# EFFECT OF SANITIZING AGENTS AND NISIN ON BIOLUMINESCENT *Salmonella typhimurium* ATTACHED ON POULTRY SKIN

**Bussagon Thongbai**

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# ผลของสารลดการปนเปื้อนจุลินทรีย์และไนซินต่อไบโอลูมิเนสเซนต์ *ซาลโมเนลลา ไทฟิมูเรียม* (bioluminescent *Salmonella typhimurium*) ที่เกาะติดบนผิวหนังไก่

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**วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต**
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# EFFECT OF SANITIZING AGENTS AND NISIN ON BIOLUMINESCENT *Salmonella typhimurium* ATTACHED ON POULTRY SKIN

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ไบโอลูมิเนสเซนต์ ***ซาลโมเนลลา ไทฟิมูเรียม*** เอส36 สร้างขึ้นโดยการทรานฟอร์เมชั่นด้วยพลาสมิดมินิ□ทีเอ็น 5 ที่ประกอบด้วยยีน *lux CDABE* และยีนที่ต้านทานกานาไมซิน (kanamycin) โดยพบว่าเซลล์ของ***ซาลโมเนลลา ไทฟิมูเรียม*** เอส36 ในช่วงสเตชั่นนารี เฟสนั้นจะมีความต้านทานต่อไตรโซเดียม ฟอสเฟต, เซทธิลไพริดิเนียม คลอไรด์ (ซีพีซี), โซเดียมแลคเตท และไนซินมากกว่าเซลล์ที่อยู่ในช่วงมิด□เอ็กโพเนนเชียล เพส ซึ่งการทดลองที่ใช้ซีพีซีและไนซินนั้น ได้พิสูจน์ให้เห็นว่ามีประสิทธิภาพมากที่สุดในการยับยั้ง***ซาลโมเนลลา ไทฟิมูเรียม*** เอส36 ยิ่งไปกว่านั้นค่าไบโอลูมิเนสเซนต์ที่วัดได้จากไบโอลูมิเนสเซนต์แบคทีเรียนี้ เป็นวิธีการที่มีประสิทธิภาพและรวดเร็วที่สามารถใช้ตรวจสอบประสิทธิภาพในการทำลายแบคทีเรีย ณ เวลานั้นของสารยับยั้งแบคทีเรียชนิดต่างๆหรือตรวจสอบประสิทธิภาพของวิธีการทำลายแบคทีเรียวิธีอื่นๆ

เนื่องจากส่วนของเนื้อเยื่อด้านนอก (outer membrane) ของแบคทีเรียชนิดแกรมลบซึ่งรวมถึงเชื้อ***ซาลโมเนลลา***ด้วยนั้น มีคุณสมบัติเป็นตัวขัดขวางความสามารถในการผ่านเข้าออกของสารต่างๆเข้าสู่เนื้อเยื่อด้านในของเซลล์ รวมถึงการป้องกันไม่ให้ไนซินผ่านเข้าไปสู่ไซโตพลาสมิค เมมเบรนได้ อย่างไรก็ตามหากความสามารถในขัดขวางการยอมให้สารต่างๆผ่านเนื้อเยื่อด้านนอกนี้ถูกทำให้มีประสิทธิภาพลดลง เชื้อแบคทีเรียชนิดแกรมลบจะสามารถถูกยับยั้งได้ด้วยไนซิน การเลี้ยงเซลล์ที่สภาวะอุณหภูมิและพีเอชที่ไม่เหมาะสมต่อการเจริญของ***ซาลโมเนลลา***ที่ระดับต่างๆกันและรวมถึงการใช้ซีพีซีและไนซินนั้น สามารถเปลี่ยนแปลงความสามารถในการยอมให้สารผ่านเข้าออกของเนื้อเยื่อส่วนนอกของ***ซาลโมเนลลา ไทฟิมู□เรียม*** เอส36ได้ ทำให้เกิดการสูญเสียการทำงานของตัวขัดขวางนี้และยังไปลดความต้านทานต่อไนซินของเซลล์ ส่งผลให้เกิดการเปลี่ยนแปลงรูปร่างของ***ซาลโมเนลลา ไทฟิมูเรียม*** เอส36 ซึ่งติดตามการเปลี่ยนแปลงเหล่านี้ได้ด้วยกล้องจุลทรรศน์อิเลคตรอนแบบส่องกราดและแบบส่องผ่าน พบว่าเซลล์ที่ถูกเลี้ยงที่อุณหภูมิและพีเอชต่างๆกันทั้งที่ใช้และไม่ใช้ซีพีซีและไนซินนั้น จะมีผลต่อการเปลี่ยนแปลงรูปร่างของเซลล์ปรากฏให้เห็น รวมถึงการเกิดรอยบุ๋มและรูในส่วนผิว

เซลล์ จนถึงขั้นมีการยุบตัวจนเสียรูปร่างของเซลล์ นอกจากนี้ยังพบการเปลี่ยนแปลงที่เกิดขึ้นภายในเซลล์เช่นมีการโปร่งพองออกหรือมีที่ว่างในส่วนไซโตพลาสซึมเกิดขึ้น

ซีพีซีและไนซินยังมีผลต่อการยับยั้งซาล*โมเนลลา ไทฟีมูเรียม* เอส36 ที่ปนเปื้อนบนซากไก่ โดยใช้น่องไก่ที่ตัดให้มีขนาด 5 × 5 ซม. และทำการเติมเชื้อซาล*โมเนลลา ไทฟีมูเรียม* เอส36 ลงไปปนเปื้อน หลังจากนั้นก็ใช้ซีพีซีและไนซิน พบว่าซีพีซีที่ความเข้มข้น 0.5% (น้ำหนัก/ปริมาตร) นาน 120 วินาที และไนซินที่ความเข้มข้น 100 ไมโครกรัม/มิลลิลิตร นาน 30 นาที สามารถลดจำนวนของเซลล์ที่มีชีวิตของซาลโมเนลลาลงได้อย่างมีนัยสำคัญทางสถิติ ($p \leq 0.05$) และยังมีผลกระทบต่อลักษณะปรากฏของซากไก่เพียงเล็กน้อย นอกจากนี้ยังพบว่าซีพีซีและไนซินนั้นมีประสิทธิภาพในการลดจำนวนของซาล*โมเนลลา ไทฟีมูเรียม* เอส36ที่ปนเปื้อนบนผิวหนังน่องไก่ในระหว่างการเก็บรักษาที่ 4°C เป็นเวลา 6 วันได้ด้วย โดยมีการเปลี่ยนแปลงสีของหนังไก่เพียงเล็กน้อย ผลจากการทดลองเหล่านี้แสดงให้เห็นว่า ซีพีซีและไนซินมีความสามารถที่เสริมฤทธิ์กันในการยับยั้งซาล*โมเนลลา*ที่ปนเปื้อนในซากไก่ ดังนั้นอาจคาดได้ว่าการใช้ซีพีซีและไนซินจะสามารถช่วยลดการปนเปื้อนของซาล*โมเนลลา*ในซากไก่ ซึ่งจะเพิ่มความปลอดภัยของซากไก่และผลิตภัณฑ์จากไก่ได้เพิ่มขึ้น

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา _____

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BUSSAGON THONGBAI : EFFECT OF SANITIZING AGENTS AND NISIN ON BIOLUMINESCENT *Salmonella typhimurium* ATTACHED ON POULTRY SKIN. THESIS ADVISOR : ASST. PROF. PIYAWAN GASALUCK, Ph.D. 159 PP. ISBN 974-533-462-6

BIOLUMINESCENT/*Salmonella*/NISIN/CETYLPIRIDINIUM CHLORIDE/ ELECTRON MICROSCOPY

*Salmonella typhimurium* S36 was constructed by transformation with the mini-Tn5 plasmid containing the *lux CDABE* cassette with kanamycin resistance gene. The stationary phase of *S. typhimurium* S36 cells are more resistant to trisodium phosphate (TSP), cetylpyridinium chloride (CPC), sodium lactate (SL) and nisin treatments than the mid-exponential phase cells. CPC-nisin proved the most effective treatment to inhibit *S. typhimurium* S36. Moreover, bacterial bioluminescence is an effective way of monitoring in real time the sensitivity of bacteria to antibacterial agents and other treatments.

Since the outer membrane (OM) of Gram-negative bacteria including *Salmonella*, act as a permeability barrier, preventing nisin from gaining access to the cytoplasmic membrane. However, if the OM permeability is reduced, Gram-negative bacteria can show nisin sensitivity. Temperature and pH stress and CPC-nisin treatment were able to alter the OM permeability of *S. typhimurium* S36, producing a loss of barrier function and reduced resistance to nisin. Appearing of morphological changes in *Salmonella* cells were examined using scanning and transmission electron microscopy. Temperature and pH stressed cells with and without treated with CPC-

nisin had perturbed cell morphology, including apparent indentations, craters in the cell surfaces and collapsed into irregular bodies. Nevertheless, blebs, cytoplasm-sparse of cells were observed.

CPC-nisin treatment has an affect on the inhibition of *S. typhimurium* S36 contaminated on chicken carcasses. The chicken drumstick sections (5 by 5 cm), inoculated with *S. typhimurium* S36, were then treated with CPC and further treated with nisin. It was found that the use of 0.5% (w/v) CPC for 120 sec and 100$\mu gml^{-1}$ nisin for 30 min decreased significantly ($p \leq 0.05$) cell viability with minimal effect on the chicken carcass appearance. Furthermore, the CPC-nisin treatment reduced the number of *S. typhimurium* S36 contaminated on chicken drumstick skin during stored at 4°C for 6 days with a slight difference in chicken skin color. These results demonstrated the synergistic activity of CPC and nisin treatment against *Salmonella* and could be expected to enhance the safety of chicken carcasses and other chicken products.

School of Food Technology

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Student's Signature Bumgon Nongbua

Advisor's Signature

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Bussagon Thongbai

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| | | |
|---|---|---|
| **$A_{600}$** | **=** | Absorption at 600 nm |
| **ATP** | = | Adenosine-5′-triphosphate |
| **CFU** | = | Colony forming unit |
| **$^{o}C$** | = | Degree Celsius |
| **DIG** | = | Digoxigenin |
| **Da** | **=** | Dalton |
| **$^{o}F$** | **=** | Degree Fahrenheit |
| **h** | = | Hour |
| **g** | = | Gram |
| **kDa** | = | Kilo dalton |
| **kV** | = | Kilo volts |
| **LPS** | = | Lipopolysaccharide |
| **M** | = | Molar [$molel^{-1}$] |
| **mg** | = | Milligram [$10^{-3}$ gram] |
| **min** | = | Minute |
| **ml** | = | Millilitre [$10^{-3}$ litre] |
| **mM** | = | Millimolar [$10^{-3}$molar] |
| **$M_r$** | = | Relative molecular mass |
| **Mw** | = | Molecular weight |
| **N** | = | Normal |

# LIST OF ABBREVIATIONS (Continued)

| | | |
|---|---|---|
| **nm** | **=** | Nanometer |
| **OD** | = | Optical density |
| **OM** | = | Outer membrane |
| **ppm** | = | Part per million |
| **RLU** | = | Relative light units |
| **RO** | = | Reverse osmosis |
| **rpm** | = | Revolution per minute |
| **rRNA** | = | Ribosomal ribonucleic acid |
| **SDW** | = | Sterile distilled water |
| **sec** | = | Second |
| **SEM** | = | Scanning electron microscope |
| **TEM** | = | Transmission electron microscope |
| **v** | = | Volume (ml) |
| **w** | = | Weight (g) |
| μ**g** | = | Microgram [$10^{-6}$ gram] |
| μ**m** | = | Micrometre [$10^{-6}$ metre] |
| μ**M** | = | Micromolar [$10^{-6}$ molar] |
| μ**l** | = | Microlitre [$10^{-6}$ litre] |

# CHAPTER I

# INTRODUCTION

Food safety has become one of the most visible issues in recent times. Outbreaks of food borne disease occur worldwide, lead to consumer concerns and erosion in public confidence regarding the safety of our food supply. Occurrence of food borne disease in the US was recently updated by the Center for Disease Control (CDC) using the most accurate information available (Mead et al., 1999). Estimated 76 million cases per year, 325,000 hospitalizations and 500 deaths, among which about 1.3 million cases of illness due to non-typhoidal *Salmonella*, 15,600 cases resulted in hospitalization. These cases represented 9.7%, 2.5% and 30.6% of the total foodborne illness, hospitalization and deaths, respectively. In an active surveillance by CDC of 7223 culture-confirmed cases of the seven foodborne diseases in 1996, salmonellosis had the second highest incidence at 16 per 100,000 population (MMWR, 1997). Economic losses due to *Salmonella* are estimated to amount to approximately $ 2.4 billion in the United States on the basis of estimates of the number of infections, related medical costs, and productivity losses (Fewnzen et al., 1999). Poultry products are implicated as one of the major vehicles of salmonellosis for spread of human *Salmonella* to proliferate in the gastrointestinal tract of chickens and subsequently survive on commercially processed broiler carcasses and edible giblets (Rabsch, Tschäpe and Bäumler, 2001; St. Louis et al., 1988; Vadhanasin, Bangtrakulnonth and Chidkrau, 2004). In different countries, the rate of *Salmonella*

contamination of broiler carcasses, either from processing plants or retail markets, has been reported at 5 to 100% (Todd, 1980). The incidence of *Salmonella* in raw poultry is estimated at 10-12 % and 35-40% in raw ground turkey and chicken (Lattuada, 2000). In Thailand, Jerngklinchan, Koowatananukul, Daengprom and Saitanu (1994) reported *Salmonella* prevalence in raw broilers and their products as high as 91%. *Salmonella* are ubiquitous in poultry houses, and these products may result in widespread transmission of this organism vertically and horizontally (Bailey et al., 2001; Roy, Dhillon, Lauerman, Schaberg, Bandli and Johnson, 2002). Thus, any new approaches to reduce or eliminate pathogen contamination in foods would decrease foodborne illness and result in savings the food industry.

In recent years, consumer demand for fresh, minimally processed safe food, in addition to concern over the use of chemical preservatives in foods, has prompted substantial interest in the application of biopreservatives. Bacteriocins produced by lactic acid bacteria are seen as alternatives to traditional preservatives for ensuring food safety. Bacteriocins have aroused great interest in the context of food preservation, due to their potential usefulness for industrial applications because of their antibacterial activities. In addition, the bacteriocin can be used as a biopreservative and bioregulator of the microflora present in fermented food. Nisin, generally recognized as a safe bacteriocin (FDA, 1988), is the only bacteriocin that has been approved for food use in many countries to prevent spoilage of a variety of different foods. Nisin disrupts the cytoplasmic membrane of vegetative cells and inhibits spore germination. It is mainly effective against Gram-positive bacteria, but also against some sublethal injured Gram-negative bacteria. Nisin is a polypeptide and

(nontoxic substance and does not involve any risk for human health Kalchayanand, Hanlin and Ray, 1992; Ray, 1992).

Since conventional microbiological methods are often inadequate for the determination of viability and cellular metabolic activity of microorganisms exposed to various conditions in foods, bacterial bioluminescence has been proposed as an effective way of monitoring bacterial growth, survival, and colonization in foods (Bautista, Chen, Barbut and Griffiths, 1998; Chen and Griffiths, 1996; Ellison, Perry and Stewart, 1991; Hudson, Chen, Hill and Griffiths, 1997; Kelana and Griffiths, 2003; Ramsaran, Chen, Brunke, Hill, and Griffiths, 1998; Tomicka, Chen, Barbut and Griffiths, 1997). With this method, the target bacterium is genetically modified to generate light by incorporating a genetic sequence that encodes the enzyme responsible for bioluminescence from naturally luminescent bacteria. The result is a genetically modified bacterium that can be directly and rapidly identified in a variety of environments in a nondestructive manner (Chen and Griffiths, 1998; Duffy, Ellison, Anderson, Cole and Stewart, 1995; Ellison et al., 1991).

Therefore, the purposes of this research project are (1) to investigate the sensitivity of bioluminescent *Salmonella typhimurium* during growth (log and stationary phase) stages and under stressed conditions (temperature- and pH-stressed), that is when exposed to sanitizing agents and nisin, (2) to optimize the concentrations and exposure times of selected sanitizing agent and nisin for eliminating *S. typhimurium* on poultry carcasses and possibly for extending the shelf life of chilled poultry at various storage temperatures.

## 1.1 Research objectives

1. To study the sensitivity of *S. typhimurium* during growth stages (log and stationary phases) to sanitizing agents and nisin.
2. To study the susceptibility of cells stressed in different environments (pH and temperature) to selected sanitizing agent and nisin.
3. To optimize the concentrations and exposure times of selected sanitizing agent and nisin for eliminating *Salmonella* on poultry carcasses.

