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Morphological changes of temperature- and pH-stressed *Salmonella* following exposure to cetylpyridinium chloride and nisin

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Abstract

The outer membrane of Gram-negative bacteria such as *Salmonella*, act as a permeability barrier, preventing nisin gaining access to the cytoplasmic membrane. If the outer membrane permeability is reduced, however, Gram-negative bacteria can show nisin sensitivity. In this study, temperature stresses (heating, chilling and freezing), pH stress (pH 4.5, 5.0, 6.0) and cetylpyridinium chloride (CPC)-nisin treatment were used to alter the outer membrane permeability of *Salmonella*, producing a loss of barrier function and reduced resistance to nisin. The morphological changes in *Salmonella* were examined using scanning electron microscopy. Temperature and pH-stressed *S. typhimurium* cells, untreated and treated with CPC-nisin had perturbed cell morphology, including apparent indentations and craters in the cell surfaces and collapsed amorphous bodies.

Keywords: *Salmonella*; Nisin; Cetylpyridinium chloride; Outer membrane

1. Introduction

Nisin is a 35-amino acid cationic peptide antimicrobial bacteriocin, produced by *Lactococcus lactis* subs. *lactis* (Delves-Broughton, Blackburn, Evans, & Hugenholtz, 1996) and has been accepted as a food additive by both the FDA and WHO. As a result to date it has found several applications in food (Schillinger, Geisen, & Holzapfel, 1996). Nisin is active against almost all Gram-positive bacteria and their germinated spores, but not against Gram-negative bacteria or fungi (Stevens, Klapes, Sheldon, & Klaenhammer, 1992; Delves-Broughton et al., 1996). The sensitivity of some Gram-negative bacteria to nisin has been reported (Stevens, Sheldon, Klapes, & Klaenhammer, 1991). The interference of nisin in the cytoplasmic membrane of susceptible species leads to pore formation and dissipation of the proton motive force. The consequence is the efflux of low-molecular-weight solutes, such as amino acids and K^+ which are involved in the maintenance of the cells; turgor pressure, enzyme activation, and regulation intracellular pH homeostasis, together with depletion of intracellular ATP (Moll, Roberts, Konings, & Driessen, 1996; Montville, Chung, Chikindas, & Chen, 1999). In some bacteria, this interference of nisin with lipid II (C55 bactoprenol pyrophosphate—a carrier involved in cell wall biosynthesis) in the membrane has been proved to be the cause of death due to loss of cell integrity (Brotz et al., 1998; Chung & Hancock, 2000).

In general, the protective outer membrane, surrounding the cytoplasmic membrane and peptidoglycan layer of Gram-negative cells cannot be damaged by nisin. The inner and outer membranes are composed of glycerophospholipids and lipopolysaccharides, respectively. Lipopolysaccharides, which are composed of a lipid part and heteropolysaccharide, partly show an anionic characteristic which is an essential property of the

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hydrophilic surface (Nikaido, 1996). Since outer membrane is a permeability barrier of hydrophobic substances and macromolecules, nisin, as a hydrophobic macromolecule ($M_r = 3353$), cannot, reach its site of action (Helander & Mattila-Sandholm, 2000). To use nisin to act as an antimicrobial reagent for Gram-negative bacteria, outer membrane of Gram-negative should be disrupted to allow the reaching of nisin to its site of target into its cell. Outer membrane can be damaged by various methods; e.g. by using chelating agents including EDTA, or by pretreatment with cell membrane disrupters such as trisodium phosphate or by physical treatments (hydrostatic pressure, heat, freezing and thawing) (Kalchayanand, Hanlin, & Ray, 1992; Cutter & Siragura, 1995; Hauben, Wuytack, Soontjens, & Michiels, 1996; Boziaris, Humpheson, & Adams, 1998; Carneiro de Melo, Cassar, & Miles, 1998). These disruption mechanisms result in the changes of morphologies and structures of outer membrane such as blebs, vesiculation and damage or release of lipopolysaccharides. These changes can alter the permeability barrier of the outer membrane, resulting in the efflux of periplasmic enzymes, and sensitivity to hydrophobic compounds, dyes and surfactants (Katsui et al., 1982; Tsuchido, Katsui, Takeuchi, Takano, & Shibasaki, 1985). However, such injury can be subsequently repaired if the cell is not severely disrupted (Boziaris & Adams, 2001).

