Kasetsart J. (Nat. Sci.) 39 : 622 - 632 (2005)

The Susceptibility of Bioluminescent *Salmonella typhimurium* Contaminating Chicken Carcasses to Cetylpyridinium Chloride and Nisin

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ABSTRACT

The effect of cetylpyridinium chloride (CPC) and nisin on the inhibition of viable bioluminescent *Salmonella typhimurium* contaminating chicken carcasses were conducted. The chicken drumstick sections (5 by 5 cm^2), inoculated with bioluminescent *S. typhimurium* S36, were treated with 0, 0.1, 0.3 and 0.5 % (w/v) CPC for 30 sec and further treated with 0, 20, 60 and 100 μg/ml nisin for 30 min. It was found that 0.5% (w/v) CPC and 100μg/ml nisin decreased ($P<0.05$) the numbers of *Salmonella lux*. Additionally, inoculated chicken drumstick sections were treated with 0.5% (w/v) CPC for 15, 30, 60 and 120 sec and then treated with 100 μg/ml nisin for 15, 30, 45 and 60 min. The use of 0.5% (w/v) CPC for 120 sec and 100 μg/ml nisin for 30 min increased ($P<0.05$) the loss of cell viability with minimal effect on the appearance of chicken carcass. These results also demonstrated the synergistic activity of CPC and nisin against *S. typhimurium* and can be expected to enhance the safety of chicken carcasses and other chicken products.

Key words: *Salmonella*, bioluminescent, cetylpyridinium chloride, nisin, chicken carcass

INTRODUCTION

The consumption of poultry increases every year and so does the concern for marketing a good quality and safe product. Bacterial contamination of poultry during commercial processing is undesirable though unavoidable. Chickens naturally carry a wide variety of bacteria into the processing plant and this microflora can be transferred onto the surface of carcasses during processing. *Salmonella typhimurium* has been reported as one of the predominant Gram-negative pathogenic bacteria in chicken meat causing serious food poisoning. It may infect poultry via the food chain and then contaminate during food processing or in food products (Uyttendaele *et al*., 1998; Vandhanasin *et al*., 2004). During the slaughtering and processing steps such as scalding, picking and chilling, poultry skin surfaces are contaminated with *Salmonella* (Medina, 2004). Various methods were implemented to reduce or eliminate of bacteria attached to poultry carcasses, for examples thermal treatments, chemical dipping and spraying and irradiation (Kim and Slavik, 1996). There are reports of various chemical methods currently in use in poultry processing, such as the use of chlorine during the chilling process and the use of chlorinated water to rinse

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Received date : 03/05/05 Accepted date : 20/07/05

poultry carcasses at the end of the evisceration line to reduce the numbers of *Salmonella*, but with limited effectiveness (Yang *et al*., 2001).

At present, instead of chemical preservatives, the interest is shifting to natural and minimally processed safe food with the application of biopreservatives such as bacteriocins for reducing bacterial contamination on the processing line. Delves-Broughton and Gasson (1994) reported that nisin, a heat-stable bacteriocin produced by *Lactococcus lactis* ssp. *lactis* inhibited Gram-positive bacteria such as *Listeria monocytogenes* and *Staphylococcus aureus* and prevented the outgrowth of spore of many *Clostridium* and *Bacillus* spp. In 1988, the U.S. Food and Drug Administration designated nisin with generally recognized as safe (GRAS) status. The C-terminal region of nisin binds to the cytoplasmic membrane of vegetative cells and penetrates into the lipid phase of the membrane, forming pores which allow the efflux of potassium ions and amino acids resulting in the dissipation of the proton motive force, together with depletion of intracellular ATP and eventually cell death (Breukink *et al*., 1999). Gram-negative bacteria become more sensitive to nisin whenever the outer membrane is penetrated by chelating agents including EDTA, or pretreated with cell membrane disrupters (Cutter and Siragusa, 1995; Carneiro de Melo *et al*., 1998). Gill and Holly (2000) reported the inhibition of gram-negative bacteria, *Escherichia coli* 0157:H7 and *S. typhimurium*, growth on ham and bologna by lysozyme, nisin and EDTA treatment. Log reductions of 1 and 1.5 for *Escherichia coli* 0157:H7 and *S. typhimurium* respectively were found.