## 1.2 Research hypothesis

1. Sanitizing agents and nisin could affect the sensitivity of *Salmonella* in different growth stages and after different stresses.
2. Concentration and exposure time of selected sanitizing agent and nisin could affect the reduction of viable *Salmonella* attached on food.
3. Selected sanitizing agent and nisin could affect the inhibition of *Salmonella* in food during storage.

## 1.3 Scope and limitation of the study

1. Construction of bioluminescent *S. typhimurium*

   Bioluminescent *S. typhimurium* as representative of *Salmonella* was used throughout the study for explanation of bacterial attachment on poultry skin.
2. Trisodium phosphate (TSP), cetylpyridinium chloride (CPC) and sodium lactate (SL) were selected as representative sanitizing agents for enhancing nisin sensitivity to Gram-negative bacteria during growth stages and in

different environments (pH and temperature). The inhibitory effects of treatments on the *Salmonella* cells were investigated by microbiological plating, luminometer, fluorescent microscopy, electron microscopy (scanning electron microscopy (SEM) and transmission electron microscopy (TEM)).

3. Appropriate concentration and exposure time of selected sanitizing agents and nisin were determined and applied on bioluminescent *S. typhimurium* inoculated chicken drumstick with various storage temperatures. Microbiological analysis, photoluminometer, digoxigenin probe, fluorescent microscopy and SEM were used to evaluate the effects of selected sanitizing agent and nisin on poultry carcasses.

## 1.4 Expected results

1. The sensitivity of *Salmonella* cells during growth stages and stressed conditions was reported.
2. The appropriate concentration and exposure time of selected sanitizing agents and nisin for eliminating *S. typhimurium* on poultry carcasses were revealed.
3. The attachment, quality attributes and storage life of chilled poultry treated with selected sanitizing agent and nisin were understood and applicable.

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# CHAPTER II

# LITERATURE REVIEW

## 2.1 *Salmonella*

*Salmonella*, a gram-negative, facultative anaerobic, rod shaped bacteria (Bergey, 1989), is one of the most important causes of foodborne disease worldwide. Outbreaks caused by this organism in humans have been mainly associated with consumption of poultry and poultry products. In the United States and other developed countries, *Salmonella* is recognized as the leading cause of bacterial gastroenteritis in humans. Preliminary data from the 1998 FoodNet surveillance indicates that 29.1% of reported bacterial gastroenteritis cases are caused by *Salmonella*. This bacterium has a broad host-spectrum, and can be isolated from a wide range of animal species, including birds and reptiles. The animals usually are healthy carriers, and contaminated feed plays an important role in the epidemiology of salmonellosis. *Salmonella* can survive for a long time in the environment. Humans are usually infected through consumption of contaminated food of animal origin. The association of *Salmonella* with raw foods of animal origin, particularly poultry products, is well established. Poultry products are considered to be the single most prevalent source of *Salmonella* among all animal-derived food products. Cross-contamination of poultry carcasses with pathogenic organisms often occurs during postmortem processing with transfer of pathogens from the skin, feathers, and intestinal tract contents of one contaminated carcass to processing equipment,

employees, and other carcass surfaces. The most prevalent site for *Salmonella* contamination of poultry carcasses is on the skin (Natrajan and Sheldon, 2000). *Salmonella typhimurium* is a foodborne pathogen which is a major concern to both the poultry industry and consumers. Virulent forms of this microorganism can cause acute and chronic illness in humans. Acute illness can include diarrhea, abdominal cramping, nausea, vomiting and fever, and in infants, the elderly and immuno-compromised individuals it can result in death. It should be noted that immune-compromised individuals include those with illness, transplant recipients, and pregnant women. Chronic illnesses have also been associated with foodborne pathogens (Archer, 1988). Hence, efforts to eliminate or reduce *Salmonella* pathogen attached to poultry during processing and retailing can ensure the safety of the food we eat.

## 2.2 Gram-negative cell envelope

The outer cell layer of Gram-negative bacteria consists of an outer membrane (envelope), a thin layer of peptidoglycan and a periplasmic space ostensibly situated between the outer membrane and the peptidoglycan/cytoplasmic membrane (Fig. 2.1). The outer envelope is a bilayer structure composed of lipopolysaccharide (LPS) and phospholipids, and possesses proteins, in particular porins, embedded in that membrane (Neidhardt, Ingraham and Shaechter, 1990).

### 2.2.1 Lipopolysaccharides (LPS)

Lipopolysaccharides characterize the outer membrane of Gram-negative bacteria. They are the principal component of the outer envelope and carry a net

negative charge. They are responsible, sometimes in large part, for the impermeability of these microorganisms to compounds (Nikaido, 1996; Wiese, Brandenburg, Ulmer, Seydel and Muller-Loennies, 1999). Lipopolysaccharides consist of three regions: (i) the lipid A; (ii) the central core polysaccharide; and (iii) the outer O-polysaccharide side chain (Fig. 2.1). This composition forms a highly ordered crystalline structure with low fluidity (Nikaido, 1996).

Lipid A is a phosphorylated glucosamine disaccharide unit to which a number of fatty acids are attached. This hydrophobic structure does not vary considerably between Gram-negative bacteria, although phosphate composition might differ. It is an important component in the structure of the LPS and its deletion results in an increased susceptibility to hydrophobic antibiotics (Vaara, 1993). The core polysaccharide is a complex oligosaccharide that is linked to the lipid A by 3-deoxy-D-manno-2-octulosonate (KDO). Appearance of free KDO in the medium has often been taken as an indicator of LPS degradation. The O-side chain of LPS is composed of many repeating units of oligosaccharides. The O-side chain is used for the identification of subspecies (e.g. serotyping) as it shows high diversity between and within bacterial species.

Lipopolysaccharides are non-covalently cross-linked and are held in position at the outer membrane surface by divalent cations; these are essential for the structural integrity and strength of the outer membrane (Nikaido and Vaara, 1985).

### 2.2.2. Porins

The Gram-negative bacterium regulates outer membrane permeability characteristics by the presence of hydrophilic channels known as porins. They can be

separated into two classes according to their function: (i) the general diffusion porins that are non-specific; and (ii) the specific porins that mediate the entry of particular solutes (e.g. ferric iron chelates, maltose, maltodextrins, nucleosides and vitamin B12) that are unable to pass through non-specific porins. Recently, these transport protein channels have been the subject of intense investigation in relation to bacterial resistance to antimicrobial agents and, particularly, to the phenomenon of efflux.

### 2.2.3 Periplasmic space

The periplasmic space is situated between the cytoplasmic and the outer membrane but its boundary is not always well defined. It contains a gel-link solution of proteins and polysaccharides that facilitate nutrition, transport and cell-wall maintenance, and that also inactivate (or detoxify) harmful compounds (Beveridge, 1995); it is increasingly believed to be one of the most active metabolic processing centres in the bacterium. The periplasm includes negatively-charged oligosaccharides that play a role in osmoregulation in Gram-negative bacteria, binding proteins that capture sugars, amino acids, vitamins and inorganic ions, degradative enzymes such as phosphatases, nucleases, proteases, catalases, and detoxifying enzymes such as β-lactamases (Beveridge, 1995).

### 2.2.4 Peptidoglycan

The peptidoglycan, similar between Gram-positive and Gram-negative bacteria, is a unique structure among prokaryotes and consists of a polymer of a disaccharide repeating unit of two different N-acetylated amino sugars (N-acetyl-muramic acid and N-acetyl glucosamine) cross-linked by short peptide linkage

between the carboxyl-group of D-alanine on one glycan strand and the free amino group of meso-diaminopimelic acid on the adjacent chain. The peptidoglycan layer is firmly bound to the outer membrane of wall by covalent linkage of an occasional side chain to murein lipoprotein, an abundant outer membrane protein.

The function of the peptidoglycan is primarily to confer shape and mechanical strength to bacteria. Burman, Nordstrom and Bloom (1972) reported that the peptidoglycan may play a role in the permeability of Gram-negative bacteria either directly by forming a permeability barrier, or indirectly by holding together the outer membrane. The latter appears the most likely function for most biocides and antibiotics, since the open network itself conveys little restriction to low molecular weight compounds (Lambert, 2002).

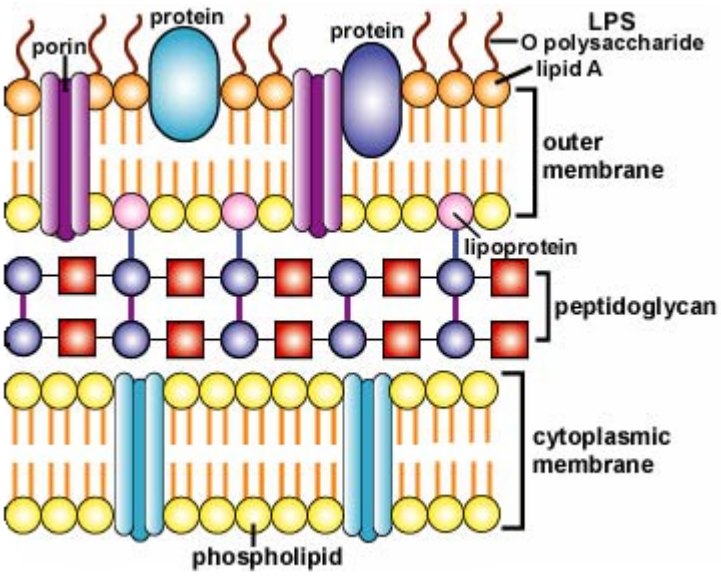



**Fig. 2.1** Structure of a Gram-negative cell wall

**(Source:**http://www.cat.cc.md.us/courses/bio141/lecguide/unit1/prostruct/gncw.html)

Based on studies of these structures, two main pathways for antibacterial agents are described, one hydrophobic through the LPS-layer and the other hydrophilic through the porin channels (Denyer and Maillard, 2002; Russell and Gould, 1988; Russell, 1999a). In addition, a third pathway called self-promoted uptake is used by the so-called membrane active agents, e.g. cationic disinfectants, of which chlorhexidine and quaternary ammonium compounds (QACs) have been much studied (Maillard, 2002; McDonnell and Russell, 1999; Russell, 1998; Russell and Gould, 1988). Membrane active agents are thought to damage the outer membrane by displacing divalent cations and thus destabilizing LPS-LPS links. The exact mechanisms remain to be elucidated, but the addition of $Mg^{2+}$ ions reverses the uptake of at least some of the active membrane agents (Russell and Gould, 1988).

## 2.3 Nisin

Nisin is a small, amphiphilic peptide (34 amino acid residues) produced by *Lactococcus lactis* subsp. *lactis* belongs to bacteriocin class I lantibiotics and has unusual amino acids, such as dehydroalanine (Dha), dehydrobutyrine (Dhb), lantionine and β-methyllanthionine (Jay, 2000; Sahl, Jack and Bierbaum, 1995). The thioether bridges of the lanthionines force the peptide into a five cycles structure (Fig. 2.2) (Guiotto, Pozzobon, Canevari, Manganelli, Scarin and Veronese, 2003). Nisin has a broad inhibitory spectrum against Gram-positive foodborne pathogens such as *Listeria monocytogenes* and *Clostridium botulinum* (Delves-Broughton, 1990) but does not inhibit the majority of Gram-negative bacteria, yeast or fungi. The outgrowth of bacterial spores is inhibited in the post-germination stages of spore development (Chan et al., 1996). It is presumed that the double bond provides a reactive group for

interaction with a sore associated factor that is essential for outgrowth of spores. Nisin is permitted as a food ingredient but remains as the only bacteriocin allowed for such purpose. The mechanism of action of nisin involves interaction with peptidoglycan precursors and formation of transient pores in the cytoplasmic membrane of the target organism, with loss of membrane potential and leakage of intracellular metabolites (Brötz et al., 1998; Moll, Roberts, Konings and Driessen, 1996; Sahl, 1991).

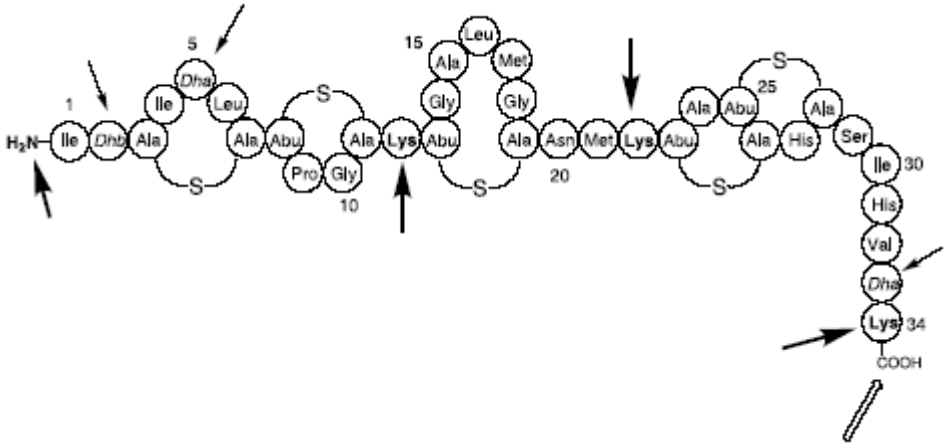
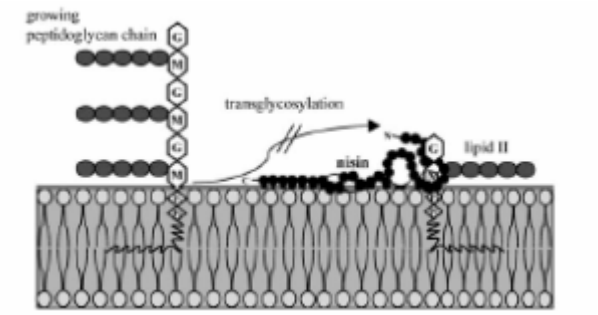



**Fig.2.2** Schematic representation of the primary structure of nisin. Ala-*S*-Ala: lanthionine, Abu-*S*-Ala: β-methyllanthionine, Dha: dehydroalanine, and Dhb: dehydrobutyrine (←, amono groups; ⇐, carboxylic group; ◄, double bonds).

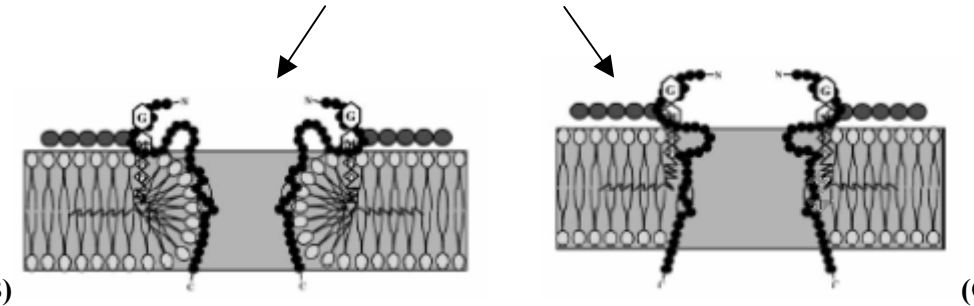
(**Source**: Guiotto et al., 2003)

**Binding of lipid II and inhibition of cell wall biosynthesis**




**(A)**

**Binding of lipid II and subsequent pore formation**



**(B)** **(C)**

**Fig. 2.3** Model of target-mediated action of nisin.

(A) Based on the results of Wiedemann et al. it has been proposed that nisin first binds to the outerwardly orientated carbohydrate moiety of lipid II in a 1:1 stoichiometry. The N-terminal segment of nisin is essential for binding whereas the C-terminal part is then assumed to be subsequently translocated across the membrane, in accordance to pore formation in the absence of lipid II. For this step the flexible hinge region between the third and the fourth ring of nisin is important. Several nisin/lipid II complexes are presumed to assemble for a functional pore. In (B) nisin is assumed to stay surface bound when translocating across the membrane, then following the wedge model. In (C) it is assumed that nisin dips into the bilayer, interacting with the acyl chains; such a situation, in essence, follows the pore formation as proposed by the barrel-stave model

(**Source:** Hoffmann, Pag, Wiedmann and Sahl, 2001).