Cetylpyridinium chloride (CPC) is a quaternary ammonium compound having antimicrobial properties against many bacteria such as *Esherichia coli* O157: H7, coliforms, *Salmonella typhimurium*, *Campylobacter jejuni*, *Aeromonas hydrophila*, *Listeria monocytogenes*, and *Staphylococcus aureus* as well as viruses (FDA, 1998; Polhman, Stivarius, McElyea, & Waldroup, 2002). It is classified as a cationic surface active agent and can therefore absorb phosphates in negatively charged bacteria cell membranes, resulting in cell wall disruption (Randford, Beighton, Nugent, & Jackson, 1997).

Salmonella, a Gram-negative bacterium, is one of the most important causes of foodborne disease. Its contamination in food products brings significant public health concern (Jung et al., 2003). Generally, during food processing, the outer membrane of Gram-negative bacteria including *Salmonella* could be disrupted by stress conditions such as temperature and pH stress occurring during food processing. Accordingly, nisin could be applied as biopreservative to inhibit an activity of these Gram-negative bacteria in food production under these stress conditions. In addition, the use of disruptive agents such as CPC to increase the damage of a outer membrane could be a significant alternative. Therefore, this study aimed to investigate the effect of temperature- and pH-stress following a CPC-nisin treatment on *Salmonella typhimurium* by determining of morphological changes of cells using scanning electron microscopy (SEM), and viability of cells.

2. Materials and methods

2.1. Bacterial strain

S. typhimurium S36 used throughout this study containing the mini-Tn5 plasmid containing the complete *lux* (CDABE) with kanamycin resistance gene cassette. It was grown in 100 ml of brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK) added with kanamycin (30 μg/ml), at 37 °C and 150 rpm for 12–14 h. Culture was transferred to a sterile tube and centrifuged at 4000*g* for 10 min, 4 °C to sediment bacterial cells. Cell pellets were washed once in sterile maximum recovery diluent (MRD; Oxoid, Basingstoke, UK) and resuspended in MRD. The populations ca. 10^7 cfu/ml (A_{600} 0.2) of cells in the suspensions were used for following study.

2.2. Cetylpyridinium chloride (CPC) and nisin

Cetylpyridinium chloride (CPC) (Sigma, UK) solution was prepared at concentration of 0.05 g in 100 ml distilled water. Nisin (Sigma, UK) solution was prepared at concentration of 100 μg nisin in 1 ml of 0.02 mol/l HCl. Each solution was filter sterilized (0.45 μm, Millipore, France) and stored at 4 °C until used.

2.3. Temperature stress

The temperature stresses (heating, chilling, and freezing/thawing) of 10 ml (10^7 cfu/ml) of *S. typhimurium* S36 were conducted in universal bottles containing MRD. For heating, the bottles containing *Salmonella* suspensions were heated at 55 ± 1 °C for 10 min in water-bath and immediately placed in cold water at 4 °C for 5 min before moving to room temperature (22 °C) for 5 min. For chilling, the cell suspensions were chilled in an ice water-bath (~0.5 °C) for 30 min and then let to room temperature for 5 min. Freezing/thawing treatment was operated by placing the cell suspensions in a freezer at −20 °C for 24 h, then placing in an incubator at 37 °C for 30 min to let its thaw before moving to room temperature for 5 min. The control cells were placed in a stationary incubator at 37 °C for 30 min and then left for 5 min at room temperature.

The viability of temperature stressed cells was determined. All suspensions of temperature stresses were serially diluted in MRD and were spread onto BHI agar. Plates were incubated at 37 °C for 16–18 h

after which colonies were counted. The temperature-stressed cells were also subjected to an examination of morphological changes using SEM.

2.4. pH stress

Portions (100 μl each) of *Salmonella* suspensions were inoculated in 100 ml of BHI broth under the following conditions: pH 4.5, 5.0, 6.0, and 7.4 (control) with shaking at 150 rpm for 12–14 h, at 37 °C. The pH-stressed cells were harvested by centrifugation with a Beckman model J2-21 centrifuge (Beckman Instruments Inc., Glenrothes, UK) (4000*g* for 10 min) at 4 °C. The harvested cells were washed with 10 ml of MRD, and then resuspended the pellet in MRD to give a cell density of ca. 10^7 cfu/ml (A_{600} 0.2). The pH-stressed cells were subjected to a determination of cell viability and morphological changes as just those described.

2.5. CPC and nisin treatments

Ten milliliters of temperature and pH-stressed *Salmonella* suspensions were treated with 1 ml of 0.05 g/100 ml CPC for 30 s then filtered through a sterile Wahtman filter paper (0.2 μm) suddenly. The filter paper was then placed into a tube containing 10 ml of MRD, and vortexed for 30 s to remove bacterial cells. The CPC-treated cells were further treated with 1 ml of nisin (100 μg/ml) for 30 min. Suspension containing CPC-nisin-treated cells were then subjected to cell viability and morphological changes determination.