Cetylpyridinium chloride (CPC) is a quaternary ammonium compound classified as a cationic surface-active agent which possesses antimicrobial properties against many microorganisms including viruses (FDA, 1998). The antimicrobial activity of CPC is due to an interaction of basic cetylpyridinium ions with acid groups of bacteria, which subsequently inhibits bacterial metabolism by forming weak ionic compounds that interfere with bacterial metabolism (Kim and Slavik, 1996). A maximum concentration of 0.1% is permitted for use in several dental products by the U.S. Food and Drug Administration. It has also been approved for limited human consumption in forms such as Cepacol RTM Lozenges (Lattin *et al*., 1994). Pohlman *et al*. (2002) and Bosilevac *et al*. (2004) revealed the application of CPC in beef trimming process before grinding that could reduce *E. coli*, coliform, and aerobic bacteria in ground beef, and microbial populations on cattle hides, respectively.

This research was performed to determine the appropriate concentrations and exposure times of CPC and nisin to decontaminate *Salmonella* on chicken carcasses, based on the OM permeability alteration by CPC solution and the resulting enhancement of nisin sensitivity. Macrodistribution of bioluminescent *S. typhimurium* cells attachment on chicken carcasses was also studied.

MATERAILS AND METHODS

1. Bacterial strain and culture preparation

Salmonella typhimurium transformed to a bioluminescent phenotype using the mini-Tn5 plasmid (kindly donated by Dr. Philip J. Hill, University of Nottingham, UK) containing the complete *lux* CDABE conferring kanamycin resistance gene cassette was used throughout the study. This plasmid was maintained within *E. coli* λPir1 (Simon *et al*., 1983). Competent *E. coli* S17-1 cells were transformed by electroporation with a purified plasmid preparation according to described by Winson *et al*. (1998). Conjugation between wild-type *S. typhimurium* (recipient cell) was isolated from a slaughter house and *E. coli* S17-lλpir was performed on a Whatman cellulose acetate membrane (0.45 μm pore size) overlaid onto a Luria-Bertani (LB) agar plate (Difco

Laboratories, Surrey, UK) that was subsequently incubated at 37°C for 14 h (De Lorenzo and Timmes, 1994). The selection of *S. typhimurium* exconjugants were selected by incubating 500 ml aliquots of conjugation mixture in 10 ml Rappaport-Vassiliadis (RV) broth (Oxoid, UK) supplemented with 30 μg of kanamycin/ml and incubated at 42°C for 24 h. The culture broths were made serial dilutions and were then plated onto Xylose Lysine Desoxycholate (XLD) agar medium (Oxoid, UK) containing kanamycin (30 μg/ml) and then incubated overnight at 37°C. *S. typhimurium* exconjugants formed black bioluminescent colonies and were imaged using a NightOwl image analyzer in conjunction with the manufacturer computer software (E. G. & G., Berthold, Munich, Germany). Growth of *lux* transformants were also determined by monitoring light output to select an exconjugant *S. typhimurium lux*. Lucy1 Photoluminometer (Anthos labtec instruments, Salzburg, Austria) and associated software was used for automated luminometry and photometry (Winson *et al.*, 1998). The bioluminescent phenotype was stable in the selected exconjugate, and growth characteristics were not significantly different from its parental strain (data not shown).

Suspensions of bioluminescent *S. typhimurium* S36 were prepared from overnight culture grown in Brain heart infusion (BHI) broth containing kanamycin (30°g/ml) to ensure plasmid stability in shaking incubator (200 rpm) at 37°C. The cells were harvested by centrifugation (4,000xg for 10 min at 4°C) and washed once in sterile maximum recovery diluent (MRD; Oxoid, UK). The cell pellet was finally resuspended in MRD to give a final cell density ca. 10^7 CFU/ml (A_{600} 0.2).

