the cell's outer membrane, allowing nisin to penetrate and access the cytoplasmic membrane (Abee et al., 1995).

4.4. Ultrasonication and antimicrobial agents

Arce-Garcia et al. (2002) reduced the intensity and duration of ultrasound treatment required to inhibit *Zygosaccharomyces rouxii* by 67% and 33%, respectively, by incorporating potassium sorbate, sodium benzoate or eugenol into the recovery media. The authors suggested that the different modes of action of the ultrasonication, mild heating (45 °C) and antimicrobial hurdles were responsible for the observed inhibition. Ahmed and Russell (1975) found the combination of ultrasonication and hydrogen peroxide was many times more lethal to both *Bacillus* and *Clostridium* spores than either treatment alone. It was believed that the ultrasonic waves improved lethality of the hydrogen peroxide by increasing the permeability of the cells, increasing the rate of reaction between the hydrogen peroxide and cell components, and dispersing cell aggregates to increase the surface area for contact.

4.5. Irradiation and antimicrobial agents

Licciardello et al. (1984) extended the shelf life of iced cod fillets from 19 to 25 days when irradiation was supplemented with dipping of the fillets in a 5% potassium sorbate solution. Another study reported a doubling of the shelf life of Dover sole when 0.19% sodium benzoate was added to samples before irradiation (Monk et al., 1995). Vacuum and modified atmosphere packaging have also been shown to work well with irradiation treatments to extend the chilled shelf life of fresh beef, pork and fish (Licciardello et al., 1984; Lambert et al., 1991, 1992).

5. Nonthermal processes combined

Successful combinations of nonthermal processes depend not only on enhanced microbial inactivation but also on the technical compatibility of the selected processes. For example, product characteristics are an important consideration, as PEF and ultrasonication are mainly restricted to treatment of liquid products, while ultraviolet radiation and pulsed light are limited to application on food or packaging surfaces (Barbosa-Canovas et al., 1998; Sizer and Balasubramaniam, 1999; U.S. Food and Drug Administration, 2000; Butz and Tauscher, 2002). The biochemical effects of nonthermal processing on the product also need consideration because molecular and biological modifications caused by some technologies (e.g. HHP) have the theoretical potential to enhance, neutralize or reduce the effectiveness of others (Earnshaw et al., 1995).

General aspects of the practical application of HHP, PEF, ultrasonication and irradiation to foods are briefly described in Sections 3.1–3.4 of this review.

5.1. Ultrasonication and high pressure

At ambient temperature and pressure, ultrasonication has little lethal effect on microorganisms (Raso et al., 1998a; Pagan et al., 1999). High treatment intensities may cause some microbial inactivation, but simultaneously produce adverse sensory changes in the food (Williams, 1994; Sala et al., 1995). A much more gentle yet effective treatment, termed manosonication (MS), utilizes moderate doses of ultrasonication under mild pressure. Manothermosonication (MTS) describes an MS process that is carried out at elevated temperatures (Williams, 1994; Manvell, 1997).

Raso et al. (1998a) studied the inactivation of *Y. enterocolitica* by combining ultrasonication, pressure and heat. The lethal effect of ultrasonication (20 kHz, 150 μm) increased with rising pressure until an optimum pressure of 400 kPa was reached, when maximum inactivation occurred. Levels of destruction of *B. subtilis* spores using MTS (20 kHz, 117 μm) followed a similar trend under increasing pressure, with maximum inactivation reached at a pressure of 500 kPa (Raso et al., 1998b). In both studies, the

lethal effect of pressurization was significantly more pronounced when the amplitude of ultrasonic waves was increased.

Pagan et al. (1999) also found that the ultrasonic (20 kHz, 117μm) inactivation of *L. monocytogenes* increased dramatically when the pressure was raised from ambient to 200 kPa. The increase in the inactivation rate became progressively smaller, however, as the pressure was raised from 200 to 400 kPa. The authors theorized that the higher lethality of ultrasound under moderate pressure was due to the higher intensity of cavitation. It should be clarified that the pressures applied during manosonication (e.g. 200–600 kPa) are not in the lethal range of pressures applied during HHP treatment (e.g. 50–1000 MPa; Williams, 1994).

Sala et al. (1995) found the lethality of MTS treatments for bacterial cells, spores and yeast to be 6–30 times greater than thermal treatments of equal temperature and concluded that the combined effects of ultrasonication, pressure and heat were synergistic. Raso et al. (1998a) and Pagan et al. (1999) also noted enhanced microbial inactivation when MS was combined with temperatures above 50 °C. Unlike the synergism between ultrasound and pressure, however, the lethality of MS plus heat was additive only and appeared to be due to two different mechanisms acting independently.

5.2. Ultrasonication and pulsed electric fields (PEF)

Su et al. (1996) observed that ultrasound enhanced the inactivation of *B. subtilis* spores under PEF; however, no details were given in their published abstract as to the intensity of the ultrasonication treatment, whether it was performed before, during or after PEF, and what the actual levels of inactivation were. Jin et al. (1998) inactivated 4-log cycles of *B. subtilis* spores using 2000 Hz sonication combined with PEF at 30–40 kV/cm.