2.6. Determination of morphological changes by SEM

In order to visualize any morphological changes in the cells following exposure to CPC-nisin, the control, and temperature and pH-stressed *Salmonella* cells were examined by SEM.

The CPC-nisin treated cell (5 ml) suspension was filtered through a cellulose nitrate membrane. The bacteria on the filter were fixed in the mixture of 3 g glutaraldehyde/100 ml solution for 2 h at 4 °C, and washed three times for 30 min each in 0.1 sodium mol/l phosphate buffer (pH 7.2). The cells were post-fixed with 1 g osmium tetroxide (OsO_4)/100 ml solution in phosphate buffer for 1 h, before rinsing twice in the same buffer for 30 min. For dehydration, the membranes were serially dehydrated in a gradient series of ethanol for 5 min each. The samples were then dried in a critical point dryer, and mounted on aluminum stubs, sputtered with gold, and viewed under a JSM-6400 scanning electron microscope (JEOL, Japan) at an operating voltage of 20 kV.

2.7. Imaging of bacterial cells stained with the BacLight nucleic acid stain

Twenty microliters of samples were dropped onto glass microscope slides and an aliquot (3 μl) of the Live/Dead BacLight stain mixture (Molecular Probes, Leiden, The Netherlands) consisting of SYTO 9 and propidium iodide (1:1) nucleic acid stains were spotted on. A coverslip was placed over the suspension. The preparation was incubated for 10–15 min in the dark at room temperature. The stained samples were examined by an epifluorescent microscope (Nikon DXM-1200/LUCIA, Japan) equipped with a mercury lamp. This staining procedure results in an appearing of fluorescent green in live cells and fluorescent red in dead cells.

2.8. Statistical analysis

Bacterial numbers were transformed to $\log_{10}$ value for statistical analysis. Data were analysed with SAS (Statistical Analysis Systems Institute, Cary, NC). The mean values and the standard deviation (SD) were calculated from the data obtained. Significant differences between means were determined by the Duncan's multiple range tests.

3. Results and discussion

3.1. Effect of CPC-nisin treatment on the cell viability

The results in Table 1 show the kill of *S. typhimurium* S36 after treatment with heating, chilling and freezing. The viability of freeze-stressed *S. typhimurium* cells was relatively different from other temperature stresses and control. After treating all temperature-stressed cells with CPC-nisin, freeze-stressed cells also showed the highest

Table 1
$\log_{10}$ colony forming units (cfu/ml) of temperature-stressed *S. typhimurium* following treatment with 0.05 g/100 ml CPC and 100 μg/ml nisin

Sample	$\log_{10}$ cfu/ml[2]		$\log_{10}$ reduction (cfu/ml)[2,3]
	Untreated	CPC-nisin treatment	
Control[1]	7.06 ± 0.14	4.15 ± 0.38	2.91 ± 0.41[c]
Heating	6.93 ± 0.10	3.50 ± 0.14	3.43 ± 0.22[bc]
Chilling	7.00 ± 0.08	4.00 ± 0.07	3.00 ± 0.09[c]
Freezing	6.44 ± 0.29	1.28 ± 1.01	5.16 ± 0.75[a]

[1]Control = 37 °C

[2]Values are mean ± standard deviation of duplicate determinations from three trial experiments.

[3]Means within a column without common following letters are significantly ($P < 0.05$) different.

kill ($P<0.05$) of 5.16 $\log_{10}$ cfu/ml. Heat stress with CPC-nisin treatment indicated higher kill ($P<0.05$) of 3.43 $\log_{10}$ cfu/ml than chill stressed with CPC-nisin treatment, which was not significantly different from control ($P>0.05$) in terms of cell viability. In general, outer membrane of Gram-negative bacteria acts as a protector for the reaching of nisin to cytoplasmic membrane. If the outer membrane is damaged or altered by physical treatments or permeabilizers, the Gram-negative bacteria could be sensitive to nisin (Boziaris et al., 1998; Helander & Mattila-Sandholm, 2000; Ray, Miller, & Jain, 2001). Tsuchido et al. (1985) reported that conformational alterations of the outer membrane protein and lipopolysaccharide, which can take place during heating, change the structure of the outer membrane and its permeability. The outer membrane response to temperature changes, therefore, it is the first component that suffers damage and it is where most cellular changes occur to prevent or repair damage.