2. Cetylpyridinium chloride (CPC) and nisin

Various concentrations (w/v) of cetylpyridinium chloride (CPC; $C_{21}H_{38}NCl$, Sigma.) solutions were made by dissolving in distilled water. The nisin (Sigma) solutions were dissolved in 0.02 M HCl. Each solution was sterilized by filtration through 0.45μm filters and stored at 4°C until required.

3. Sample inoculation and treatment application

3.1 Chicken drumstick inoculation

Fresh chicken drumsticks were purchased from a local supermarket (Nottingham, UK). They were rinsed in reverse osmosis water to remove any extraneous material, and then cut into individual squares (approximately 5 by 5 cm) with a flesh thickness of about 1.5 cm.

The samples were submerged in *S. typhimurium* S36 (10^{7}CFU/ml) suspensions for 5 min and were held at room temperature (~22-24° C) for 20 min to allow cell adherence.

3.2 Effect of CPC and nisin concentrations treatment

Inoculated chicken drumstick sections (from 3.1) were submerged in 0, 0.1, 0.3, and 0.5 % (w/v) CPC for 30 sec followed by 30-min dips in 0, 20, 60, and 100 μg/ml nisin at room temperature. Bacterial enumeration was performed. Each experiment was duplicated.

3.3 Effect of exposure times of CPC and nisin treatment

The inoculated chicken drumstick sections were dipped in an appropriate CPC concentration drawn from previous experiment (3.2) for 15, 30, 60 and 120 sec and then treated with appropriate nisin concentration from previous trials (3.2) for 15, 30, 45 and 60 min at room temperature. Surviving cells were enumerated.

4. Bioluminescent images

Bioluminescent images were performed in a NightOwl image analyzer after chicken drumstick sections had been inoculated and also following treated with CPC and nisin to demonstrate the distribution of cells attachment on chicken carcasses.

5. Scanning electron microscopy

S. typhimurium S36 contaminated chicken drumstick skins (1 by 1cm) were removed with sterile scissors and forceps, treated then with appropriate CPC, nisin concentration (from 3.2) and appropriate exposure times (from 3.3). The samples were cut into small pieces with a sterile surgical blade, and fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.2), finally post-fixed in 1% OsO_4. Specimens were taken though a graded series of ethanol and dried in critical point dryer, and then mounted on aluminum stubs. The samples were sputter coated with gold and examined in a JSM-6400 scanning electron microscope (JEOL, Japan) at 15 kV.

6. Imaging of *Salmonella* on chicken drumstick skin with the baclight nucleic acid stain

The sections of chicken drumstick skin (untreated and CPC-nisin treated) were removed aseptically and cut (1 by 1 cm) using a sterile scissors. Aliquots (50 μl) of the Live/Dead® baclight stain mixture of SYTO 9 and propidium iodide (PI) (1:1) nucleic acid stains (Molecular Probes, The Netherlands) were added on the surface chicken skins and then stored in a dark place for 10-15 min. The stained samples were submerged in liquid nitrogen, sliced (6-12 μm) using cryomicrotome (Bright starlet cryostat, UK), mounted on clean glass microscope slide, covered with a coverglass and incubated in a dark place. The attached *Salmonella* cells were viewed using a Nikon epifluorescent microscope (DXM-1200/ LUCIA, Japan) equipped with a mercury lamp. The excitation/emission maxima of these dyes for PI and SYTO9 are about 490/635 nm and 480/ 500 nm, respectively. Green fluoresce on intact cell membrane was interpreted as live cell, whereas dead cells due to the injury shows red fluoresce.

7. Microbiological analysis

Enumeration of *S. typhimurium* cells was done by surface spread plating. The samples were blended with MRD by a Seward Lab-Blender 400 for 2 min. Serial dilutions of homogeneous samples were made, and 100 μl aliquot was spread onto BHI agar supplemented with kanamycin. Inverted plates were then incubated at 37°C for 16-18 h prior to counting.