5.3. High hydrostatic pressure (HHP) and pulsed electric fields (PEF)

Pressurization may reduce the effectiveness of a PEF treatment, as Knorr (2001) found that electric field pulse treatment under high pressure (200 MPa) exerted a protective effect against permeabilization of bacterial cell membranes although each treatment alone cause major damage at this site.

Pagan et al. (1998) studied the possibility of germinating *Bacillus* spores using HHP, then inactivating the germinated cells with a PEF treatment. They found that germination of more than 5-log cycles of spores was initiated by pressurization, and while the germinated cells did become sensitive to a subsequent heat treatment, they were not sensitized to PEF application below 40 °C. It was suggested that spore inactivation by these combined processes could be improved by adding an intermediate holding step to allow germinated spores to outgrow into vegetative cells.

Shimada and Shimahara (1991) treated *E. coli* cells with a 50-Hz alternating current separately and before to HHP treatment. The mechanism of cell inactivation by alternating current is not the same as for PEF, as alternating current causes cell death at sublethal temperatures by the formation of toxic substances in the treatment suspension as a result of electrolysis (Shimada and Shimahara, 1981). The survival of *E. coli* was reduced by 50% to 90% by the combined treatment, which was attributed to the two-step degradation of the bacterial membrane barrier by the slightly different mechanisms of the electric current and high pressure (Shimada and Shimahara, 1991).

5.4. Irradiation and high hydrostatic pressure (HHP)

Sale et al. (1970) investigated the use of gamma irradiation before to HHP treatment of *B. coagulans* spores and found that mildly lethal doses of radiation increased the pressure sensitivity of survivors. Crawford et al. (1996) also found that irradiation sensitized *Clostridium sporogenes* spores to pressure and that mild doses of irradiation and high pressure combined was more effective for inactivating *Clostridium* spores than application of either process alone. Gould and Jones (1989) reported the effects of simultaneously applying pressure and ionizing radiation to spores. The two treatments were additive in their sporicidal effect; thus, the intensity of one or both treatments could be lowered while retaining a degree of inactivation. The mechanisms of spore destruction were postulated as either the germination of spores by HHP and consequent sensitization to radiation, or the disruptive effect of irradiation on peptidoglycan in the

spore cortex, allowing partial rehydration of the core and increased sensitivity to both radiation and pressure (Gould and Jones, 1989).

6. Will nonthermal processing make heat obsolete?

Nonthermal processing technologies were designed to eliminate the use of elevated temperatures during processing and so avoid the adverse effects of heat on the flavour, appearance and nutritive value of foods (Barbosa-Canovas et al., 1999). Heat remains a powerful and reliable hurdle, however, whenever microbial inactivation is desired. Temperatures of 40 to 70 °C applied during HHP, PEF, manosonication and irradiation have been shown to cause significantly greater inactivation of vegetative microorganisms (Garcia et al., 1989; Jayaram et al., 1992; Williams, 1994; Patterson et al., 1995; Sala et al., 1995; Vega-Mercado et al., 1996; Alpas et al., 1999; Garcia-Graells et al., 1999; Sepulveda et al., 2002) and bacterial spores (Timson and Short, 1965; Sale et al., 1970; Burgos et al., 1972; Gould and Jones, 1989; Hayakawa et al., 1994; Earnshaw, 1996; Roberts and Hoover, 1996; Balasubramaniam et al., 2001) in foods, compared with treatments at ambient temperatures.

According to Ohlsson (1994), increased product safety is much in demand by food manufacturers and distributors. It could be proposed, therefore, that the role of emerging nonthermal technologies need not depend solely on the production of minimally processed foods, as they may also be used to complement traditional heat processes for production of safer and commercially sterile foods. Conversely, combining lethal heat treatments with novel nonthermal processes might help eradicate problematic microbial subpopulations that show high resistance to some nonthermal technologies (Patterson et al., 1995; Rademacher et al., 1998; Raso et al., 1998c).

While there are potential benefits to food safety of combining high-temperature conditions with emerging nonthermal processes, the use of high heat negates the original goals of nonthermal processing. Moreover, such an approach does not address the modern consumer's concerns, as the desire for long life with ambient storage of products has been replaced by the trend towards fresh, natural and minimally processed foods (Ohlsson, 1994).

7. Conclusion

Most novel nonthermal technologies are still in their early stages of development although some emerging nonthermal processes have now been implemented in industrial-scale systems for commercial and research applications (Mermelstein, 1997; Kempkes, 2001, personal communication; Satin, 2002). Other than irradiation, however, it is safe to say that not one nonthermal process has been developed to a point where its use alone can guarantee the safety of low-acid foods.

Effective combinations of two or more preservation hurdles may be chosen once the modes of action and cellular targets of each treatment are known (Leistner, 2000). For the intelligent selection of nonthermal processing combinations, therefore, target elements within cell and the effects of treatments on those elements need to be determined. The treatment intensities required for cell inactivation need quantification and standardization also.

The small number of scientific studies summarized in this review show that combining nonthermal treatments has great potential for improving the safety and quality of foods although many technological and regulatory barriers still need to be overcome before the food supply can receive these benefits.

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