Exposure of bacteria to low temperature (chilling) may induce phospholipids and fatty acid alterations, resulting in alteration of outer membrane (Berry & Foegeding, 1997). According to results in this study, the viability of chill-stressed cell of *S. typhimurium* S36 was not different from control. This might indicate that chill stress did not induce the change in the outer membrane of cells, which agrees with the Boziaris and Adams's (2001) study, who found that chilling did not cause a significant damage of outer membrane of *Pseudomones aeruginosa, S.* Enteritidis PT4 and *S. Enteritidis* PT7.

As for freezing, during freezing of aqueous suspensions of bacterial cells the microbes become concentrated in the unfrozen portion of the solution where they are subjected to the effect of concentrated solutes. As the temperature is reduced and more water is frozen, the increase in solute concentration in the unfrozen liquid results in diffusion of water from the microbial cells. Injury to *Salmonella* during freezing is probably due to formation of ice crystals from outside the cell (extracellular ice) and draw water out of the cell with a resulting dehydration or concentration effect. Intracellular crystals may form and grow or crystallize right through the cell, resulting in altered permeability or holes in the membrane and cell wall (Frazier & Westhoff, 1988; Lund, 2000). After thawing leakage of cell material occurs and there is an increased permeability of compounds into the cells (Macleod & Calcott, 1976). As a result in this study, the viability of freeze-stressed *S. typhimurium* S36 cells was relatively lower than control. This could be an effect of freeze/thaw on cell wall and/or cell membrane damages. Consequently, this kind of cells could significantly induce CPC-nisin sensitivity. Hence, number of death cells from freeze stress treated with CPC-nisin treatment was higher than heat and chill stress treatment. This is supported by reports of Stevens et al. (1991) and Kalchayanand et al. (1992), who suggested that the application of sublethal stress in particular freezing was shown to disrupt the permeability barrier of the lipopolysaccharide leading to an increased sensitivity of *Salmonella enterica* and *E. coli* towards nisin.

Microorganisms could expose to dramatic pH fluctuations in nature. Exposure to acidic pH is of major importance in food microbiology because contaminating microorganisms commonly face low external pH in many acidic or acidulated foods and at some stages in food production. In this study, *S. typhimurium* was stressed at the lower pH (6.0, 5.0, and 4.5) than its optimal pH (7–7.5) for *Salmonella* spp growth (Lund & Eklund, 2000). *S. typhimurium* S36 at the lowest used pH (4.5) and treated with CPC-nisin showed the highest value of log reduction of 5.36 $\log_{10}$ cfu/ml ($P<0.05$) (Table 2). Also, pH-stressed (pH 5.0) cells treated with CPC-nisin presented higher log reduction of 3.77 $\log_{10}$ cfu/ml ($P<0.05$) than unstressed *Salmonella* (pH 7.4) cells. On contrary, pH stressed (pH 6.0) and unstressed cells treated with CPC-nisin did not give significantly different ($P>0.05$) log reductions (Table 2). Damage of outer membrane is one kind of damage to enteric bacteria like *E. coli* and *Salmonella* spp. which is caused by stresses. Stress due to acidity might damage the outer membrane, and even if bacteria are not killed by damage, it allows other lethal agents (for example, nisin), which are normally unable to penetrate, to pass through to the cytoplasmic membrane and destroy the cell (Rowbury, 2003). Our results demonstrated that cells treated at pH 4.5, which is the lowest pH, and treated with CPC-nisin might increase ruptured of outer membrane and enhance cells sensitivity to CPC-nisin treatment.

Both temperature and pH-stressed cells were treated with CPC-nisin, bacteria cell membrane can be increase perturbed, possibly alter their outer membrane and enhance permeability results in increasing injury of cells later (Randford et al., 1997; Hamouda & Baker, 2000).

Table 2
Log_{10} colony forming units (cfu/ml) of pH-stressed *S. typhimurium* following treatment with 0.05 g/100 ml CPC and 100 μg/ml nisin

pH	$\log_{10}$ cfu/ml[1]		Log_{10} reduction (cfu/ml)[1,2]
	Untreated	CPC-nisin treatment	
7.4	7.07±0.12	3.87±0.19	3.20±0.05^{c}
6.0	7.06±0.11	3.50±0.24	3.56±0.19^{bc}
5.0	7.09±0.11	3.32±0.23	3.77±0.01^{b}
4.5	7.00±0.12	1.64±0.22	5.36±0.72^{a}

[1]Values are mean±standard deviation of duplicate determinations from three trial experiments.

[2]Means within a column without common following letters are significantly ($P<0.05$) different.