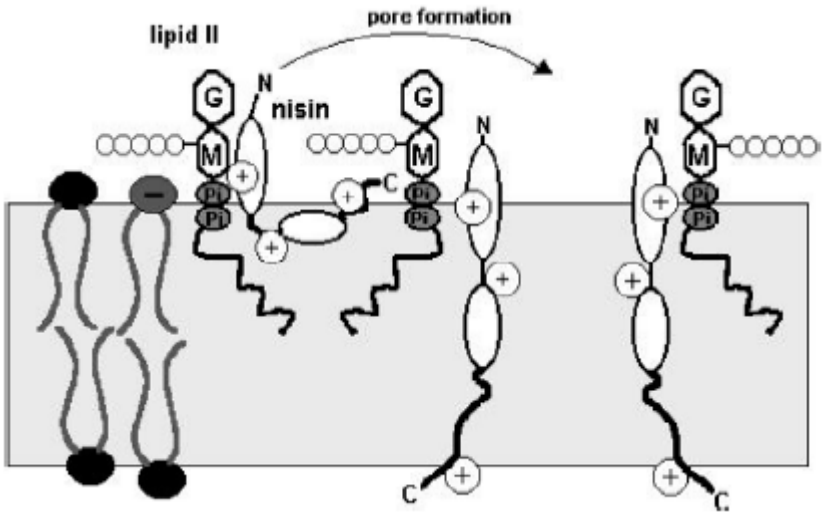



**Fig. 2.4** Model for the lipid II-mediated nisin pore formation

(**Source:** Wiedemann et al., 2001)

From the model proposed by Wiedemann et al. (2001) in Fig.2.3A and 2.4, it is clear that the carboxylic function has to migrate through the cell membrane for pore formation. The C- terminal portion of the protein is also rather flexible if compared with the N-portion, allowing higher accessibility to the carboxylic moiety (Guiotto et al., 2003). Initially, a barrel-stave model may adequately describe the activity of nisin in Fig.2.3C (Kodel, Benz and Sahl, 1988; Ruhr and Sahl, 1985; Sahl and Brandis, 1983). According to such a model, α-helical amphipathic peptides initially bind via electrostatical interaction to the outer leaflet in a parallel orientation to the membrane surface. In order to avoid the unfavorable position of polar residues to the lipid acyl chains, several monomers of peptides have to assemble forming a bundle of helices. After insertion of the peptides into the membrane the non-polar side-chains of the peptides interact with the hydrophobic lipid core of the membrane and the hydrophilic

side-chains point inward, which results in the formation of a water-filled pore. When conformational data derived from NMR studies applying nisin in the presence of membrane-mimicking micelles (van den Hooven, Doeland, van de Kamp, Konings, Hilbers and van de Ven, 1996a; van den Hooven, Spronk, van de Kamp, Konings, Hilbers and van de Ven, 1996b) became available, a “wedge model” (Fig. 2.3B) for pore formation by lantibiotics was proposed (Moll et al., 1996; van den Hooven et al., 1996b). Upon contact with the membrane, the peptides adopt an amphiphilic conformation with the charged residues aligned to one face of the molecules and the hydrophobic residues aligned to the other. The cationic peptides interact with the phospholipids head groups by ionic forces causing a locally disturbed bilayer structure, while the hydrophobic residues insert into the membrane (Demel, Peelen, Siezen, de Kruijff, Kuipers and Nisin, 1996; van den Hooven et al., 1996a; van den Hooven et al., 1996b). Studies with nisin demonstrated that the C-terminal region as well as the overall negative surface charge of the membrane was important for binding and pore formation (Breukink, van Kraaij, Demel, Siezen, Kuipers and de Kruijff, 1997; El-Jastimi and Lafleur, 1997; Giffard, Ladha, Mackie, Clark and Sanders, 1996; van Kraaij et al., 1998; Winkowski, Ludescher and Montville, 1996). It is assumed that several molecules have to associate within the membrane in order to form a pore (Moll et al., 1996), since nisin is too short to transverse the membrane more than once. The formation of pores leads to dissipation of small metabolites such as amino acids or ATP, which in turn immediately stops all cellular biosynthesis processes of macromolecules such as DNA, RNA, proteins and polysaccharides (Fig. 2.5). Furthermore, bacterial cells are unable to actively take-up amino acids and become leaky for inorganic ions and small metabolites (Benz, Jung and Sahl, 1991;

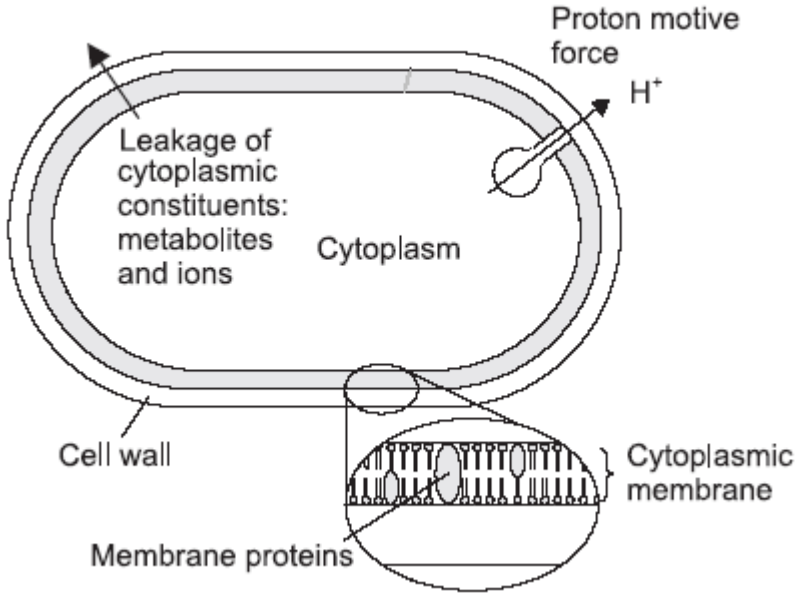



**Fig. 2.5** Mechanisms in the bacterial cell thought to be sites of action for nisin and dipping solutions: degradation of the cell wall; damage to cytoplasmic membrane; leakage of cell contents and depletion of the proton motive force.

**(Source:** modified from Burt, 2004)

Jack, Tagg and Bibek, 1995; Ruhr and Sahl, 1985; Sahl and Brandis, 1983).

The inability of nisin to attack Gram-negative bacteria is due to the protective OM, which covers the cytoplasmic membrane and peptidoglycan layer of Gram-negative cells. This asymmetrical membrane contains glycerophospholipids in its inner leaflet, but the outer leaflet is built of LPS molecules. LPS, which are composed of a lipid part and a complex heteropolysaccharide with partly anionic character, form a tight layer endowed with a hydrophilic surface (Nikaido, 1996). As a result, OM is a penetration barrier that excludes hydrophobic substances and macromolecules. Nisin, as a hydrophobic macromolecule ($M_r$ = 3353), is unable to traverse a normal OM and

thus cannot reach its target of action. The cytoplasmic membrane of Gram-negative cells is also sensitive to nisin when the outer membrane is disrupted e.g. by ion chelators such as Ethylene diaminetetra acetic acid (EDTA) or citrate (Stevens, Sheldon, Klapes and Klaenhammer, 1991) causing an aberration in its lipopolysaccharide component. Nisin, which would increase the microbial safety of foods that do not receive a severe processing treatment, may become important in the preservation of minimally-processed foods, even though regulatory obstacles currently make these natural preservatives unavailable for commercial use.

## 2.4 Sanitizing agents

To reduce or eliminate bacteria attached to poultry carcasses, various methods, including thermal treatment, chemical dipping and spraying, electrical pasteurization and irradiation have been evaluated. Antimicrobial agents presently applied in poultry processing include the use of chlorine in chiller water to prevent cross-contamination of poultry carcasses during the chilling process. A chlorinated water spray has also been used to rinse chicken carcasses at the end of an evisceration line. However, chlorinated water does not reduce the number of *Salmonella*-positive carcasses, indicating that S*almonella* already on the carcass are not accessible to the chlorine (Yang, Li and Slavik, 1998). Many compounds, including hydrogen peroxide, organic acids, phosphates and other food grade compounds, have been explored for controlling bacterial populations on poultry by dipping or spraying.

### 2.4.1 Trisodium phosphate (TSP)

Trisodium phosphate (TSP, $Na_3PO_4$), a generally recognized as safe (GRAS) food additive according to the FDA classification, has been approved by USDA for use during poultry processing (Wang, Li, Slavik and Xiong, 1997). USDA/FSIS has approved its use with poultry in concentrations of 8 to 12% w/v when maintained at a temperature between 45 and 55$^{o}$F. It is typically used in concentrations of 10% due to its proven effectiveness and reduced phosphate waste at this level (Arritt, 2000). If the TSP is consumed in pure form the oral $LD_{50}$ is 7400 $mgkg^{-1}$ (rat) (Rhodia Inc., 1996). It has been shown to kill gram-negative organisms artificially inoculated on surfaces of variety of foodstuffs and *Listeria monocytogenes* on stainless steel (Carneiro de Melo, Cassar and Miles, 1998). Wang et al. (1997) used a 10% solution of TSP with varying spray pressures to reduce the amount of *S. typhimurium* on breast skin by between 1.5 and 2.3 $log_{10}$ CFU. Salvat et al. (1997) also reported a reduction in the incidence and concentration of *Campylobacter* spp. from chicken neck skin samples after treatment with a 10% TSP solution. Whole carcasses were used to test another 10% solution of TSP for reduction of *S. typhimurium* and total aerobes by Yang et al. (1998) with net losses of 2.0 $log_{10}$ CFU and 0.74 $log_{10}$ CFU respectively.

### 2.4.2 Cetylpyridinium chloride (CPC)

Cetylpyridinium chloride (CPC) (Fig. 2.5), or 1-hexa-decyl pyridinium chloride, is a quaternary ammonium compound with antimicrobial properties against many microorganisms including viruses (FDA, 1998). It is classified as a cationic surface-active agent and contains acetyl radical substituted for hydrogen atom on position 1. In hydrochloric acid it forms a chloride salt. The cetyl radical renders the

molecule lipophilic, contributing to the lipophilic/hydrophilic balance which is necessary for the antimicrobial activity of such quaternary nitrogenous compounds (FDA, 1998). Currently it is used in some commercial mouthwashes to prevent the formation of dental plaque (Frost and Harris, 1994). 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).

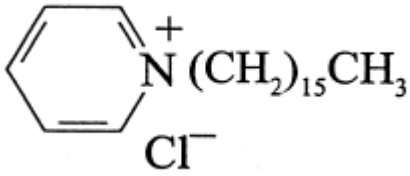


**Fig. 2.6** Chemical structure of cetylpyridinium chloride

(**Source:** Zhou, Handie, Salari, Fifer, Breen and Compadre, 1999)

The $LD_{50}$ for rats is 200 $mgkg^{-1}$ given orally as a pure compound (FDA, 1998). Wang et al. (1997) showed that with a temperature of 10, 35, or 60$^{o}$C, spray pressure from 0 to 1034.2 kPa, and spray duration of 30 sec, *Salmonella* reductions with 0.1% CPC were 1.5 to 2.5 $log_{10}$ CFU. A 0.5% CPC solution maintained a pH of 7.6 and reduced *Salmonella* by 2.16 $log_{10}$ CFU (Yang et al., 1998). Reaction times of 1, 3, and 10 min. at CPC levels of 1, 2, 4 and 8 $mgml^{-1}$ resulted in *Salmonella* reductions of between 0.59 and 4.91 $log_{10}$ CFU (Breen, Salari and Compadre, 1997). Arritt (2000) found that reduction of *Campylobacter jejuni* > 1.0 $log_{10}$ $CFUskin^{-1}$ was achieved with the 0.1% CPC, when bacteria were applied before treatment. When

bacteria were applied after treatment, a reduction of *Campylobacter jejuni* > 1.0 $\log_{10}$ CFUskin$^{-1}$ was achieved with 0.5% CPC.

### 2.4.3 Sodium lactate (SL)

Sodium lactate (SL) is generally recognized as safe. In 1988, the Food-Safety Inspection Service (FSIS) of the United States Department of Agriculture approved the use of sodium lactate (2g/100g of meat product) as a flavor enhancer and flavoring agent in various meat and poultry products (Houtsma, Wit and Rombouts, 1996; Nuñez de Gonzalez, Keeton, Acuff, Ringer and Lucia, 2004). There have been many reports of sodium lactate's antimicrobial activity in meat products (Shelef, 1994). One possibility is that the undissociated form moves across the cell membrane, and dissociates inside the cell thus acidifying the cell cytoplasm resulting in disruption of cell functions (Long and Phillips, 2003). As sodium lactate inhibits toxin formation by *Clostridium botulinum*, it could also be used to improve the microbiological safety of chicken and turkey products (Houtsma, 1994). Lactate was believed to lower the $a_w$ of the environment and thus exert its antimicrobial effect (Loncin, 1975). Furthermore, sodium lactate has been reported to exhibit antioxidant activity. Addition of up to 3 % lactate results in a shelf life extension of cured meat products, fish and uncured meat without negatively affecting sensory characteristics (Pelroy, Peterson, Holland and Eklund, 1994; Shelef, 1994). Papadopoulos, Miller, Acuff, Vanderzant and Cross (1991) showed that cooked beef containing 3 or 4% sodium lactate had 2 log units reduction in its microbial population during storage. The addition of 2 or 3% sodium lactate to fresh pork sausages extended the lag phase and the log phase of the growth of aerobic microorganisms, thus increasing the storage stability and reducing off-

odors (Shelef, 1994). Brewer, Mckeith and Sprouls (1993) reported that compared with the control, an additional week of shelf life was achieved for fresh pork sausage containing 1% sodium lactate and packaged under vacuum. Bunic, Fitzerald, Bell and Hudson (1995) found that 4% sodium lactate completely inhibited the growth of *Listeria monocytogenes*. Lin and Chuang (2001) reported that pork loin chop dipped in 10% sodium lactate solution had lower total plate counts and psychrotrophic counts and had better visual appearance at 6 days of cold storage than the non-treated chops.

## 2.5 Microbial contamination during poultry processing

Since the 1960s poultry has been transformed from a luxury item to a cheap and plentiful source of protein. This has arisen primarily as a result of the industrialization of poultry farming. Production units are now fewer in number yet significantly larger, while throughput at slaughter and processing plants has increased greatly due to mechanization. For example, a local poultry processing plant has the ability to process almost 500,000 chickens in a week. Consequently, once pathogens are introduced under such conditions, they can be transferred to other birds at an increased rate. Furthermore, the technical equipment used in poultry processing makes it almost impossible to prevent cross contamination of carcasses. The responsibility of poultry processors therefore, must involve all practical steps include reduce to a minimum level of contamination associated with the final product.

It is thought that live birds become infected with *Salmonella* through contact with contaminated food and water, vermin, transportation crates and contaminated workers. In the case of *S. enteritidis*, trans-ovarian infection was a major contamination issue for some time but this has now been addressed. Intensive farming

establishments, responsible for production of the majority of poultry consumed in the UK, represent a high contamination risk. The prevailing cramped conditions in which the birds live provide ample opportunity for the spread of contaminating organisms, once introduced, from bird to bird. If it is accepted that such practices are a commercial reality and contamination is likely to remain an issue, then treatment of birds in the processing plant may provide a satisfactory compromise in dealing with *Salmonella* contamination of poultry meat.

On arrival at the processing plant, birds commonly undergo the treatments, where there is potential for contamination and/or cross contamination with *Salmonella*.

### 2.5.1 Transportation

Contamination of birds has been reported during transportation due to a combination of increased faecal shedding and the close physical proximity of birds. Contaminating organisms may also be picked up from the vehicle. Indeed, in 1982, Rigby, Pettit, Bentley, Spencer, Salomons and Lior found 99% of transport crates in their study tested positive for *Salmonella*. Since then, the introduction of more readily cleaned transportation crates has ameliorated this problem rather than eliminated it. Increased stress in the animals may also increase the risk of infection by reducing immune system competence while increasing the rate of faecal shedding. Therefore, there is a strong possibility that specific batches of birds will arrive at the processing plant in an internally and externally contaminated state, thus introducing pathogens to this environment. External contamination by *Salmonella*, i.e. on feather skin and feet,

for example, can reach levels as high as $1.7x10^7$ $CFUg^{-1}$. This provides an important reservoir of contamination with implications for the subsequent processing.

### 2.5.2 Hanging

When the live birds arrive at the processing plant they are suspended by their feet from a rail which transports them to the stunning stage. Should they arrive in a contaminated state, wing flapping and general struggling will create microbial aerosols further contaminating the unloading zone. The likely outcome here is additional cross-contamination of arriving birds.

### 2.5.3 Stunning

Stunning of birds prior to slaughter commonly involves immersion of their heads in a charged water bath. Defecation into this medium has the potential to further contaminate the birds either externally, or internally by ingestion of the contaminated water.

### 2.5.4 Neck cutting and ex-sanguination

Neck cutting and ex-sanguination follows rapidly after stunning to ensure the birds are dead before they have the chance to regain consciousness. Necks are cut mechanically or manually and the birds bleed to death. Since knife blades are not washed between birds there is a potential for cross contamination here. Manual cutting provides the additional risk of contamination from the slaughterman.