8. Statistical analysis

Colony counting numbers were transformed to $\log_{10}$ data before statistical analysis. The log counts were then calculated and analyzed using SAS software (SAS Institute, Cary, N.C.) When the analysis of treatment effects indicated a significant difference ($P \geq 0.05$), mean separation would be conducted using the Duncan's multiple range tests.

RESULTS AND DISCUSSION

Effect of CPC and nisin concentrations

The effect of CPC and nisin concentrations in reducing the number of attached *S. typhimurium* S36 on chicken drumstick sections was determined, as shown on Table 1. The most effective ($P<0.05$) decontamination of *S. typhimurium* S36 on chicken samples was observed at 0.5% (w/v) CPC and 100 μg/ml nisin. The concentration of CPC required to kill attached cells was higher than that for suspension cells (from preliminary experiment). Furthermore, attached cells of *S. typhimurium* S36 appeared less susceptible to nisin than did suspended cells (data not shown). This may be due to the microtopograghy of the surface and the presence of feather follicles and folds on the skin surface which, along with oils and fats present, offer some protection. In food systems the effect of nisin was far less pronounced. Binding of nisin by lipids and protein has been identified as the reason for its decreased activity in other complex food systems such as meat and poultry skin (Delves-Broughton, 1990; Rose *et al*., 1999). However, a synergy between CPC and nisin treatment to kill *S.*

typhimurium S36 cells was found. CPC was able to disrupt the outer membrane of cell wall of *S. typhimurium* S36, thus enabling nisin to reach the peptidoglycan layer of the cell wall and the cytoplasmic membrane.

Effect of exposure times of CPC and nisin

The cell viability was obviously decreased by treatment with 0.5% (w/v) CPC followed by 100 μg/ml nisin as shown in Table 2. The most marked effects were observed with 0.5% (w/v) CPC for 60 sec and 100 μg/ml nisin for 60 min, 0.5% (w/v) CPC for 120 sec and 100 μg/ml nisin for 30, 45 and 60 min. These 4 treatments did not differ significantly (P>0.05). Nevertheless, the exposure time of 0.5% (w/v) CPC for 120 sec and 100 μg/ml nisin for 30 min combined the reduction of viable cells with minimal effect on the appearance of chicken samples. Longer exposure times to nisin can change the appearance of chicken drumstick skin to look bloat. This result can be supported by Thomas and McMeekin (1982), who reported that water-immersion cleaning and chilling of poultry carcasses caused the skin swell by taking up water and exposed deep channels and crevices in the skin surfaces. As a result, the appropriate concentration and exposure time of CPC and nisin for reducing *S. typhimurium* S36 populations on chicken carcasses were 0.5% CPC for 120 sec and 100 μg/ml nisin for 30 min, which do not cause undesirable carcass characteristics such as bleaching and swelling of the skin.

Bioluminescent *S. typhimurium* on chicken carcasses

Bioluminescence can be a valuable mean to identify directly the distribution of bacterial cells *in situ*. When *lux* (*CDABE*) carrying *S. typhimurium* S36 was inoculated onto chicken drumstick cuts (Figure 1A). *Salmonella lux* expressing the *lux* operon could be readily

Table 1 Viable cells of *S. typhimurium* on chicken drumstick sections treated with 0, 0.1, 0.3 and 0.5% (w/v) CPC and 0, 20, 60 and 100 μg/ml nisin.