Thus, under these circumstances nisin can gain access to the cytoplasmic membrane where it exerts its effect, favoring the formation of pores through which a rapid efflux of ions and cytosolic solutes (amino acids and nucleosides principally) take place (Driessen et al., 1995). The synergistic action of physical stresses: temperature, pH stress and CPC-nisin may reflect its ability to alter membrane fluidity and hydrophobic interactions between phospholipid acyl chains in the bilayer. Any increase in membrane fluidity made the membrane more sensitive to nisin (Li, Chikindas, Ludescher, & Montville, 2002). Boziaris and Adams (2001) have reported that injury during physical stresses was measured by changes in cell surface hydrophobicity and loss of lipopolysaccharide. This is presumably due to the temporary appearance of phospholipids in the outer membrane filling the void space (Nikado & Vaara, 1985). Increases in cell surface hydrophobicity were found in the heating and freezing stress treatments, and also increased hydrophobicity appeared to be related to lipopolysacharide release. By contrast, there was no change in hydrophobicity in chill stress treatment. These results demonstrated that physical stresses: temperature and pH stress could induce sublethal injury in the cell wall and membrane which act synergistically with CPC-nisin and producing larger numbers of cell death by enhancing these damages.

3.2. Scanning electron micrographs

The changes in cell morphology and topography due to temperature stress and CPC-nisin treatment were examined by SEM (Fig. 1). The control *S. typhimurium* cells have a normal rod shape with a smooth surface (Fig. 1A). Whereas, the cells treated with CPC-nisin lost their original shape and showed indentations on the surface (Fig. 1B). The control heat-stressed celled show minor indentations on the surface (Fig. 1C), in contrast, the CPC-nisin heat-stressed cells were completely collapsed (Fig. 1D). The control chill-stressed cells and CPC-nisin chill-stressed cells showed no significant change in shape, although there was some surface ruffling and some craters on the surface, respectively (Fig. 1E and F). Fig. 1G showed the freeze-stressed cells have similar appearance as the chill-stressed cells. Irregular changes in shape and collapse into amorphous bodies observed under freeze stress with CPC-nisin, as shown in Fig. 1H, may be due to cytoplasmic leakage.

Furthermore, Fig. 2 illustrated the changes of morphology caused by pH stress and treatment with CPC-nisin. The untreated controls and cells stressed at pH 6.0, 5.0, and 4.5 (Figs. 2A, C, E, and G, respectively) showed no significant change in the original smooth surface and rod shape. *Salmonella* cells treated with CPC-nisin showed ruffling and craters on the surface (Fig. 2B). As Fig. 2D showed the result of pH stress (pH 6.0), cells treated with CPC-nisin has morphological changes with some ruffling and some indentations on the cell surface. However, the craters on the surface were present after pH stress (pH 5.0) and treatment with CPC-nisin (Fig. 2F). Cells stressed at pH 4.5 and treated with CPC-nisin completely lost their shape, resulting during CPC-nisin treatment in indentations and amorphous collapsed bodies (Fig. 2H). These results indicate the possibility that the exopolysaccharide on the outer membrane of the cells might be untangled and released or the peptidoglycan or the cytoplasmic membrane partially perturbed (Slavik, Kim, & Walker, 1995). The presence of cracks in the outer membrane allowed penetration of cationic substances (Helander, Kilpeläinen, & Vaara, 1994). All of these scanning electron micrographs revealed the changes in the cell morphology caused by CPC-nisin treatment on temperature and pH-stressed cells. These results are in agreement with the report of Kalchayanand, Dunne, Sikes, and Ray (2004), who suggested the morphological changes of two Gram-negative pathogens, *E. coli* O157:H7 and *S. typhimurium*, were exposed to hydrostatic pressure and bacteriocin mixture (nisin and pediocin). Pressurization produced extensive changes in the cell envelope. Although the bacteriocin mixture did not cause any change in cell morphology of the two Gram-negative bacteria, when present during pressurization, they enhanced changes in the cell morphology. As our results presented above demonstrated that temperature stress, particularly freeze and heat stress, and pH stress at pH 4.5 and 5.0, could be caused obviously greater morphological changes on cell surfaces of *Salmonella*, allowed CPC and nisin to gain access to cytoplasmic membrane where pores were formed. Such changes in the cell envelope could account for the additive effect of the combination of temperature or pH stress treatment and CPC-nisin treatment on cell morphology that increase in killing *Salmonella*.