### 2.5.5 Scalding

Scalding eases the removal of feathers. Birds are immerged in a water bath for 1.5 to 4 minutes at 50-52$^{o}$C (soft scald, for air chilled carcasses) or 60-63$^{o}$C (hard scald, for water chilled carcasses). Greater numbers of salmonellae are destroyed at higher scald temperatures although these conditions are generally used only for production of birds for freezing since unsightly skin blotching may occur. The consumer regards such cosmetic faults as undesirable so 'fresh' birds are often scalded at lower, less lethal, temperatures (50-52$^{o}$C), increasing the risk of sustaining contamination. These sub-lethal temperatures fail to eliminate fully external contamination. Furthermore, the water bath itself provides a medium for cross contamination. Some studies have shown, however, that the degree of external contamination may actually be reduced at this point (Mead, Hudson and Hinton, 1993). Nevertheless, viable salmonellae have been isolated from scald water and carcasses subsequent to this treatment. Humphrey and Lanning (1987) also found that scalding leads to changes in the birds' skin, allowing greater opportunity for colonization of the carcass by contaminating organisms both in the scald water and at subsequent processing points. Specifically, it was noted that damage or removal of the epidermal layer exposed a smother and less hydrophobic surface, increasing the potential for contamination. Also there was the possibility of exposing microscopic channels and crevices, again providing a more favorable environment for bacterial colonization.

The greater potential for contamination created here is compounded further by the emergence of the carcass covered in a film of scald water contaminated with organic matter and large numbers of bacteria. Such bacteria now have favorable

conditions for attachment to, or entrapment within the skin of the chicken carcass. These attached organisms are much more difficult to remove and express increased resistance to heat.

### 2.5.6 Mechanical defeathering

Rubber flails attached to counter-rotating disks carry out defeathering mechanically. Water is introduced (often with sodium hypochlorite added) to facilitate the removal of feathers. The circulation of water within the plucker provides the potential for cross contamination of carcasses. In addition, there is the opportunity for bacteria to attach to the rubber fingers and stainless steel of the plucking machine providing a long-term reservoir of contamination. It is likely that plucking represents the primary source of contamination of the final product. Indeed, a number of studies have shown that bacteria recovered from the final carcass reflect the population within the plucking machine.

The evidence for the ability of chlorine to reduce the level of contamination is equivocal (Mead et al, 1993). Furthermore, it has been shown that *Salmonella* from a poultry abattoir had acquired resistance to hypochlorite concentrations of 72 ppm (Mokgatla, Brozel, and Gouws, 1998), suggesting that this treatment alone may not be sufficient to control contamination.

### 2.5.7 Evisceration

Following the removal of head and feet, evisceration is carried out mechanically or manually, by cutting a ‘vent’ followed by spooning out the viscera. These are then allowed to hang outside the bird or are placed on a tray. The latter is

believed to lead to a lower degree of contamination. It is not practical to clean cutting blades between birds, so they may, therefore, act as media for cross contamination. Since the reservoir of *Salmonella* in birds entering the factory is likely to be in the ceca or crop, the degree to which viscera are kept intact will have a bearing upon the subsequent level of contamination of the carcass. When this occurs it has been suggested that rinsing the carcass with 20 ppm chlorine would be an appropriate precautionary measure (Dickens and Whittemore, 1994). This technique, however, is not widespread due to the increased costs associated with reprocessing.

Subsequent to evisceration the crop, trachea and thymus are removed. Internal organs such as the lungs and heart are then taken out mechanically by suction. The carcass is then spray-washed inside and out with (usually chlorinated) water. This process has been shown to improve the visual and microbiological quality of the bird (Abu-Ruwaida, Husseuni and Banat, 1995). It is much less effective, however, at removing attached or entrapped bacteria.

### 2.5.8 Chilling

At this point the carcasses pass on to the final chilling stage. The speed of contemporary poultry processing means that birds can emerge from evisceration at a temperature of 30$^{o}$C. This is a favorable growing temperature for *Salmonella* so prompt chilling at this stage is not only sagacious but a legal requirement under the poultry meat hygiene regulations. A number of chilling methods are employed, each being associated with specific contamination risks. Most commonly, carcasses are moved through counter-flowing chilled water. The water represents a potential reservoir of contaminating organisms and often has chlorinated compounds added. It

is possible, however, that this method of chilling may have no effect at all on level of *Salmonella* contamination (Cason, Bailey, Stern, Whittemore and Cox, 1997). On the other hand, a Puerto Rican study demonstrated that immersion of carcasses in a water bath at 45$^{o}$C gave rise to an increase in the number of contaminated carcasses from 28 to 49% (James, Brewer, Prucha, Williams and Parham, 1992). Low- level chlorination (18-30 mgl$^{-1}$) of this water has been shown to reduce rather than eliminate this cross contamination (Mead, Hudson and Hinton, 1994).

A method alternative to chilling, particularly popular in the turkey industry, is to immerse the carcasses in a static slush ice tank. The colder temperatures associated with this method are ineffective at eliminating contamination, although the static nature of the bath may reduce the degree of cross contamination. Some poultry processors use spray chilling to avoid the cross contamination risk of chiller tanks while retaining the benefit of washing. This method, however, uses a large amount of water and has been associated with aerosol based cross-contamination (Fries and Graw, 1999).

Another popular chilling method originally used in the turkey industry but now by most of the poultry industry, is air chilling. Here the birds are chilled by a blast of cold air or in a chill room. This technique does not have the washing benefit associated with water-based methods nor does it have the cross contamination risk. This balance is borne out in evaluation studies that tend to show little difference in contamination when air-based are compared with water-based methods (Abu-Ruwaida et al., 1995). This is primarily due to a balance within the processes themselves. Birds destined to be frozen were exposed to higher scald temperatures and were chilled by water-based methods, whereas ‘fresh’ birds were exposed to

lower scald temperatures and air-chilled. Consequently the benefit of the higher scald temperature was offset by water chilling and *vice versa*.

## 2.6 Attachment

The attachment mechanism initially involves retention of bacteria in a liquid film on the skin from which they migrate and become entrapped in ridges and crevices. The process of retention begins with the live birds and is exacerbated during scalding. It continues during processing, and the level of contamination is directly related to the microbial concentration in the processing water. The scalding operation opens feather follicles to aid feather removal, and the follicles remain open throughout processing until chilling during immersion. Certain microorganisms (e.g. *Campylobacter*, *Salmonella*) adhere to polysaccharide material and material surrounding collagen fibers. The nature of bacterial attachment to skin is complex and involves many elusive factors (Arritt, 2000).

## 2.7 Bioluminescence

Conventional microbiological techniques are often inadequate for determining the viability and extent of injury of microorganisms when exposed to certain stimuli, e.g., bactericidal treatments. Generally, conventional microbiology relies on the ability of microorganisms to grow from a single cell into a visible colony under aerobic conditions. This methodology relies on the ability of microorganisms to completely recover under defined culture conditions. However, there is now substantial evidence indicating that sublethally injured cells can be nonculturable. As a result, conclusions drawn from antimicrobial, challenge and storage studies by

culture methods may not be entirely valid (Bautista, Chen, Barbut and Griffiths, 1998). Bacterial bioluminescence could be a novel approach to monitor viability of microorganisms in samples. In this method, the bacterium of interest is genetically modified to emit light by incorporating a genetic sequence that encodes the enzyme responsible for light production by naturally luminescent bacteria (Bautista et al., 1998).

The ability of organisms to emit light is termed bioluminescence. It is believed that bioluminescence, which is visible with the naked eyed plays an important role in the survival or reproduction of species. Bioluminescent organisms have been seen in both prokaryotes and eukaryotes except in mammalian cells. Bioluminescent organisms commonly live in the sea with one genus existing in the terrestrial environment. They range from dinoflagellates to fungi, fireflies, jellyfish and bacteria (Campbell, 1988).

### 2.7.1 The native bioluminescent bacteria

All different species of bioluminescent bacteria fall into four different genera: *Vibrio, Photobacterium, Alteromonas* and *Xenorhabdus*. They are Gram-negative, motile rods and function as facultative anaerobes (Meighen, 1994). *Vibrio, Photobacterium* and *Alteromonas* are originally from marine environments such as seawater. *Xenorhabdus* is a terrestrial organism and some of its species have a parasitic life in some insects. In fact the process of luminescence is found in organisms with different life-styles including parasitic, symbiotic, saprophytic and free-living bacteria. The ecological benefit for a fish or a squid living in symbiotic association with luminescent bacteria has been established (Nealson and Hastings,

1979). Light emitted by bioluminescent bacteria can be used for attraction of prey, escape from predators or interspecies communication (Bassler and Silverman, 1995; Morin, Harrington, Nealson, Krieger, Baldwin and Hastings, 1975)

### 2.7.2 Biochemical and genetic basis of bioluminescence

In bioluminescent bacteria the *lux* operon contains a number of genes, with five structural genes (*CDABE*) required for the bioluminescent phenotype common in almost all of them (Meighen, 1991). Genetic engineering techniques have facilitated the conversion of natively dark bacteria to bioluminescent by transformation of either the entire *lux* operon or just *luxAB* with an aldehyde substrate being added exogenously (Ulitzur and Kuhn, 1988). This has great potential for employing the *lux* genes as a means of reporting growth and gene expression. Bioluminescence involves a luciferase-catalysed oxidation of the reduced form of flavin mononucleotide ($FMNH_2$) by molecular oxygen, with the concomitant oxidation of a long-chain aliphatic aldehyde such as dodecanal and results in the emission of blue green light (Jay, 2000; Stewart and William, 1992).

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + \text{light (490 nm)}$$

For bioluminescent assays in microbiology it is necessary to have instruments capable of detecting low levels of light, and the ability to transfer the genes (luminescence genes) encoding the enzymic components for light production into normally dark bacteria. These genetically manipulated bacteria must be identical to their parents in

all other physiological respects. The *lux* genes from *Vibrio* spp. have been transferred into other species by plasmid transformation, transposition or conjugation. Transformation involving plasmids, to transfer the luminescence genes into a host, contain purified DNA comprising appropriately modified bioluminescence genes. At high population densities ($10^7$ to $10^8$ cells per ml) cells of *E. coli* transformed with specific plasmids exhibited light emission within 2 to 5 h (Ulitzer and Kuhn, 1987). However, the time of onset of luminescence was inversely proportional to the cell concentration when transformed with a constant amount of DNA. Conjugation involves the transferring of DNA from one bacterial cell to another by plasmids containing the transfer genes. Transformation and conjugation are the methods most often used in transfer of *lux* genes into various gram-positive and gram-negative bacteria. Ulitzer and Kuhn (1987) found that transformation or conjugation of *lux* DNA was approximately 1000-fold less sensitive for detection of bacteria than was transduction. Transformation by *lux* genes has achieved light emission from a wide diversity of bacterial species which are normally non-bioluminescent. Light emission by these recombinant organisms may be exploited from a number of perspectives (Stewart, Aldsworth, Sharman, Gibson and Dodd, 1997). By monitoring light output from these bacteria over time, it can be easily determined if the organism is growing (resulting in an increase in light emission), is dead (causing the loss of light production) or is injured (light output remains constant or declines). The use of imaging systems allows the response of bioluminescent bacteria to be studied directly on the food, making the technique even more useful (http://www.pulsus.com/Infdis/1103/grifed.htm, 2001; Warriner, Eveleigh, Goodman, Betts, Gonzales and Waites, 2001).

### 2.7.3 Application of *lux* genes

As in marine bioluminescent bacteria, bioluminescence is a function of bacterial metabolism. Consequently an injury in the cells causing an interruption in the biochemical pathways, causes a loss of light in the bacteria. The power of bioluminescence to report on bacterial injury and recovery, to respond quickly to the activity of antibacterial agents and to signal the detection of living pathogens comes from the relationship between cellular viability and living reaction (Hill, Rees, Winson and Stewart, 1993). The gene encoding bioluminescence can be engineered into non-bioluminescent bacteria, allowing them to produce light. This has enabled a vast range of bacterial species to become light producers. The bioluminescent sensors have been made by using transformation technology. Since the production of light from recombinant organisms depends on a functional intracellular biochemistry, agents that disrupt or kill the *lux*-containing bacteria destroy their metabolism and directly or indirectly affect the intracellular production of $FMNH_2$, so reducing the bioluminescence of the culture. Therefore, this principle can be used to evaluate the biological efficacy of food-grade preservatives. Bioluminescence is reported as a relative light unit (RLU) or $\log_{10}$ RLU (LRLU) (Green, Russell and Fletcher, 1998). Luciferase reactions are rapid, requiring only minutes to complete, and the amount of photons quantified with a luminometer can be converted to the number of bacterial cells per milliliter (Swaminathan and Feng, 1994; White, Leifert, Ryder and Killham, 1996).

Food contamination can be detected in less than 15 min using bioluminescence (Kricka, 1988). Although the dark terrestrial organisms that need to be monitored in food microbiology (pathogens, starter cultures, hygiene indicators)

could not produce luciferase or fatty acid reductase, they can produce $FMNH_2$. For their detection all that is needed is the transfer of the genes for only luciferase and fatty acid reductase (Stewart, Smith and Denyer, 1988). In addition, the study of bacteria attached to carcass surfaces facilitated by *lux* reporter systems has produced detailed knowledge of the interaction of foodborne pathogens with food animal tissue surfaces. In this study beef carcass surface tissue inoculated with bioluminescent *E. coli* 0157:H7 harbouring *luxAB* were visualized in real-time using a sensitive proton counting camera to obtain *in situ* images (Siragusa, Nawotka, Spilman, Contag and Contag, 1999). With this method there is no need to obtain, excise, homogenize and culture multiple samples from the tissue surface. The need to add exogenous aldehyde was eliminated by using the complete *lux* operon as the bioluminescent reporter system. This allowed detection and quantification of bacterial inocula and rapid evaluation of adherence of a potential human pathogen to tissue surfaces.

The major advantage of this approach is that the metabolic activity of the modified bacteria can be monitored in real time directly since light emission decreases with time when the organism is exposed to an inhibitory stimulus. The opposite is true for favorable conditions. Since samples can be continuously monitored with minimal disturbance during analysis, bacterial bioluminescence may be more useful for challenge and storage tests than cultural techniques. Monitoring metabolic activity might provide a better indication of the organism's ability to recover from injury or stress than conventional cultural techniques. As a result, more accurate interpretations of an organism's response to antibacterial treatments can be obtained on a single sample during the entire course of the study. Therefore, bioluminescence may be used

as a tool to investigate the effectiveness of bactericides and biopreservatives for removing bacterial contaminants from food products.

## 2.8 BacLight nucleic acid stain

The dual staining BacLight stain has been demonstrated to distinguish accurately between dead and live cells (Haugland, 1996). This method is based on the retention of the cell membrane integrity as a characteristic of the bacterial viability status (Decamp and Rajendran, 1998). This stain contains a mixture of two fluorochromes, SYTO9-green fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide (PI). The excitation/emission maxima of these dyes are about 490/635 nm for PI and 480/500 nm for SYTO9. The SYTO9 stain generally labels all bacteria in a population, those with intact and damaged membranes, thus acting as total cell stain. On the contrary, PI is membrane impermeant and generally excluded from viable cells; it penetrates only bacteria with damage2d membranes and quenches the green SYTO 9 fluorescence. Thus, bacteria with intact or 'live' cells will fluoresce green and damaged bacteria cells will fluoresce red (Alonso, Mascellaro, Moreno, Ferrus, and Hernandez, 2002; Ananta and Knorr, 2004; Auty et al., 2001; Boulos, Pre′vost, Barbeau, Coallier, and Desjardins, 1999; Bunetel et al., 2001; Moleacular Probes, 2001; Que′ric, Soltwedel, and Arntz, 2004).

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# CHAPTER III

# CONSTRUCTION OF BIOLUMINESCENT *Salmonella typhimurium* AND ITS SENSITIVITY TO TRISODIUM PHOSPHATE, CETYLPYRIDINIUM CHLORIDE, SODIUM LACTATE AND NISIN

## 3.1 Abstract

*Salmonella typhimurium* S36 was constructed by transformation with the mini-Tn5 plasmid containing the *lux CDABE* cassette with kanamycin resistance gene. Sensitivity studies were performed by determining cell viability and bioluminescence output following exposure to trisodium phosphate, cetylpyridinium chloride (CPC), sodium lactate and nisin treatments at mid-exponential and stationary phase of bioluminescent *S. typhimurium* S36. The stationary phase of *S. typhimurium* S36 is more resistant to the treatments than the mid-exponential phase cells. Especially, CPC-nisin proved to be the most effective treatment to inhibit *S. typhimurium* S36. Also, the study demonstrated that bacterial bioluminescence is an effective way of monitoring in real time the sensitivity of bacteria to antimicrobial agents and other treatments by measuring bioluminescence output.