CPC% (w/v)	Log_{10} CFU/g[1/]			
	Nisin (μg/ml)			
	0	20	60	100
0	$4.54^{a} \pm 0.00$	$4.32^{abc} \pm 0.13$	$4.10^{cde} \pm 0.09$	$4.11^{cde} \pm 0.08$
0.1	$4.01^{bcd} \pm 0.00$	$3.97^{de} \pm 0.08$	$4.05^{ed} \pm 0.13$	$3.99^{de} \pm 0.16$
0.3	$4.44^{ab} \pm 0.00$	$4.37^{ab} \pm 0.08$	$4.22^{bcd} \pm 0.23$	$3.86^{ef} \pm 0.19$
0.5	$3.99^{de} \pm 0.16$	$3.87^{ef} \pm 0.01$	$3.70^{f} \pm 0.03$	$3.16^{g} \pm 0.04$

Note: initial inoculum = 7.10 log_{10} CFU/ml

[1/] Means with the same superscript are not significantly different (P>0.05).

Table 2 Cell viability of *S. typhimurium* on chicken drumstick sections treated with 0.5% (w/v) CPC for 15, 30, 60 and 120 sec and 100 μg/ml nisin for 15, 30, 45 and 60min.

0.05% (w/v)CPC (s)	Log_{10} CFU/g[1/]			
	100 μg/ml nisin (min)			
	15	30	45	60
15	$4.43^{a} \pm 0.03$	$4.01^{bcd} \pm 0.04$	$3.89^{de} \pm 0.03$	$3.95^{cd} \pm 0.01$
30	$4.03^{bc} \pm 0.03$	$3.96^{bcd} \pm 0.08$	$3.94^{cd} \pm 0.01$	$3.98^{bcd} \pm 0.03$
60	$4.07^{b} \pm 0.04$	$3.94^{cd} \pm 0.03$	$3.83^{e} \pm 0.01$	$3.62^{f} \pm 0.11$
120	$3.81^{e} \pm 0.06$	$3.70^{f} \pm 0.16$	$3.70^{f} \pm 0.04$	$3.65^{f} \pm 0.03$

[1/] Means with the same letter are not significantly different (P>0.05).

visualized macroscopically directly on chicken drumstick section. Emitted photons were detected immediately over the entire inoculated surface area of the sample. For example, there was a clear preference of cells to aggregate on chicken skin crevices and on feather follicles as seen by areas of high luminescent intensity (Figure 1B and 1C). When the same samples were treated with 0.5% (w/v) CPC for 15 sec, followed by 60 μg/ml nisin for 30 min (Figure 1D), a low intensity luminescence of *S. typhimurium* S36 cells attached on chicken skins (Figure 1E and 1F) was observed.

The use of luciferase as the reporter ensures that the signal observed is from viable, metabolically active cells which can be detected as they exist in or on a food matrix (Siragusa *et al*., 1999). A comparison of the light intensities before and after treatment with CPC and nisin showed that *S. typhimurium* S36 cells preferentially adhered to chicken skin surface, and indicated that there were more viable cells directly on the rougher parts of the surface of chicken drumstick skin. On the contrary, CPC-nisin treated chicken drumstick samples revealed a reduced light emission since *Salmonella lux* could be killed when exposed to CPC and nisin and provided low viable cells on chicken sample, hence, the "real time" response was a decrease in light output.