3.3. Imaging of bacterial cells stained with the BacLight nucleic acid stain

Epliflurescent micrographs of BacLight staining in Fig. 3 illustrated live and dead cells of *S. typhimurium* S36 treated with CPC-nisin. Control cells under untreated control condition, all cells reveal the green fluorescence of the SYTO9 stain, which represents live or intact cells (Fig. 3A). On the other hand, Fig. 3B shows both green (live) and red (dead) fluorescent cells after CPC-nisin treatment. Freeze-stressed control (Fig. 3C) and CPC-nisin-treated freeze-stressed (Fig. 3D) cells show some red cells when compared with the control cells (Fig. 3A) while CPC-nisin treatment after freeze stressing (Fig. 3D) showed a majority of red (dead) cells probably due to the alteration of outer

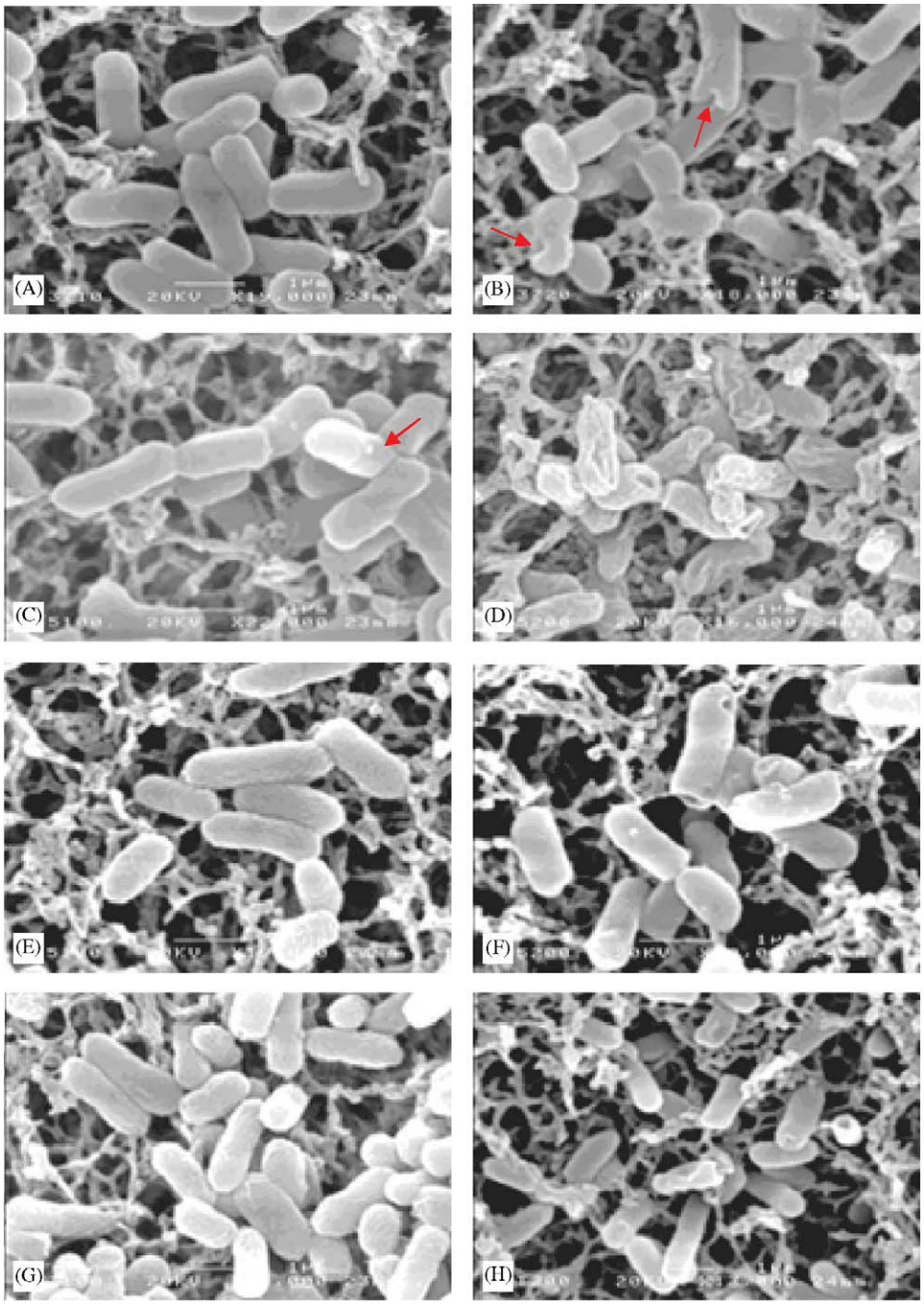

Fig. 1. Scanning electron micrographs of temperature-stressed *S. typhimurium* treated with 0.05 g/100 ml CPC and 100 μg/ml nisin. (A) Untreated control; (B) control treated with CPC and nisin; (C) heat-stressed control; (D) heat-stressed cells treated with CPC and nisin; (E) chill-stressed control; (F) chill-stressed cells treated with CPC and nisin; (G) freeze-stressed control; (H) freeze-stressed cells treated with CPC and nisin (the arrows indicated the indentations).