## 3.2 Introduction

Salmonellosis is one of the most frequent foodborne diseases, being an important public heath problem worldwide. In the last decades, a significant increase in the incidence of salmonellosis has been reported in Europe by □□ H□ □ur□e□llan□e programme for Control of Foodborne Infections and Intoxications in Europe□ (Schmidt, 1998). In the United States, between 1993 and 1997, *Salmonella* accounted for the largest number of outbreaks and cases of foodborne disease (CDC, 2000).

Bacterial bioluminescence may be a novel approach to monitor viability of microorganisms in samples. In this method, the interest bacterium is genetically modified to emit light by incorporating a genetic sequence that encodes the enzyme responsible for light production by naturally luminescent bacteria (Baker, Griffiths, and Collins-Thompson, 1992; Chen and Griffiths, 1996; Stewart and Williams, 1992). The luminescence genes can be introduced into the target organism by transformation, conjugation, or transduction. The result is a genetically modified organism that can be easily identified and detected in a variety of environments (Chen and Griffiths, 1996; Duffy, Ellison, Anderson, Cole, and Stewart, 1995). The major advantage of this approach is that the metabolic activity of the modified bacteria can be monitored in real time directly in samples. For example, light emission decreases with time when the organism is exposed to an inhibitory stimulus. The opposite is true for favorable conditions. Since samples can be continuously monitored with minimal disturbance during analysis, bacteria bioluminescence may be more useful for challenge and storage tests than cultural techniques. Monitoring metabolic activity might provide a better indication of the organism ability to recover from injury or stress than conventional cultural techniques. As a result, more accurate interpretations of an

organism response to antimicrobial treatments can be obtained on a single sample during the entire course of the study.

Nisin, a bacteriocin, exerts rapid bactericidal effects against Gram-positive bacteria but Gram-negative bacteria are generally resistant (Stevens, Klapes, Sheldon and Klaenhammer, 1992). However, if the OM is penetrated by chelating agents including EDTA or pretreated with cell membrane disrupters such as trisodium phosphate then the cells become more sensitive to nisin (Carneiro de Mero, Cassar and Miles, 1998; Cutter and Siragusa, 1995).

Trisodium phosphate (TSP) has been approved for use as a food ingredient (Federal Register, 1982). At 8 to 15% w/v (0.2 to 0.4 M), it has been demonstrated to kill gram-negative bacteria artificially inoculated on surfaces of a variety of foodstuffs (Dickson, Nettles-Cutter and Siragusa, 1994; Zhuang and Beuchat, 1996). Possible mode of action of TSP include exposure of microorganisms to high pH, which might particularly affect cell membrane components (Mendonca, Amoroso, and Knabel, 1994), sequestration of metal ions (Lee, Hartman, Olson and Williams, 1994), and its role as a surfactant, enhancing the detachment of bacteria from food surfaces. The USDA has approved TSP to be utilized in the poultry industry to reduce possible contamination of *Salmonella* (Giese, 1993) and *E. coli* O157:H7 (Kim and Slavik, 1994).

Cetylpyridinium chloride (CPC) is a versatile ingredient that can be used in ready-to-cook, ready-to-eat, and processed products manufactured from poultry, meat, and fish as well as fruits and vegetables (Anonymous, 2000). CPC is effective against many pathogens, including *Salmonella, Campylobacter*, and *E. coli* O157:H7, and does not adversely affect the flavor, texture, appearance, or odor of foods. As

germicidal surfactant agent, the antibacterial activity of CPC is caused by its cation. CPC has both hydrophilic and lipophilic properties derived from the chloride and cetylpyridinium cations, respectively. Along with its amphiphilic properties, the low surface tension of CPC allows it to effectively penetrate cells (Lim and Mustapha, 2004).

Sodium lactate (SL) is used as a humectant or flavor enhancer (CFR 184.1768) in cooked meat and poultry products, cured or fresh poultry products (Federal Register, 1990; Shelef, 1994). Buncic, Fitzerald, Bell and Hudson (1995) found that 4% sodium lactate completely inhibited the growth of *Listeria monocytogenes*. Wang (2000) demonstrated that addition of 3% sodium lactate to Chinese-style sausage maintained low microbial numbers.

The purposes of this study were (1) to construct bioluminescent *Salmonella. typhimurium* for using through experiments, (2) to evaluate the effectiveness of TSP, CPC, SL and nisin, alone and in combination against a bioluminescent *S. typhimurium* strain during different growth phases.

## 3.3 Materials and methods

### 3.3.1 Construction of bioluminescent *S. typhimurium*

*S. typhimurium* was 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*) gene cassette and the kanamycin resistance gene. This plasmid was maintained within *E. coli* λPir1 (Simon, Priefer and Puhler, 1983).

#### 3.3.1.1 Preparation of Plasmid DNA

Large scale preparation of plasmid DNA was carried out using QIAGEN 500 maxi prep kits or QIAGEN 100 midi kits according to the manufactures instructions (Qiagen GmbH), indicated in appendix A.

#### 3.3.1.2 Preparation of competent *E.coli* cells

To prepare the competent cells of *E. coli*. A 5 ml overnight culture of *E. coli* S17-1 was inoculated into 500 ml of Luria-bertani-Lennox (LB) broth in a 2 L flask. The flask was then inoculated in a shaking incubator at 37$^{o}$C until the absorbence reading at 600 nm ($OD_{600}$) of the culture reached 0.5-0.6 in a spectrophotometer (CECIL 2021). Immediately the culture was placed on ice for 15 min and then harvested in a Beckman J2-21 centrifuge (Beckman Instruments Inc, Glenrothes, United Kingdom) using a JA-10 rotor at 7000 x g at 4$^{o}$C. The supernatant was discarded and the pellet was washed twice in ice-cold sterile distilled water (SDW), a first time in 500 ml and a second time in 250 ml. Then the cells were resuspended in 20 ml ice-cold 10% glycerol (Sigma) and were centrifuged as above. The cells were resuspended in a final volume of 0.5 ml in ice cold 10% glycerol. This suspension was aliquoted, in 40 portions, into sterile Eppendorf tubes and frozen rapidly in liquid nitrogen. The competent cells were stored at -70$^{o}$C.

#### 3.3.1.3 Transformation of competent cells by electroporation

Competent *E. coli* S17-1 cells were transformed by electroporation with a purified plasmid preparation as described by Winson et al. (1998). To remove the excess of salts from the DNA to be introduced into the cells, the DNA was dialysed

by pipetting into a 0.025 μm dialysis filter (Millipore; catalogue number VSWP 013) floating on distilled water and allowing the salts in the DNA sample to dissipate for 30 min. The DNA was recovered from the filter by pipetting using a Gilson pipetman (Anachem, UK). The DNA (0.5-2 μg in 15 μl) was added to the 40 μl aliquot of competent cells and the mixture was transferred to an ice-cold 0.2 cm electroporation cuvette (Bio-Rad, Richmond CA). The cuvette was then placed in an ice-cold safety chamber slide and a pulse of 25 μF, 2.5 kV and 200 Ω was delivered using the Bio-Rad gene pulsar$^{TM}$ electroporator (Fig. 7.2A. in appendix B). Immediately after the pulse, 1 ml of sterile LB broth was added to the cuvette and gently mixed by pipetting action. The cuvette was then incubated in stationary incubator at 37$^{o}$C for 1 hour to allow the introduced genes to be expressed. The cuvette contents were spread out onto LB agar plates supplemented with kanamycin to isolate cells containing recombinant DNA and incubated overnight at 37$^{o}$C.

#### 3.3.1.4 Conjugation

Conjugation between the donor strain, *E. coli* S17-λpir1, and wild-type *S. typhimurium* (recipient cells) strain isolated from a slaughter house, was performed on a Whatman cellulose acetate membrane (0.45 μm pore size) overlaid onto a LB agar plate (Difco Laboratories, Surrey, UK) that was subsequently incubated at 37$^{o}$C for 14 h (De Lorenzo and Timmis, 1994). The selection of *Salmonella* exconjugants were selected by incubating 500 μl aliquots of conjugation mixture in 10 ml Rappapart-Vassiliadis (RV) broth (Oxoid, UK) containing kanamycin (30 μgml$^{-1}$) and incubated at 42$^{o}$C for 24 h. Serial dilutions of this overnight culture broth were then plated onto xylose lysine desoxycholate (XLD) agar medium (Oxoid, UK) containing kanamycin

and incubated overnight at 37$^{o}$C. *S. typhimurium* exconjugants formed black bioluminescent colonies. Bioluminescent colonies on agar plates were imaged using a Night-Owl image analyzer in conjunction with the manufacturer computer software (E.G. & G. Berthold, Munich, Germany).

**3.3.1.5 Bioluminescence of growing cultures.**

Growth of *lux* transformants was also determined by monitoring light output to select an exconjugate *S. typhimurium lux*. For automated luminometry and photometry, Lucy1 Photoluminometer (Fig. 7.2B in appendix B) (Anthos labtech, Salzburg, Austria) and associated software was used as described by Winson et al (1998). The overnight cultures in brain heart infusion (BHI) broth were diluted 1:100 and 1:1000 in fresh BHI broth containing kanamycin (30 $\mu gml^{-1}$) and the 200 µl samples of each dilution were loaded into a clear-bottomed 96-well microtitre plate. This was set to 37$^{o}$C and was programmed to measure OD at approximately 590nm. Optical density and luminosity readings were taken at 30 min intervals for 24 h. Data were written to a spreadsheet format compatible with Microsoft Excel for further analysis. In each case the optical density data were written in milli-absorbance units ($OD_{590}$). Plots of $\log_{10}$ (luminescence/optical density) and optical density ($OD_{590}$) against time (h) are used to represent the data. All the plots shown were done in at least triplicate.

### 3.3.2 Sensitivities of phase of growth of bioluminescent *S. typhimurium* treated with sanitizing agents and nisin.

#### 3.3.2.1 Bacterial preparation

Suspensions of mid-log phase bioluminescent *S. typhimurium* were obtained from cultures grown in BHI broth containing kanamycin (30 $\mu gml^{-1}$) to ensure plasmid stability, in a shaking incubator (150 rpm) at 37$^{o}$C until the $OD_{600}$ of the culture reached 0.4-0.5 (Fig. 7.1 in appendix A) in a spectrophotometer (CECIL 2021). Immediately the culture was placed on ice for 15 min and then harvested in a Beckman J2-21 centrifuge (Beckman Instruments Inc, Glenrothes, United Kingdom) using a JA-10 rotor at 4000 x g at 4$^{o}$C. The supernatant was discarded and the pellet was washed once in ice-cold sterile MRD. The cell pellets were finally resuspended in MRD to yield a suspension containing ca. $10^{7}$ $CFUml^{-1}$ ($A_{600}$ 0.2). Suspensions of stationary phase bioluminescent *S. typhimurium* were prepared from overnight culture grown at 37$^{o}$C in BHI broth supplemented with kanamycin. The cells were harvested and resuspended as previously described.

#### 3.3.2.2 Trisodium phosphate, cetylpyridinium chloride, sodium lactate and nisin preparation

Trisodium phosphate (TSP), cetylpyridinium chloride (CPC) and sodium lactate (SL) were obtained from Sigma. Each solution was made by dissolving in distilled water. Nisin was obtained from Sigma. The nisin solutions were dissolved in 0.02 M HCl. Each solution was sterilized by filtration through 0.45 μm filters (Gelman Sciences, Michigan) and stored at 4$^{o}$C until required.

### 3.3.2.3 Effect of TSP, CPC, SL and nisin on mid-log and stationary phases of bioluminescent *S. typhimurium*

Ten milliliters of mid-log and stationary phase of bioluminescent *S. typhimurium* suspensions containing approximately $10^5$ $CFUml^{-1}$(Tomicka, Chen, Barbut and Griffiths, 1997) were treated alone with 1 ml of 0.5 % (w/v) TSP, 0.05% (w/v) CPC, 1.0% (w/v) SL for 30 sec and 100 $\mu gml^{-1}$ nisin for 30 min. Combination treatments between 0.5 % (w/v) TSP, 0.05% (w/v) CPC, 1.0% (w/v) SL for 30 sec each followed by filtering through sterile filter membrane (0.45 μm). The bacteria-retaining filters were added into 10 ml MRD and bacteria revealed by vortexing for 1 min. The mixtures were then treated with 1 ml nisin (100 $\mu gml^{-1}$) for 30 min as TSP-nisin, CPC-nisin and SL-nisin treated cells. After 30 min, the mixtures were serially diluted in sterile MRD and plated (100 μl) in duplicate on BHI agar containing kanamycin to determine their viability. Plates were incubated for 16-18 h at 37$^o$C before colonies were counted.

#### 3.3.2.3.1 Determination of bioluminescence

Bioluminescence reading was taken using the Turner TD-20e luminometer serial 0583 (Fig. 7.2C in appendix B) (Turner designs, Inc., M.T. View, CA). Culture aliquots and treatments (from 3.3.2.3) were placed in scintillation vial inserts and the light output was expressed as relative light units (RLU).

**3.3.2.3.2 Imaging of bacterial cells stained with the BacLight nucleic acid stain**

Ten microlitters of *S. typhimuirum* cell suspensions were dropped onto glass microscope slides, aliquots (3 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 spotted on, and a coverslip was placed over the suspensions. After incubation for 15 min in the dark at room temperature, stained samples were observed under an epifluorescent microscope and images captured on a Nikon DXM-1200/LUCIA digital imaging system (Japan).

### 3.3.3 Statistical analysis

Microbiological counts were transformed to $\log_{10}$ data before statistical analysis. All experiments were performed in triplicates. Statistical analysis was evaluated in Completely Randomized Design (CRD) with Statistical Analysis System (SAS Institute, Inc., 1995.). Analysis of variance (ANOVA) and mean values comparison by Duncan□□New Multiple Range Test (DMRT).

## 3.4 Results and discussions

### 3.4.1 Construction of bioluminescent *S. typhimurium*

The conjugation between *E.coli* S17-1λPir carrying the mini-Tn5 vector and wild-type *S. typhimurium* generated a large number of exconjugants with a bioluminescent phenotype (Figs. 3.1A to 3.1C). The bioluminescent signal intensities of exconjugant colonies were initially determined with a Night-Owl image analyzer.

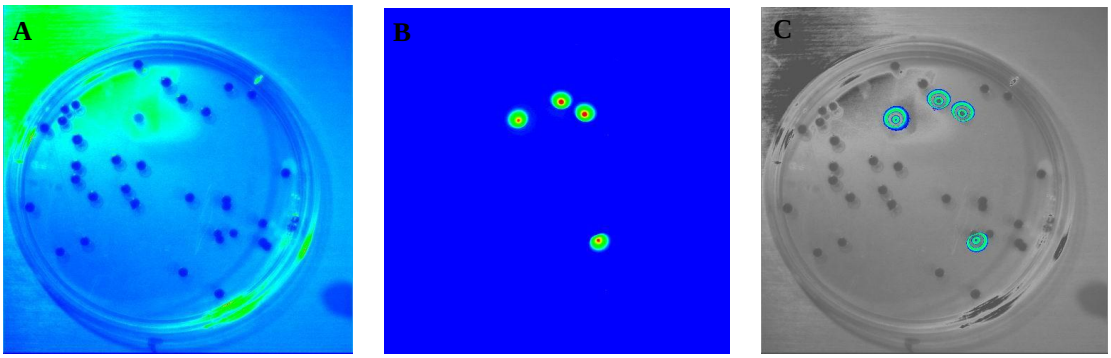


**Fig. 3.1** Bioluminescence images of exconjugant colonies. Visible colonies appearance of exconjugant colonies (A), bioluminescence pseudocolor of the same colonies of A (B), and color overlaid of exconjugant colonies to reveal the bioluminescent colonies (C).