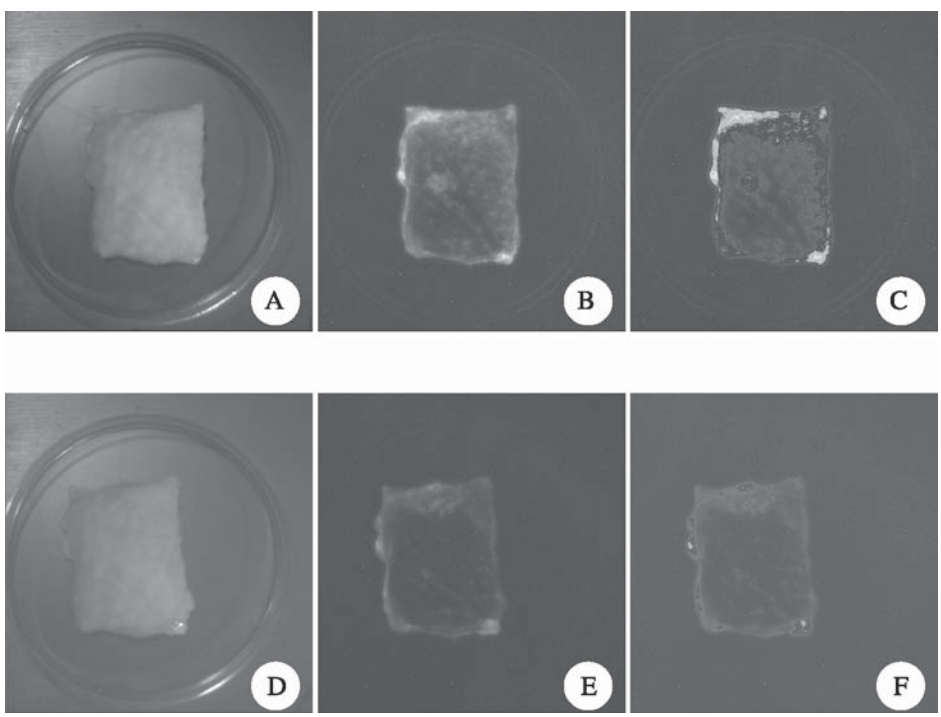

Figure 1 Bioluminescence images of *S. typhimurium* S36 distribution on chicken drumstick sections derived from untreated control (A-C) and CPC-nisin treated chicken drumstick sections (D-F). Chicken drumstick sections were inoculated with *S. typhimurium* S36 and images taken under illumination (A), bioluminescence in the dark (B) and bioluminescence pseudocolor image (C). The chicken drumstick sections were then treated with CPC and nisin and image capture repeated under illumination (D), bioluminescence in the dark (E) and bioluminescence pseudocolor image (F) to determine changes in bioluminescence distribution.

Consequently, it can be concluded that the CPC-nisin treatment studied was effective for eliminating *S. typhimurium* S36 attached on chicken drumstick. Although bioluminescence monitoring will not entirely replace culture methods in the study of attachment and decontamination the need for excision sampling, it has distinct advantages as a screening tool for selecting decontamination protocols for validation.

S. typhimurium attachment on chicken drumstick skin observed by SEM

Chicken skin had many folds and deep crevices with structures projecting inward from the skin surface. In this study, *S. typhimurium* S36 attachment was observed on the surface of chicken drumstick skin as viewed by SEM (Figure 2). Scanning electron micrograph of *S. typhimurium* S36 contaminated on chicken drumstick skin untreated control (Figure 2A and 2B) indicated *Salmonella* cells adhered on the flat portion of the skin surface area (Figure 2A) and were mostly located in crevices or surface grooves (Figure 2B). After treatment with CPC and nisin, *Salmonella* cells remained on the poultry skin surface (Figure 2C), but the cells lost their original shapes and showed surface raffling and craters on the surface (Figure 2D). In addition, the SEM method showed that CPC and nisin did not detach cells from the chicken skin. Moreover, CPC was reported to