membrane permeability by ice crystal formation. Subsequently, the larger molecules of red flurochrome (propidium iodide) can gain access to damaged membranes, bind to DNA and stain cells in fluorescent red. Several reports (Haugland, 1996; Boulos, Pre′vost, Barbeau, Coallier, & Desjardins, 1999; Que′ric, Soltwedel, & Arntz, 2004) have revealed that propidium iodide with full access to cells with ruptured membrane is greater than that of SYTO9. The red fluorescence indicates that there is an increase in permeability of CPC-nisin treatments. Hence, greater propidium iodide uptake was observed in temperature and pH-stressed cells treated with CPC-nisin (data not shown). Overall the results of the BacLight nucleic acid stain correlate with cell viability and morphological changes from SEM.

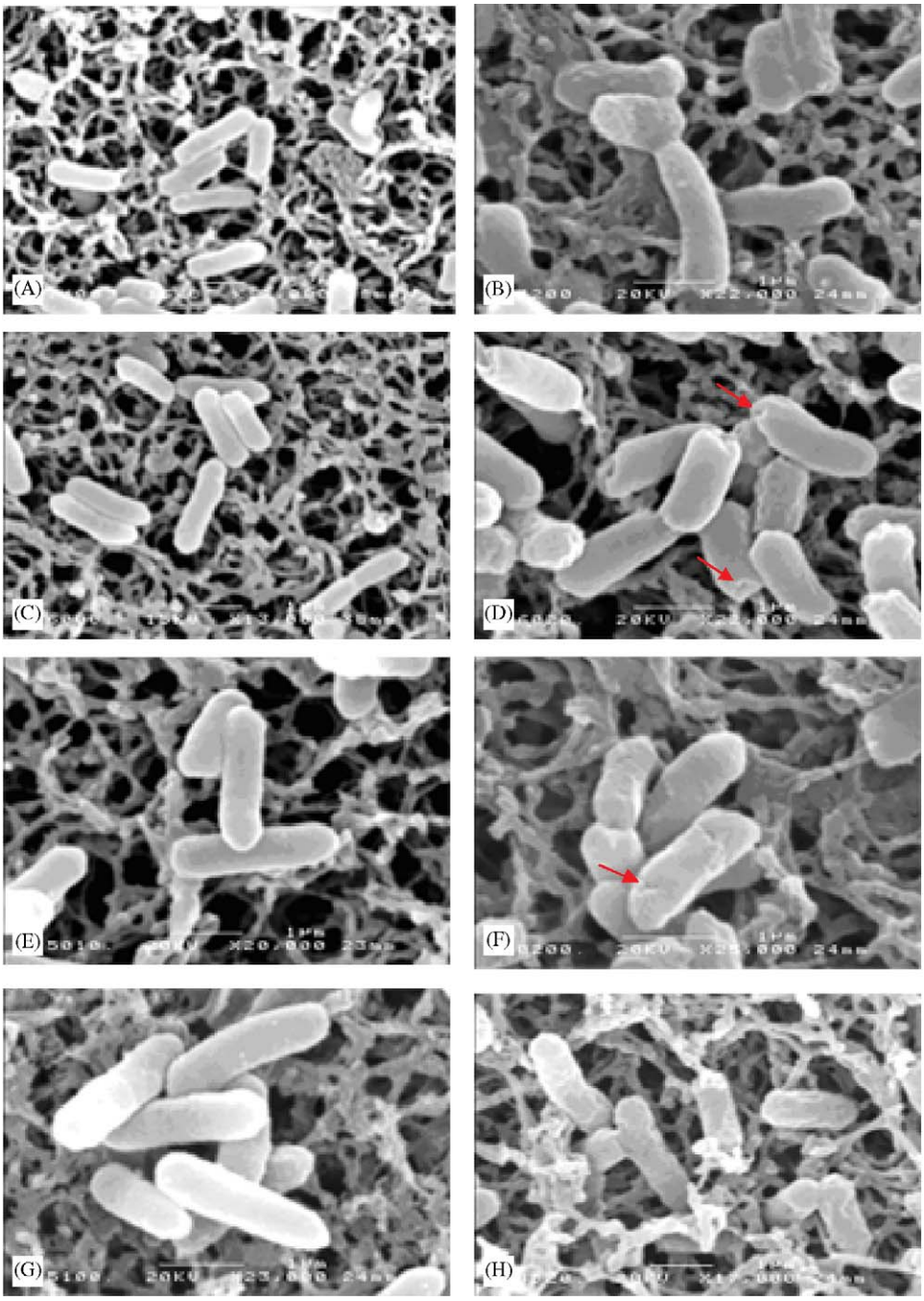

Fig. 2. Scanning electron micrographs of pH-stressed *S. typhimurium*-treated with 0.05 g/100 ml CPC and 100 μg/ml nisin. (A) Control (pH 7.4) cells; (B) cells treated at pH 7.4 and then with CPC and nisin; (C) cells treated at pH 6.0; (D) cells treated at pH 6.0 and then with CPC and nisin; (E) cells treated at pH 5.0; (F) cells treated at pH 5.0 and then with CPC and nisin; (G) cells treated at pH 4.5; (H) cells treated at pH 4.5 and then with CPC and nisin (the arrows indicated the indentations).