Bioluminescent signals of bioluminescent images were represented as pseudocolor images (Fig. 3.1B), where color was indicative of light intensity (red represented the most intense signal, and green represented the least intense signal). Then the pseudocolor images were overlaid on the reference images (illuminate images) (Fig. 3.1A) to reveal the bioluminescent colonies (Fig. 3.1C). One exconjugate expressing high bioluminescence was selected from the range of exconjugants generated and further characterized. The phenotypic bioluminescent characteristic of the exconjugates was retained following several transfers onto BHI agar indicating that the label was stable within the chromosome of the host. The growth of the tagged *S. typhimurium* S36 did not differ in BHI broth compared to parental strain (Fig. 3.2). The extent of bioluminescence of *S. typhimurium* S36 was found to be high during the exponential phase of growth but decreased upon entering the stationary phase. Nevertheless, the intensity of light production in *Salmonella* S36

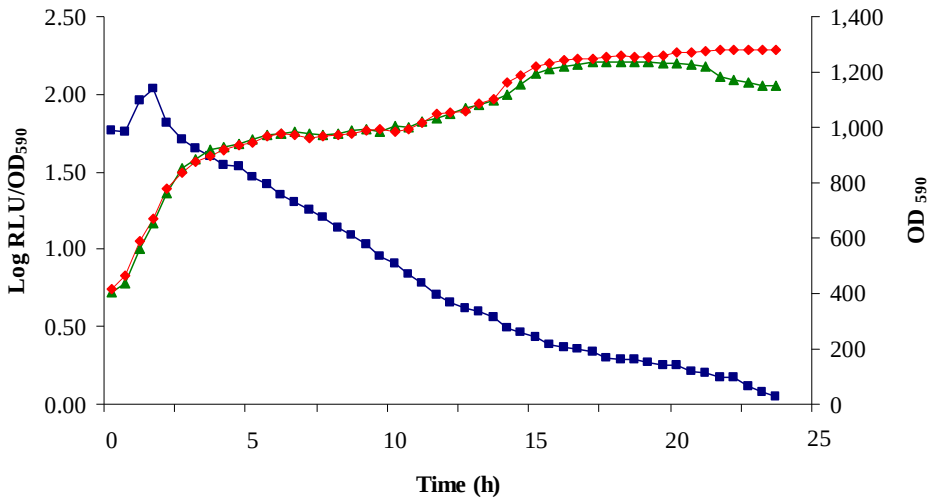


**Fig 3.2** Growth and bioluminescent of *S. typhimurium* in BHI broth at 37°C. The wild-type and exconjugate S36 were inoculated into BHI broth and optical density measurements ($OD_{590}$) performed at 30 min intervals for 24h. In parallel the luminosity (relative light units, RLU) of the exconjugate culture was measured and subsequently converted to specific bioluminescence activity (■). Optical density of wild-type (▲) and Optical density of exconjugate S36 (♦) are given in milli-absorbance units ($OD_{590}$).

was dependent on the growth phase bioluminescence activity and was detected throughout growth. The *Salmonella* exconjugates S36 generated exhibited a range of bioluminescent activities. This finding has also been previously observed with *Pseudomonas aeruginosa* GP41B exconjugants, and was attributed to the transposon inserting behind different promoters of the recipient chromosome (Dandie et al., 2001). In terms of tracing studies the criteria for selection was based on the transposon insert having no major impact on the physiology of the cell and on the bioluminescence activity being detectable at different stages of growth. Although the

precise insertion point within the *S. typhimurium* S36 chromosome was not determined there was no significant influence over the extent or rate of growth in BHI broth. The highest bioluminescence activity was observed at different times during exponential growth, suggesting that the insert was controlled by promoter activation during this phase. As cells entered the stationary phase of growth the bioluminescence decreased reflecting the down regulation of metabolic activity of the cell (Rattray, Prosser, Killham and Glover, 1990). However, a low level of bioluminescence was still maintained by *S. typhimurium* S36 in late stationary phase of growth.

### 3.4.2 Sensitivities of phase of growth of bioluminescent *S. typhimurium* treated with sanitizing agents and nisin

#### 3.4.2.1 Viable cells of mid-log and stationary phases bioluminescent *S. typhimurium* treated with sanitizing agents and nisin

The present study was conducted to evaluate the effect of 0.5% (w/v) TSP, 0.05% (w/v) CPC, 1.0% (w/v) SL and 100 $\mu gml^{-1}$ nisin against wild-type and bioluminescent *S. typhimurium*. Cell suspensions ($10^5$ $CFUml^{-1}$) of wild-type and bioluminescent *S. typhimurium* were exposed to sublethal concentration of TSP, CPC and SL for 30 sec or/and were then treated with 100 $\mu gml^{-1}$ nisin for 30 min. As shown in Table 3.1, viable cells of mid-log phase of wild-type and bioluminescent *S. typhimurium* treated with CPC, nisin, TSP-nisin, CPC-nisin and SL-nisin could not be detected. In contrast the viable cells of mid-log phase of wild-type and bioluminescent *S. typhimurium* treated with TSP, SL and untreated control were detected, and their quantities did not significantly differ from each other ($p>0.05$). Similarly, the viability

of stationary phase of wild-type *S. typhimurium* untreated control and treated with TSP, CPC, SL and nisin did not differ significantly ($p>0.05$) compared with bioluminescent *S. typhimurium* S36 (Table 3.2). In contrast, TSP-nisin, CPC-nisin and SL-nisin had an effect in reducing viable cells of wild-type and bioluminescent *S. typhimurium* compared with untreated control and TSP, CPC, SL and nisin treatments. Especially the CPC-nisin treatment showed the highest susceptibility ($p\leq0.05$) to stationary phase of wild-type and bioluminescent strains. These results suggest that CPC greatly increased the susceptibility of growth phases (log and stationary phases) of wild-type and bioluminescent *S. typhimurium* to nisin. It may due to CPC, a cationic surfactant, is capable of adsorbing onto bacterial cell membranes, possibly disrupting the cell wall and increasing permeability of Gram-negative bacterial cells. Moreover, CPC could change the outer membrane and also produce nisin sensitivity in *Salmonella*. In comparison, mid-log phase of wild-type and bioluminescent *S. typhimurium* S36 were more sensitive to sanitizing agents and nisin treatments than stationary phase of both strains. The results were in agreement with Dodd, Sharman, Bloomfield, Booth and Stewart (1997), who explained that exponentially growing cells are more sensitive than stationary phase cells to inimical processes such as heating, freezing and the presence of biocides and antibiotics. This difference in resistance is currently explained by the differential enzyme systems that provide an adaptive advantage to the stationary phase cell.

**Table 3.1** Viable cells of mid-log phase wild-type *S. typhimurium* and bioluminescent *S. typhimurium* S36 treated with 0.5% (w/v) TSP, 0.05% (w/v) CPC, 1.0% (w/v) SL and 100 μgml$^{-1}$nisin

| Treatments | $Log_{10}$ CFUml$^{-1}$* | |
|---|---|---|
| | Wild-type *S. typhimurium* | *S. typhimurium* S36 |
| Control$^{ns}$ | 5.20±0.10$^{a}$ | 5.20±0.08$^{a}$ |
| TSP$^{ns}$ | 4.94±0.02$^{b}$ | 5.00±0.03$^{c}$ |
| CPC$^{ns}$ | ND$^{c}$ | ND$^{d}$ |
| SL$^{ns}$ | 5.01±0.03$^{b}$ | 5.05±0.06$^{b}$ |
| Nisin$^{ns}$ | ND$^{c}$ | ND$^{d}$ |
| TN$^{ns}$ | ND$^{c}$ | ND$^{d}$ |
| CN$^{ns}$ | ND$^{c}$ | ND$^{d}$ |
| SN$^{ns}$ | ND$^{c}$ | ND$^{d}$ |

*Means with different letters in the same column are significant different (P≤0.05)

$^{ns}$Not significantly different (p>0.05) between wild-type and bioluminescent *S. typhimurium* were evaluated with the paired Student *t*-test.

TSP = 0.5% (w/v) trisodium phosphate, CPC = 0.05% (w/v) cetylpyridinium chloride

SL = 1.0% (w/v) sodium lactate, Nisin = 100 μgml$^{-1}$nisin

TN = 0.5% (w/v) TSP and 100 μgml$^{-1}$nisin, CN = 0.05% (w/v) CPC and 100 μgml$^{-1}$nisin

SN = 1.0% (w/v) SL and 100 μgml$^{-1}$nisin

ND = No viable cell detected ($log_{10}$ < 1CFUml$^{-1}$)

**Table 3.2** Viable cells of stationary phase wild-type *S. typhimurium* and bioluminescent *S. typhimurium* S36 treated with 0.5% (w/v) TSP, 0.05% (w/v) CPC, 1.0% (w/v) SL and 100 μgml$^{-1}$nisin

| Treatments | $Log_{10}$ CFUml$^{-1*}$ | |
|---|---|---|
| | Wild-type *S. typhimurium* | *S. typhimurium* S36 |
| Control$^{ns}$ | 5.20±0.10$^{a}$ | 5.11±0.12$^{a}$ |
| TSP$^{ns}$ | 4.99±0.31$^{ab}$ | 5.01±0.14$^{a}$ |
| CPC$^{ns}$ | 4.81±0.51$^{ab}$ | 4.91±0.25$^{a}$ |
| SL$^{ns}$ | 4.97±0.26$^{ab}$ | 5.05±0.16$^{a}$ |
| Nisin$^{ns}$ | 4.51±0.42$^{bc}$ | 4.80±0.46$^{a}$ |
| TN$^{ns}$ | 3.82±0.34$^{d}$ | 3.81±0.36$^{b}$ |
| CN$^{ns}$ | ND$^{e}$ | ND$^{c}$ |
| SN$^{ns}$ | 3.99±0.28$^{dc}$ | 4.18±0.37$^{b}$ |

*Means with different letters in the same column are significant different (P≤0.05)

nsNot significantly different (p>0.05) between wild-type and bioluminescent *S. typhimurium* were evaluated with the paired Student *t*-test.

TSP = 0.5% (w/v) trisodium phosphate, CPC = 0.05% (w/v) cetylpyridinium chloride

SL = 1.0% (w/v) sodium lactate, Nisin = 100 μgml$^{-1}$nisin

TN = 0.5% (w/v) TSP and 100 μgml$^{-1}$nisin, CN = 0.05% (w/v) CPC and 100 μgml$^{-1}$nisin

SN = 1.0% (w/v) SL and 100 μgml$^{-1}$nisin

ND = No viable cell detected ($log_{10}$ < 1CFUml$^{-1}$)

#### 3.4.2.2 Bioluminescence output of mid-log and stationary phases bioluminescent *S. typhimurium* treated with TSP, CPC, SL, and nisin

The bioluminescent *Salmonella* is able to produce bioluminescence output, which was measured using a luminometer. Light output was reported as relative light units (RLU). The efficacy of TSP, CPC, SL and nisin for reducing viability of *S. typhimurium* S36 in different growth stages was estimated. Bioluminescent output of mid-log phase *S. typhimurium* S36 (Table 3.3) demonstrated that CPC treatment dramatically decreased the light output of *Salmonella* compared with TSP and SL treatments. As for the nisin treatment, it increased the bioluminescence output. This result indicated that nisin had no effect on *S. typhimurium* S36, which is in agreement with the previous assumption that Gram-negative bacteria, including *Salmonella*, are generally resistant to nisin since their protective OM (Cutter and Siragusa, 1995). When TSP, CPC and SL were used with nisin, however, minimal light output was observed in the case of the CPC-nisin treatment. In contrast, the light emission of mid-log phase *S. typhimurium* S36 treated with TSP-nisin and SL-nisin increased.

The effects of TSP, CPC, SL and nisin on light emission by the stationary phase of bioluminescent *S. typhimurium* S36 are summarized in Table 3.4. Apparently, CPC treatment resulted in less bioluminescence output than TSP, SL and nisin treatments, while CPC-nisin treatment produced the least light output. It could be explained that the emission of light by a bioluminescent strain can be used to monitor cell viability, measured as the immediate reduction in light emission in the treatment with antimicrobial agents. Cell viability is thus quantifiable by measuring light production of the intracellular reaction catalyzed by the luciferase enzyme, which requires a metabolically active cell (Bautista, Chen, Barbut and Griffiths,

**Table 3.3** Bioluminescence output of mid-log phase bioluminescent *S. typhimurium* treated S36 with 0.5% (w/v) TSP, 0.05% (w/v) CPC, 1.0% (w/v) SL and 100 $\mu gml^{-1}$ nisin

| Treatment | RLU | |
|---|---|---|
| | Control | Treated |
| TSP | 100.3±2.40 | 99.5±3.39 |
| CPC | 105.6±0.00 | 1.876±0.62 |
| SL | 111.6±0.85 | 109.6±0.71 |
| Nisin | 68.25±4.10 | 122.0±3.96 |
| TN | 75.15±2.84 | 135.0±1.13 |
| CN | 84.41±9.47 | 0.006±0.01 |
| SN | 93.05±1.63 | 137.6±1.41 |

TSP = 0.5% (w/v) trisodium phosphate, CPC = 0.05% (w/v) cetylpyridinium chloride

SL = 1.0% (w/v) sodium lactate, Nisin = 100$\mu gml^{-1}$nisin

TN = 0.5% (w/v) TSP and 100$\mu gml^{-1}$nisin, CN = 0.05% (w/v) CPC and 100$\mu gml^{-1}$nisin

SN = 1.0% (w/v) SL and 100$\mu gml^{-1}$nisin,

RLU = Relative light units

**Table 3.4** Bioluminescence output of stationary phase bioluminescent *S. typhimurium* S36 treated with 0.5% (w/v) TSP, 0.05% (w/v) CPC, 1.0% (w/v) SL and 100 $\mu gml^{-1}$ nisin

| Treatment | RLU | |
|---|---|---|
| | Control | Treated |
| TSP | 4404.0±84.48 | 4501.7±74.51 |
| CPC | 4649.3±183.15 | 107.9±9.18 |
| SL | 4956.0±105.06 | 4980.3±86.75 |
| Nisin | 3256.3±65.84 | 3005.3±290.66 |
| TN | 3493.3±36.61 | 5943.3±144.72 |
| CN | 3821.7±118.26 | 0.024±0.01 |
| SN | 4108.0±102.12 | 5893.3±157.64 |

TSP = 0.5% (w/v) trisodium phosphate, CPC = 0.05% (w/v) cetylpyridinium chloride

SL = 1.0% (w/v) sodium lactate, Nisin = 100$\mu gml^{-1}$nisin

TN = 0.5% (w/v) TSP and 100$\mu gml^{-1}$nisin, CN = 0.05% (w/v) CPC and 100$\mu gml^{-1}$nisin

SN = 1.0% (w/v) SL and 100$\mu gml^{-1}$nisin, RLU = Relative light units

1998). In this model, a reduction in light output due to a low physiological activity of the cells or a decrease in cellular metabolic activity (Ramos, Molina, M□l□a□, □amo□ and Molin, 2000) indicated injured and dead cells. Also, these results showed the advantage of bioluminescence which could detect the viable-but-nonculturable cells. Contrary, the detection limit of culture viable count method could not detect the viable-but-nonculturable cells. The results of viable cells and bioluminescence outputs

suggested that the CPC-nisin treatment was the most effective treatment for reducing population of *S. typhimurium* S36 in both growth phases. In the present study, CPC and nisin showed strong synergistic effect against the Gram-negative *S. typhimurium* S36.