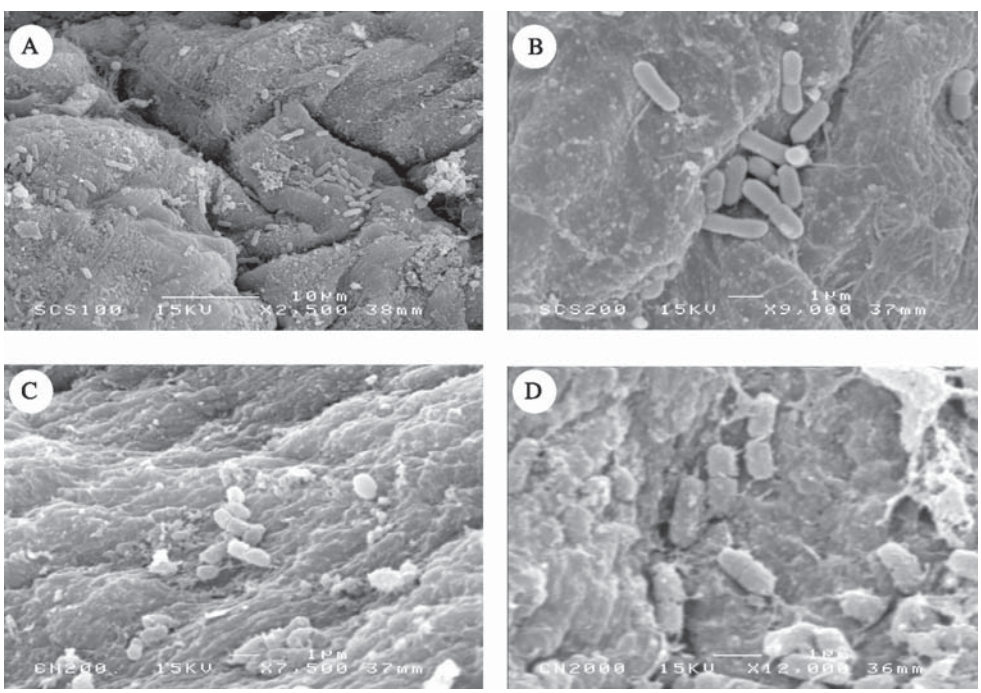

Figure 2 Scanning electron micrographs of *S. typhimurium* S36 contaminated on chicken drumstick skin untreated control (A and B) and treated with CPC and nisin (C and D). (A) Intact *S. typhimurium* cells attached on the chicken skin surface (magnification × 2,500); (B) *S. typhimurium* cells entrapped in crevices or wrinkles on chicken skin (magnification x 9,000); (C) CPC-nisin treated *S. typhimurium* cells remained to attach on chicken skin surface (magnification × 7,500); (D) CPC-nisin treated *S. typhimurium* cells attached on chicken skin showed indentations and fibril appeared on their cell surfaces (magnification × 12,000). Bar (A)=10 μm, (B-D)=1 μm.

enhance microbial attachment to hydrophobic surface by diminishing surface charge and increasing cell surface hydrophobicity (Goldberg *et al*., 1990). Kim and Slavik (1996) reported that CPC may kill salmonellae cells by causing metabolic damage, with little effect on the morphological structure. On Figure 2D also, unlike the control (Figure 2B), some fibril connecting the cells to each other or attaching to the chicken skin began to appear. These fibrils are believed to be cytoplasmic leakage caused by CPC and nisin. It is also possible that exopolysaccharide materials on the outer membrane of the cells were untangled and released during CPC-nisin treatment.

Visualization of *S. typhimurium* on chicken drumstick skin

On the screen of the SEM (Figure 2), furthermore, it is difficult to differentiate live and dead cells of *S. typhimurium* S36 contaminated on chicken skin after treatment with CPC and nisin. Consequently fluorescein staining was selected to determine viable cells. The baclight probe provides a method for effective differentiation between the fluorescence patterns of viable and non-viable cells and for identifying cells positioned horizontally along an attachment surface by viewing under epifluorescence microscopy (Korber *et al*., 1996). Epifluorescent micrograph (Figure 3A) shows the majority of live *S. typhimurium* S36 cells adhered on untreated control chicken skin surface. All live cells emitted green fluorescence of SYTO9 which stained all cell membranes, whereas cells whose cell membrane has been ruptured were stained with the red fluoresence of propidium iodide (PI) (Boulos *et al*., 1999), Since PI is membrane impermeable and can only penetrate into damaged membrane cells (Alonso *et al*., 2002). Thus, baclight stain produced a clear color difference to distinguish dead cells from live cells. With this technique, CPC-nisin treated chicken skin (Figure 3B) appears with a mixture of live and dead *S. typhimurium* S36 cells attached to its surface.