4. Conclusion

A variety of stresses (temperature and pH stress) can produce transient injury to the outer membrane permeability barrier of Gram-negative bacteria. Particularly, freeze-thaw and low pH (pH4.5) stressed *S. typhimurium* S36 cells were exposed to CPC-nisin treatment, produced very high numbers of cell death and extensive morphological changes in the cell envelope. These changes may due to the lost of outer membrane integrity, allow nisin access to the cytoplasmic membrane and pore formation resulting in leakage of cellular materials. The combination of CPC-nisin treatment during food processing could be used as a lethal process for Gram-negative bacteria, enhancing microbiological safety in food products.

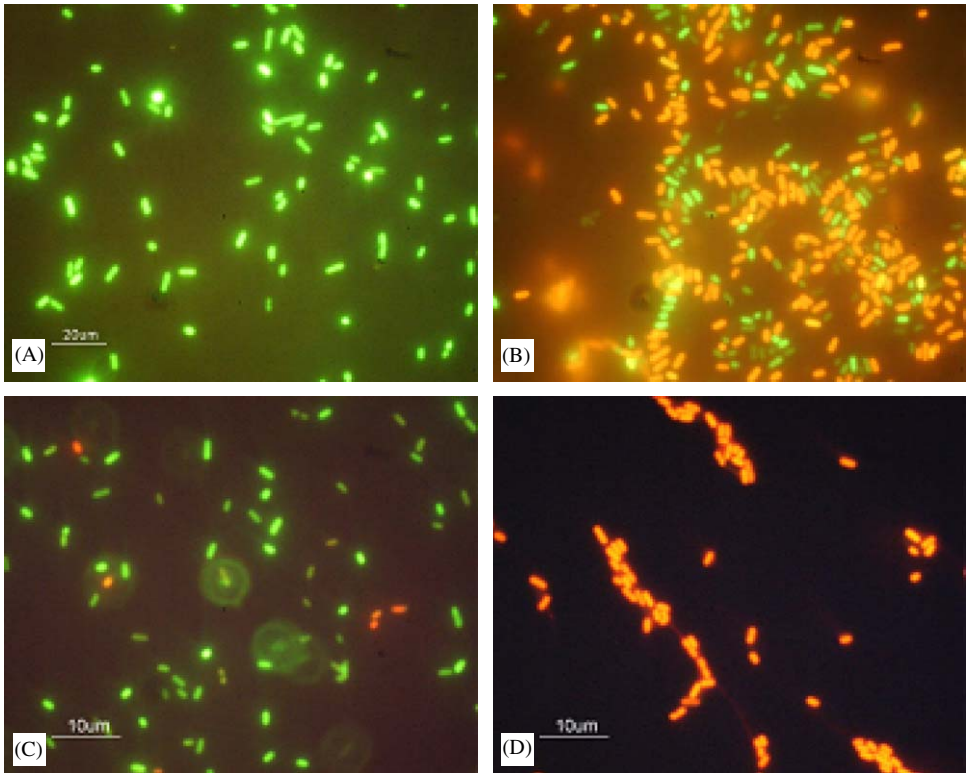

Fig. 3. Epifluorescent micrographs of live/dead cells of control and freeze-stressed *S. typhimurium* cells stained with the BacLight nucleic acid stain. (A) Untreated control cells; (B) control cells treated with CPC-nisin; (C) control cells after freezing; (D) freeze-stressed cells treated with CPC-nisin.

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