### 3.4.3 Visualization of *S. typhimurium* cells using BacLight nucleic acid stain

BacLight assesses membrane integrity by selective stain exclusion (Haugland, 1996). The BacLight-stained *S. typhimurium* cells were viewed under an epifluorescent microscope. This probe is useful for the assay of bacterial viability using dual staining methodology: On one hand, the green fluorescent nucleic acid stain SYTO 9 is membrane permeable and labels bacteria with both intact and damaged membranes. On the other hand, the red fluorescent nucleic acid stain propidium iodide (PI) penetrates only bacteria with compromised membranes, in which case it competes with SYTO 9 for nucleic acid binding sites (Haugland, 1999). Consequently, those bacteria with healthy membranes appear fluorescent green, while bacteria with damaged membranes have a fluorescencent red. Epifluorescent micrographs (Fig. 3.3) showed the staining of *S. typhimurium* treated with CPC-nisin with the BacLight nucleic acid stain. The cells within untreated control samples appeared fluorescent green, indicating living *Salmonella* cells (Fig. 3.3A) since the PI cannot transverse intact cell membranes. The CPC treatment showed a mixture of green and red cells (Fig. 3.3B) indicating that there were live and dead cells respectively. The CPC-nisin treatment showed a majority of red cells (dead cells or ruptured cells) (Fig. 3.3C). This would suggest that CPC treatment caused sufficient

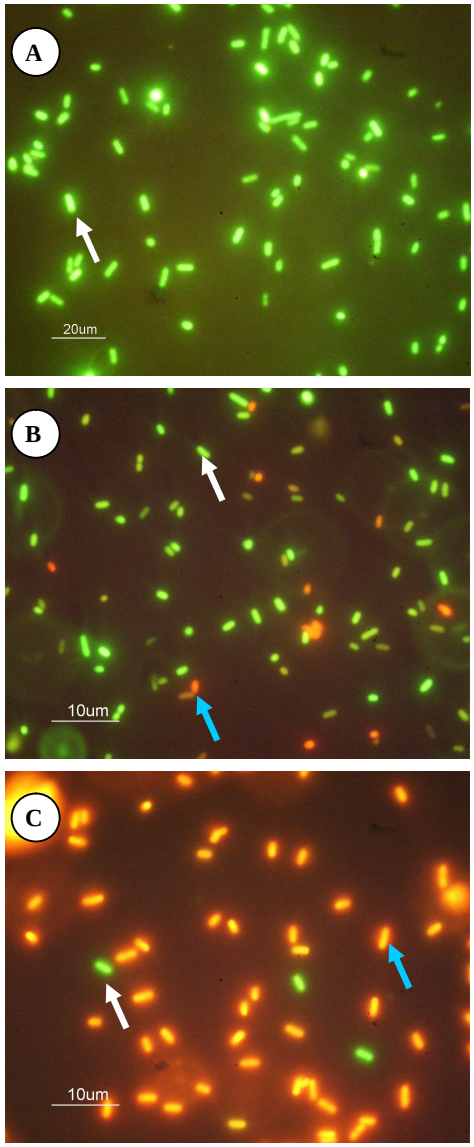


**Fig. 3.3** Epifluorescent micrographs show the BacLight staining of viable and nonviable *S. typhimurium* S36 cells treated with 0.05%(w/v) CPC and 100 μg.ml$^{-1}$ nisin. (A) Arrow shows live cell (green cell) of untreated control. (B) After treated with CPC, white arrow shows viable (green) bacterium, with blue arrow pointing to a nonviable (red) bacterium. (C) Treated with CPC-nisin, white arrow indicates live cell, blue arrow indicates dead cell (x1000).

disrupting of the cell membrane, thus allowing the PI stain to penetrate. This finding also correlated with the viable counts and bioluminescence output in Table 3.1-3.4. Obviously, the CPC-nisin treatment resulted in more dead cells, since CPC caused morphological damage to *Salmonella* cells in suspensions (Kim and Slavik, 1996) and altered the outer membrane permeability of *Salmonella*, producing a loss of barrier function and nisin sensitivity. The effect of CPC and nisin observed in the bioluminescent strain of *S. typhimurium* S36 was similar to that found in wild-type *Salmonella* strains (Bautista, Chen, Barbut and Griffiths, 1998). In addition, these results seem to confirm the synergistic effect of CPC and nisin against *Salmonella* cells. Besides this, epifluorescent micrographs of TSP and SL solutions with nisin treatments (Fig. 7.3, in appendix C) also revealed similar images of both live and dead cells compared to CPC-nisin treatment images (Fig. 3.3). Finally, the effectiveness of the sanitizing agents to enhance nisin sensitivity on stages of growth of bioluminescent *S. typhimurium* S36 was considered. TSP and SL solutions were less effective in reducing viable cells and light output of bioluminescent *Salmonella*, while the CPC solution was the most effective.

## 3.5 Conclusions

Bioluminescent *S. typhimurium* S36 can be a valuable tool for studying antimicrobial agent effects on the inactivation of bacteria populations in growth phases. CPC had the most synergistic effect with nisin against *Salmonella* cell suspensions, as shown by the decrease in luminescence activity of the bioluminescent strain. Hence, the CPC solution was selected to be used with nisin in an effort to

enhance antimicrobial efficacy on stationary phase of bioluminescent *S. typhimurium* in the further experiments (Chapters IV and V).

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# CHAPTER IV

# SUSCEPTIBILITIES OF TEMPERATURE AND PH-STRESSED BIOLUMINESCENT *S. typhimurium* TO CETYLPYRIDINIUM CHLORIDE AND NISIN

## 4.1 Abstract

The outer membrane (OM) of Gram-negative bacteria such as *Salmonella*, act as a permeability barrier, preventing nisin from gaining access to the cytoplasmic membrane. If the OM permeability is reduced, however, Gram-negative bacteria can show nisin sensitivity. In this study, temperature stress (heating, chilling and freezing), pH stress (pH 4.5, 5.0, 6.0) and cetylpyridinium chloride (CPC)-nisin treatment were used to alter the OM permeability of *Salmonella typhimurium* S36, producing a loss of barrier function and reduced resistance to nisin. The morphological changes in *Salmonella* were examined using scanning and transmission electron microscopy. Temperature- and pH-stressed *S. typhimurium* S36 cells, untreated and treated with CPC-nisin had perturbed cell morphology, including apparent indentations, craters in the cell surfaces and collapsed amorphous bodies. Moreover, blebs, cytoplasm-sparse and an abnormal shape of cells were observed.

## 4.2 Introduction

Nisin is a 35-amino acid cationic peptide antimicrobial, bacteriocin, produced by *Lactococcus lactis* subs. *lactis* (Delves-Broughton, Blackburn, Evans, and Hugenholtz, 1996). It has been accepted as a food additive by both the FDA and WHO and has found several applications in food to date (Schillinger, Geisen, and Holzapfel, 1996). Nisin is active against almost all Gram-positive bacteria and their spores, excluding Gram-negative bacteria or fungi (Delves-Broughton et al., 1996; Stevens, Klapes, Sheldon, and Klaenhammer, 1992). Sensitivity of some Gram-negative bacteria to nisin has been reported (Stevens, Sheldon, Klapes and 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 cell turgor pressure maintenance, enzyme activation, intracellular pH homeostasis regulation, and together with depletion of intracellular ATP (Moll, Roberts, Konings, and Driessen, 1996; Montville, Chung, Chikindas, and Chen, 1999). In some bacteria, the interference of nisin with lipid II (C55 bactoprenol pyrophosphate-a carrier involved in cell wall biosynthesis) in the membrane is proved to be the cause of cell death due to loss of cell integrity (Chung and Hancock, 2000).

In general, the protective outer membrane (OM), 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 (LPS) respectively. LPS, which are composed of a lipid part and a heteropolysaccharide part, partly show anionic characteristic which is essential

property of hydrophilic surface (Nikaido, 1996). Because OM is a permeability barrier of hydrophobic substances and macromolecules, nisin, as a hydrophobic macromolecule, cannot reach its site of action (Helander and 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). (Boziaris, Humpheson and Adams, 1998; Carneiro de Melo, Cassar and Miles, 1998; Cutter and Siragura, 1995; Hauben, Wuytack, Soontjens and Michiels, 1996; Kalchayanand, Hanlin and Ray, 1992). 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, Tsuchido, Hiramatsu, Fujikawa, Takano and Shibasaki, 1982; Tsuchido, Katsui, Takeuchi, Takano and Shibasaki, 1985). However, such injury can be subsequently repaired if the cell is not severely disrupted (Boziaris and Adams, 2001).

Cetylpyridinium chloride (CPC) is a quaternary ammonium compound having antimicrobial properties against many bacteria such as *Escherichia coli* O157:H7, coliforms, *Salmonella typhimurium*, *Campylobacter jejuni*, *Aeromonas hydrophila*, *Listeria monocytogenes*, and *Staphylococcus aureus* as well as viruses (FDA, 1998; Pohlman, Stivanrius, McElyea and 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 and 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 outer membrane, could be an significantly 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 transmission electron microscopy (TEM), and viability of cells.

## 4.3 Materials and methods

### 4.3.1 Bacterial strain

*S. typhimurium* S36 containing the complete *lux CDABE* gene (constructed in Chapter III) was used throughout this study and cultured overnight in BHI broth supplemented with kanamycin to ensure plasmid stability in shaking incubator (150 rpm) at 37$^{o}$C. The cells were harvested by centrifugation for 10 min, 4,000 x g at 4$^{o}$C, washed once with maximum recovery diluent (MRD; Oxoid, Basingstoke, UK)

mixed, and centrifuged. The resulting pellets were finally resuspended in MRD to give a suspension.

### 4.3.2 Cetylpyridinium chloride (CPC) and nisin

Cetylpyridinium chloride (CPC) (Sigma, UK) was dissolved in distilled water. Nisin (Sigma,UK) was dissolved in 0.02 M HCl. Each solution was filter sterilized (0.45 μm, Gelman Sciences, Michigan) and stored at 4$^{o}$C until used.

### 4.3.3 Temperature and pH stresses

#### 4.3.3.1 Temperature stress

The temperature stresses (heating, chilling and freezing/thawing) of 10 ml ($10^{7}$ CFUml$^{-1}$; Boziaris and Adams, 2001) of *S. typhimurium* S36 were conducted in universal bottles. For heating treatment, the bottles containing *Salmonella* suspensions were transferred to a water-bath operating at 55 $\pm$ 1$^{o}$C and left for 10 min. They were then placed immediately in cold water at 4$^{o}$C for 5 min before moving to room temperature. For chilling, the cell suspensions were placed in an ice water-bath (~0.5$^{o}$C) for 30 min and then left at room temperature for 5 min. Freeze/thaw treatment was performed by placing the cell suspensions in a freezer at -20$^{o}$C for 24 h. The suspensions were transferred to a static incubator at 37$^{o}$C for 30 min where they were thawed and then left for 5 min at room temperature. The control cells were placed in a stationary incubator at 37$^{o}$C for 30 min and then left for 5 min at room temperature.

#### 4.3.3.2 pH stress

Portions (100 μl each) of *Salmonella* suspensions were inoculated in 20 ml of BHI broth under the following conditions: pH 4.5, 5.0, 6.0, and 7.4 (control) at 37$^{o}$C with shaking at 150 rpm for 12-14 h. The pH-stressed cells were harvested by centrifugation with a Beckman model J2-21 centrifuge at 4,000 x g for 10 min at 4$^{o}$C. The harvested cells were then washed with 10 ml of MRD, centrifuged, and then resuspended in MRD to give a cell density of ca. $10^{7}$ CFUml$^{-1}$ ($A_{600}$ 0.2).

### 4.3.4 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 sec 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 sec to remove bacterial cells. The CPC treated cells were further treated with 1 ml of nisin (100 μgml$^{-1}$) for 30 min. The viability of temperature and pH 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$^{o}$C for 16-18 h after which colonies were counted. Nonetheless, the measurement of bioluminescence output as described in 3.3.2.3.1 (Chapter III). The temperature and pH stressed cells were also subjected to an examination of morphological changes using SEM and TEM.

### 4.3.5 Electron microscope

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 and TEM.

#### 4.3.5.1 Scanning electron microscope (SEM)

After CPC-nisin treated cells (5 ml) as described, were filtered through a cellulose nitrate membrane. The bacteria on the filter were then fixed for 2 h in 3 % (w/v) glutaraldehyde at 4$^{o}$C, and washed three times for 30 min each in 0.1 M sodium phosphate buffer, pH 7.2. Cells were then post-fixed with 1% (w/v) osmium tetroxide ($OsO_4$) solution 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 (50, 70, 90, 95 and 95 % (v/v) for 5 min each. Samples were then dried in a critical point dryer, and mounted on stubs, sputtered with gold, and viewed under a JSM-6400 scanning electron microscope (JEOL, Japan) at an operating voltage of 20 kV.

#### 4.3.5.2 Transmission electron microscope (TEM)

The pellets were resuspended in 3 % (w/v) glutaraldehyde. After 2 h at 4$^{o}$C, the cells were centrifuged to gain compact cell pellets. Following washing in 0.1M sodium phosphate buffer (pH 7.2), the cell pellets were prepared for electron microscopy by post-fixing with 1% (w/v) $OsO_4$ in sodium phosphate buffer for 1 h. After three washes with the same buffer, the pellets were embedded in 2% melted agar, dehydrated through a graded series of ethanol (35, 50, 70, 95, 100 % (v/v) for 15 min per immersion), infiltrated in a propylene oxide for 15 min twice followed

working mixture (propylene oxide: Spurr's resin, 1:1) for 30 min and Spurr's resin for 60 min. They were then embedded in 100% Spurr's resin and polymerized at 60°C in a hot air oven for 72 h. The thin sections of the polymerized samples were cut with a RMC/MTX 75500 ultramicrotome and double-stained with uranyl acetate and lead citrate. The grids were examined in a JEM 2010 transmission electron microscope (JEOL, Japan) at an operating voltage of 80 kV.

### 4.3.6 Imaging of bacterial cells stained with the BacLight nucleic acid stain

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

### 4.3.7 Statistical analysis

Bacterial counts were performed to $\log_{10}$ value before statistical analysis. All experiments were performed in triplicates. Statistical analysis was evaluated in Completely Randomized Design (CRD) with Statistical Analysis Systems (SAS Institute, Inc., 1995). Analysis of variance (ANOVA) was analyzed and mean values comparison was done by Duncan's New Multiple Range Test (DMRT).

## 4.4 Results and discussions

### 4.4.1. Effect of CPC-nisin treatment on the cell viability

The results in Table 4.1 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 treated all temperature stressed cells with CPC-nisin, freeze stressed cells also showed the highest kill ($p \leq 0.05$) of 5.16 $\log_{10}$ $CFUml^{-1}$. Heat stress with CPC-nisin treatment indicated higher kill ($p \leq 0.05$) of 3.43 $\log_{10}$ $CFUml^{-1}$ than chill stressed with CPC-nisin treatment, which was not significantly different from control ($p > 0.05$) in term 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, Humpheson and Adams, 1998; Helander and Mattila-Sandholm, 2000; Ray, Miller and 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 and 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 is agree with

**Table 4.1** $Log_{10}$ reduction ($log_{10}$ $CFUml^{-1}$) of temperature-stressed *S. typhimurium* following treatment with 0.05% (w/v) CPC and 100 $\mu gml^{-1}$ nisin

| Sample | $Log_{10}$ reduction ($log_{10}$ $CFUml^{-1}$)[2,3] |
|---|---|
| Control[1] | $2.91\pm0.41^{c}$ |
| Heating | $3.43\pm0.22^{b}$ |
| Chilling | $3.00\pm0.09^{c}$ |
| Freezing | $5.16\pm0.75^{a}$ |

[1]Control = 37°C

[2]$Log_{10}$ reduction = $Log_{10}$ ($N_0/N$), where $N$ is the colony count after treatment and $N_0$ is the colony count for the untreated sample.

[3]Means with different letters within a column are significantly different ($p\leq0.05$).

**Table 4.2** $Log_{10}$ reduction ($log_{10}$ $CFUml^{-1}$) of pH- stressed *S. typhimurium* following treatment with 0.05% (w/v) CPC and 100 $\mu gml^{-1}$ nisin

| pH | $Log_{10}$ reduction ($log_{10}$ $CFUml^{-1}$)[1,2] |
|---|---|
| 7.4 | $3.20\pm0.05^{c}$ |
| 6.0 | $3.56\pm0.19^{bc}$ |
| 5.0 | $3.77\pm0.01^{b}$ |
| 4.5 | $5.36\pm0.72^{a}$ |

[1]$Log_{10}$ reduction = $Log_{10}$ ($N_0/N$), where $N$ is the colony count after treatment and $N_0$ is the colony count for the untreated sample.

[2]Means with different letters within a column are significantly different ($p\leq0.05$).

Boziaris and Adams (2001), 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 and Westhoff, 1988; Lund, 2000). After thawing leakage of cell material occurs and there is an increased permeability of compounds into the cells (Macleod and Calcott, 1976). As a results 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 Kalchayanand et al. (1992) and Stevens et al. (1991), 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 and 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 $\log_{10}$ $CFUml^{-1}$ ($p \leq 0.05$) (Table 4.2). Also, pH stressed (pH 5.0) cells treated with CPC-nisin presented higher log reduction of 3.77 $\log_{10}$ $CFUml^{-1}$ ($p \leq 0.05$) 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 4.2). Since damage of outer membrane is one kind of damage to enteric bacteria like *E. coli* and *Salmonella* spp. caused by stresses. Stress due to acidity might be damaged 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 (Hamouda and Baker, 2000; Randford et al., 1997). 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 increasing in membrane fluidity made the membrane more sensitive to nisin (Li, Chikindas, Ludescher and 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 (Nikaido and 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. In 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.

### 4.4.2 Bioluminescence output of temperature- and pH- stressed bioluminescent *S. typhimurium* treated with CPC and nisin.