Data from these experiments could explain that *S. typhimurium* S36 cells attached on chicken skin following treatment with CPC and nisin were observed using SEM. Notably, SEM micrographs revealed many remaining *Salmonella* cells attached on skin. The baclight staining allowed to determine viable and nonviable cells

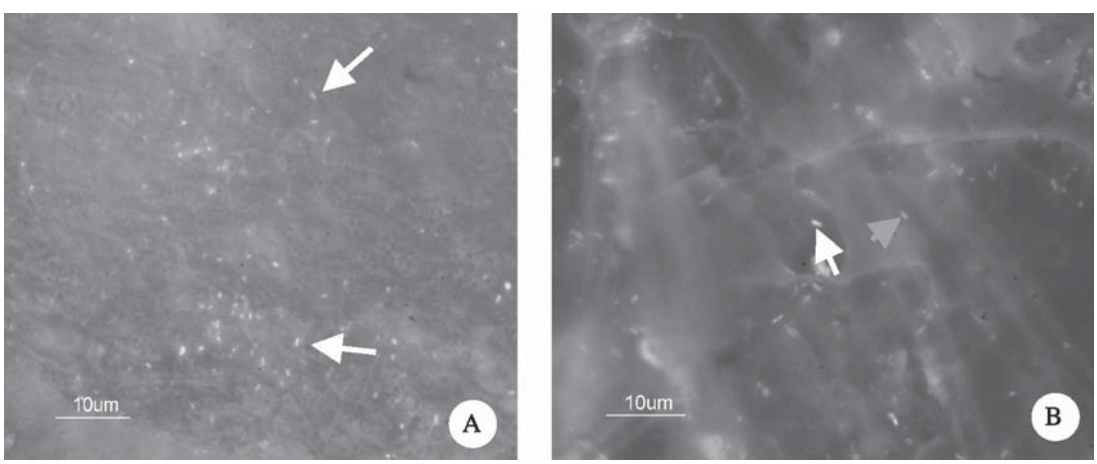

Figure 3 Photomicrographs show attachment of *S. typhimurium* S36 on chicken drumstick skin. Bacteria were stained using the baclight stain. (A) Untreated control, arrows indicate live cells (green cells) of *S. typhimurium* S36 adhesion onto chicken skin; (B) White arrow shows a viable cell (green), blue arrow shows a dead cell (red) on chicken drumstick skin after treated with CPC and nisin (magnification × 400)

attached on the chicken skin. It appeared that both living (green) and dead (red) cells adhered on the skin. The strong attachment of microorganisms to poultry appears to be a surface interaction phenomenon (Lillard, 1990). Thus, *S. typhimurium* S36 can become very firmly attached to poultry skin. Those cells that do not attach can be washed off easily, while those attached cells become strongly attached can not be removed by rinsing because of capillary action or irreversible attachment to the skin tissue and become more resistant to removal by chemical or physical means (Breen *et al*., 1995). However, these data demonstrate that CPC and nisin were more effective to inhibit or kill viable *S. typhimurium* S36 cells attached on chicken skin as seen with the numbers of red cells in Figure 3B. It can be concluded that CPC and nisin could not detach *Salmonella* on chicken skin but that they are extremely effective to kill or reduce viable attachment of *Salmonella* cells on chicken skin. Furthermore, baclight nucleic acid stain proved to have the advantage to indicate bacterial viability by confirming the status of the cells.

CONCLUSION

The study has shown that CPC has potential applications in poultry processing because it exhibited strong antimicrobial activity against *Salmonella* and also did not affect the physical appearance of the poultry products. Moreover, there was a synergistic effect to inhibit *S. typhimurium* S36 cells between CPC and nisin. The appropriate conditions of CPC and nisin for reducing *S. typhimurium* S36 contaminated on chicken carcasses were 0.5% (w/v) CPC for 120 sec and 100μg/ml nisin for 30 minutes. CPC and nisin might be beneficial agents in reducing *Salmonella* and Gram-negative bacteria contamination of poultry carcasses during poultry processing, particularly, in reducing the risk of cross-contamination.

ACKNOWLEDGEMENTS

The authors would like to gratefully acknowledge the Thai Government for financial support and would also like to thank Dr. Keith Warriner for technical assistance.

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