Viable *S. typhimurium* S36 can produce light output, and their light emission can be measured by luminometer as relative light units (RLU) to evaluate the efficacy of temperature, pH stressed and CPC-nisin treatment on viability of *Salmonella*. Light (2408.0, 2405.5 and 2416.0 respectively) as shown in Table 4.3. The bioluminescence output of untreated control (6971.5) was higher than before stressed (2407.0) because

**Table 4.3** Bioluminescence output of temperature stressed bioluminescent *S. typhimurium* treated with CPC and nisin

| Condition[1] | RLU | | |
|---|---|---|---|
| | Before stressed[2] | After stressed[3] | CN[4] |
| Control | 2407.0±82.02 | 6971.5±23.33 | 0.004±1.00 |
| Heating | 2408.0±0.001 | 0.267±0.001 | 0.010±0.01 |
| Chilling | 2405.5±23.33 | 351.3±320.0 | 4.434±4.70 |
| Freezing | 2416.0±16.97 | 2371.0±214.96 | 0.036±0.045 |

[1]control = 37°C, heating = 55°C, chilling = 0.5°C, freezing = -20°C

[2]$10^7$CFUml$^{-1}$ of *S. typhimurium* S36 cells before stressed with mild temperatures

[3]After stressed of temperature stressed *S. typhimurium* ($10^7$ CFUml$^{-1}$)

[4]CN = 0.05% (w/v) CPC and 100 μgml$^{-1}$ nisin

RLU = relative light units

of its metabolism. On the contrary, CPC and nisin had a dramatic effect on unstressed control, heat, chill and freeze stressed cells by giving lower light output levels (0.004, 0.01, 4.434 and 0.036 respectively). As indicated by the difference in light output levels, temperature stress affected the activity of bioluminescent cells, additionally to the effect of CPC-nisin treatment.

As can be seen from Table 4.4, light output of bioluminescent cells after exposure to different pHs (pH 4.5, 5.0 or 6.0) resulted in a decrease in the bioluminescence output compared to that observed for unstressed control cells (pH7.4). At pH 4.5 and 5.0, which are higher acidity conditions than the optimal pH

**Table 4.4** Bioluminescence output of pH stressed bioluminescent *S. typhimurium* treated with CPC and nisin

| pH | RLU | |
|---|---|---|
| | Control[1] | CN[2] |
| 7.4 | 1205.0±16.97 | 0.011±0.00 |
| 6.0 | 351.5±10.61 | 0.029±0.00 |
| 5.0 | 18.04±0.16 | 0.009±0.00 |
| 4.5 | 12.70±0.39 | 0.014±0.00 |

[1]Control = untreated pH stressed *S. typhimurium* S36 ($10^7$ CFU$ml^{-1}$)

[2]CN = 0.05% (w/v) CPC and 100 μg$ml^{-1}$ nisin

RLU = relative light units

(7-7.5) for *Salmonella* growth, a rapid decline in light emission was observed. Furthermore, CPC-nisin treatment could have a synergistic effect on unstressed and pH stressed cells by producing lower bioluminescence output levels.

These phenomena can be explained by the fact that the light production of bioluminescent cells depends on a functional intracellular biochemistry. Consequently, physical stresses: temperature-, pH-stress and CPC-nisin that perturb or kill the *Salmonella lux,* destroy its metabolism and directly or indirectly affect the intracellular production of $FMNH_2$, reducing the light emission of cells. Because the $FMNH_2$ production depends on functional electron transport, only living cells can produce light, and sublethally injured cells produce less than maximal light (Farkas, Anrássy, Beczner, Vidács and Mészáros, 2002). Furthermore, as pointed out already

by Stewart (1990), a changing luminometric activity of bioluminescent cells in parallel with changes in their metabolic intensity may be utilized to follow sensitively changes in their physiological and metabolic status, as a response to environmental stresses and inimical processes. Moreover, Farkas et al. (2002) reported that the light output of *lux* transformant is influenced by the pH of the culture which is probably due to the effect of pH on the catalytic activity of bacterial luciferase. Thus, the decrease in bioluminescence was thought to be a consequence of the down regulation of *S. typhimurium* S36 metabolism which was an effect of both temperature and pH stress including the synergistic effect of CPC and nisin.

### 4.4.3 Electron micrographs

#### 4.4.3.1 Scanning electron micrographs

The changes in cell morphology and topography due to temperature stress and CPC-nisin treatment were examined by SEM (Fig. 4.1). The control *S. typhimurium* cells have a normal rod shape with a smooth surface (Fig. 4.1A). Whereas, the cells treated with CPC-nisin lost their original shape and showed indentations on the surface (Fig. 4.1B). The control heat stressed celled show minor indentations on the surface (Fig. 4.1C), in contrast, the CPC-nisin heat stressed cells were completely collapsed (Fig. 4.1D). 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 (Figs. 4.1E and 4.1F). Fig. 4.1G showed the freeze stressed cells have similar appearance as the chill stressed cells. Irregular changes in shape and collapsed into amorphous bodies, can be observed under freeze stress with CPC-nisin as shown in Fig. 4.1H may due to cytoplasmic leakage.

Furthermore, Fig. 4.2 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. 4.2A, 4.2C, 4.2E, and 4.2G respectively) showed no change in the original smooth surface and rod shape. *Salmonella* cells treated with CPC-nisin showed ruffling and craters on the surface (Fig. 4.2B). As in Fig. 4.2D 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. 4.2F). 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. 4.2H). 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 and Walker, 1995). The presence of cracks in the outer membrane allowed penetration of cationic substances (Helander, Kilpeläinen and 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,

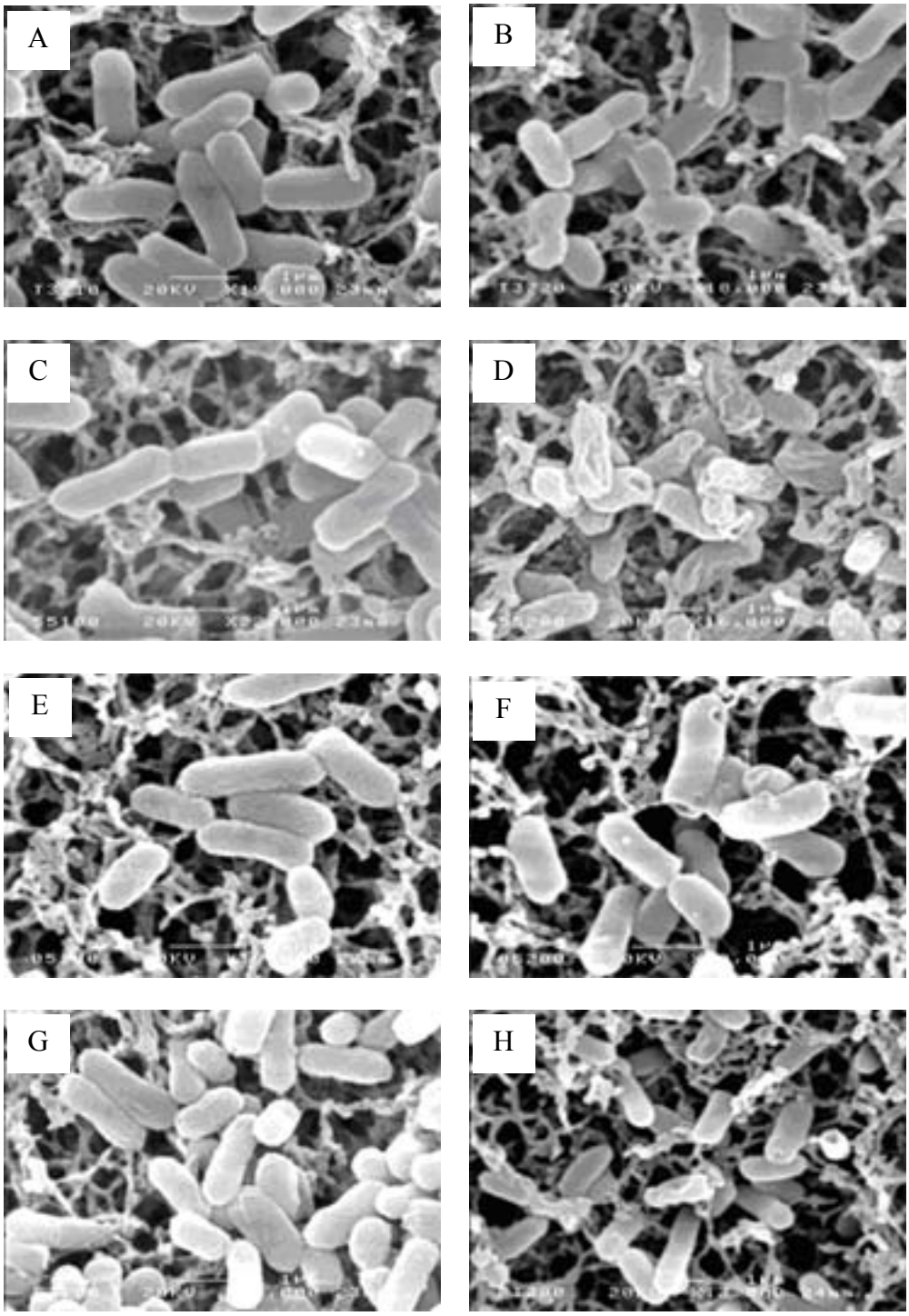


**Fig. 4.1** Scanning electron micrographs of temperature-stressed *S. typhimurium* S36 treated with 0.05% (w/v) CPC and 100 μgml$^{-1}$ nisin. (A) Untreated control; (B) Control treated with CPC-nisin; (C) Control heat stressed; (D) Heat stressed cells treated with CPC-nisin; (E) Chill stressed control; (F) Chill stressed cells treated with CPC-nisin; (G) Freeze stressed control; (H) Freeze stressed cells treated with CPC-nisin.

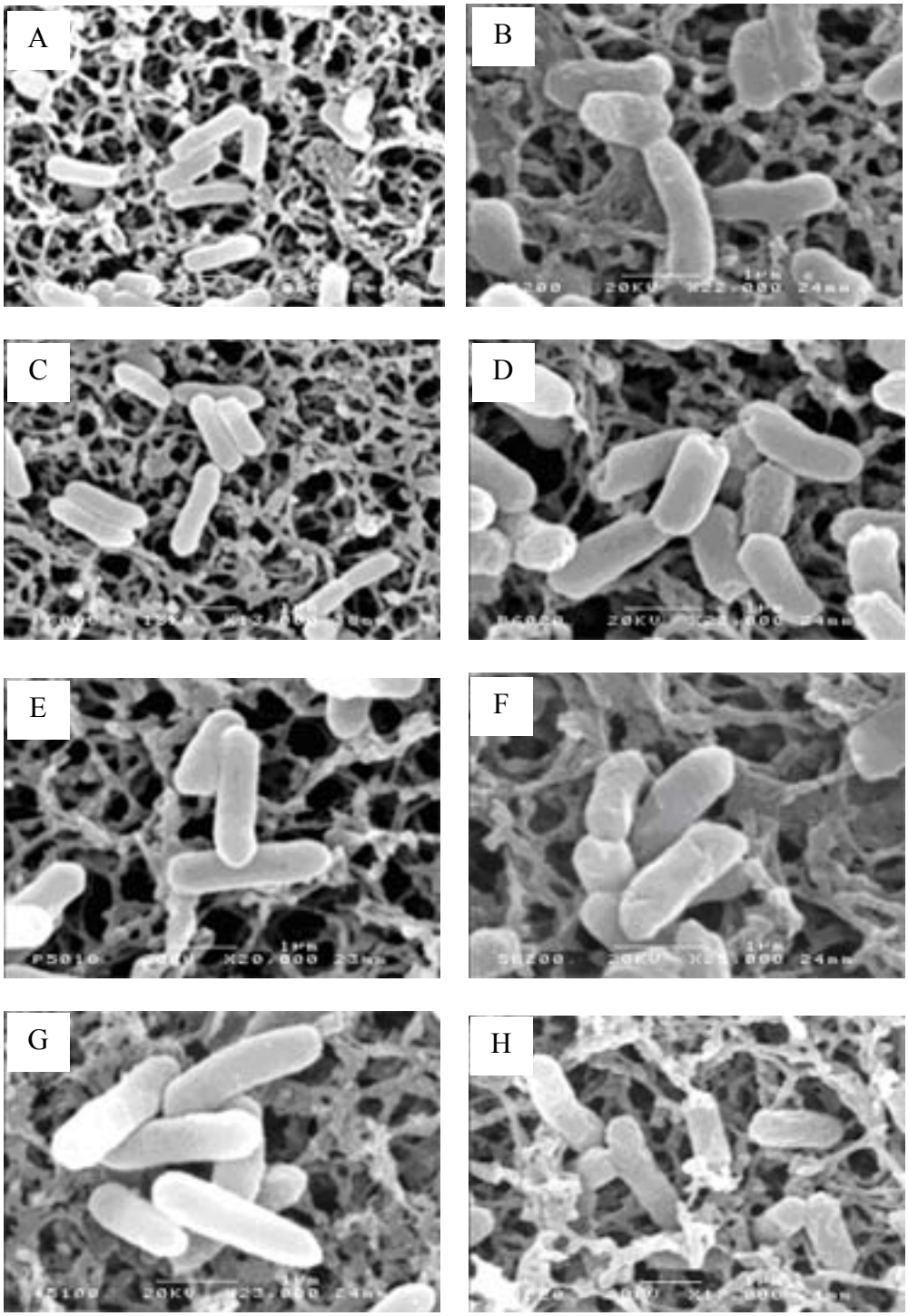


**Fig. 4.2** Scanning electron micrographs of pH-stressed *S. typhimurium* S36 treated with 0.05% (w/v) CPC and 100 $\mu gml^{-1}$ nisin. (A) Control (pH 7.4) cells; (B) Cells treated at pH 7.4 and then with CPC-nisin; (C) Cells treated at pH 6.0; (D) Cells treated at pH 6.0 and then with CPC-nisin; (E) Cells treated at pH 5.0; (F) Cells treated at pH 5.0 and then with CPC-nisin; (G) Cells treated at pH 4.5; (H) Cells treated at pH 4.5 and then with CPC-nisin.

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*.

#### 4.4.3.2 Transmission electron micrographs

The morphological changes of *S. typhimurium* S36 due to temperature stress and CPC-nisin treatment were further examined by transmission electron microscopy (TEM) (Fig. 4.3). Thin sections of control cells are presented in Fig. 4.3A. It shows the appearance of normal cell (smooth cell wall). The outer membrane and the cytoplasmic membrane were fairly distinct. The micrograph of control cells after exposure to CPC-nisin treatment (Fig. 4.3B) shows the cell membrane blebs or separation of cytoplasm from the cell membrane and less dense cell contents than for regular cells. This may result from damage of cell wall and plasma membrane and expulsion of some cell contents. Fig. 4.3C exhibits the micrograph of heat stressed control. The surface roughness of the cell wall of the cell exposed to heat stress treatment increased slightly. There were lacks of cytoplasm or sparse cytoplasm in the cells, as a result of the decrease of the cell membranes functionality as a barrier. In Fig. 4.3D, the effect of CPC-nisin on heat stressed cells can be seen: Membrane blebs, cytoplasm-sparse and irregular shape of cells were observed. Chill stressed control (Fig. 4.3E) indicates no desirable morphological cell damage except the cell wall surface roughness. Fig. 4.3F exhibits the micrograph of chill stressed cells treated

with CPC-nisin. It shows an increase in the cell wall surface roughness, membrane blebs and lack of cytoplasm. Freeze stressed control (Fig. 4.3G) showed a partial detachment of the outer membrane from the cytoplasmic membrane in some cells. Fig. 4.3H shows the micrograph of freeze stressed cells treated with CPC-nisin. A ghost cell wall without cell contents was observed. The extracellular ice crystals may have ruptured that cell. The outer membrane is altered, thus CPC and nisin were able to access to cytoplasmic membrane and form pores through which the cytoplasm moved outside of the cell wall. Also, these bacteria exhibited electron-transparent areas devoid of ribosomes (holes) normally clearly visible within the cytoplasm, suggesting that damaged plasma membrane can be seen indirectly by presence of less dense cell contents than found in normal cells. Besides, the evidence gathered indicates that the action of CPC-nisin with temperature stress induced damage to the cell wall and cell membrane. In addition, Fig. 4.4 reveals the morphological changes of the cells caused by pH stress and CPC-nisin treatment, as observed with transmission electron microscopy (TEM). Fig. 4.4A shows normal appearing control (pH 7.4) cells. The outer membrane and the cytoplasmic membrane can be distinguished. After treated with CPC and nisin, the cell has a larger gap between the cytoplasmic and outer membrane and has a membrane bleb (Fig. 4.4B). Cells treated at pH 6.0 (Fig. 4.4C) reveals no observable morphological cell damage. Cells treated at pH 6.0 and then with CPC-nisin (Fig. 4.4D). This micrograph shows cytoplasm-sparse and an abnormal shape of cell. Cells treated at pH 5.0 show lack of cytoplasm in the cell (Fig. 4.4E). Whereas cells treated at pH 5.0 and then CPC-nisin (Fig. 4.4F) exhibit a larger gap between the cytoplasmic and outer membrane, sparse cytoplasms and membrane blebs in the cells. The micrograph of cells treated at pH 4.5, as shown

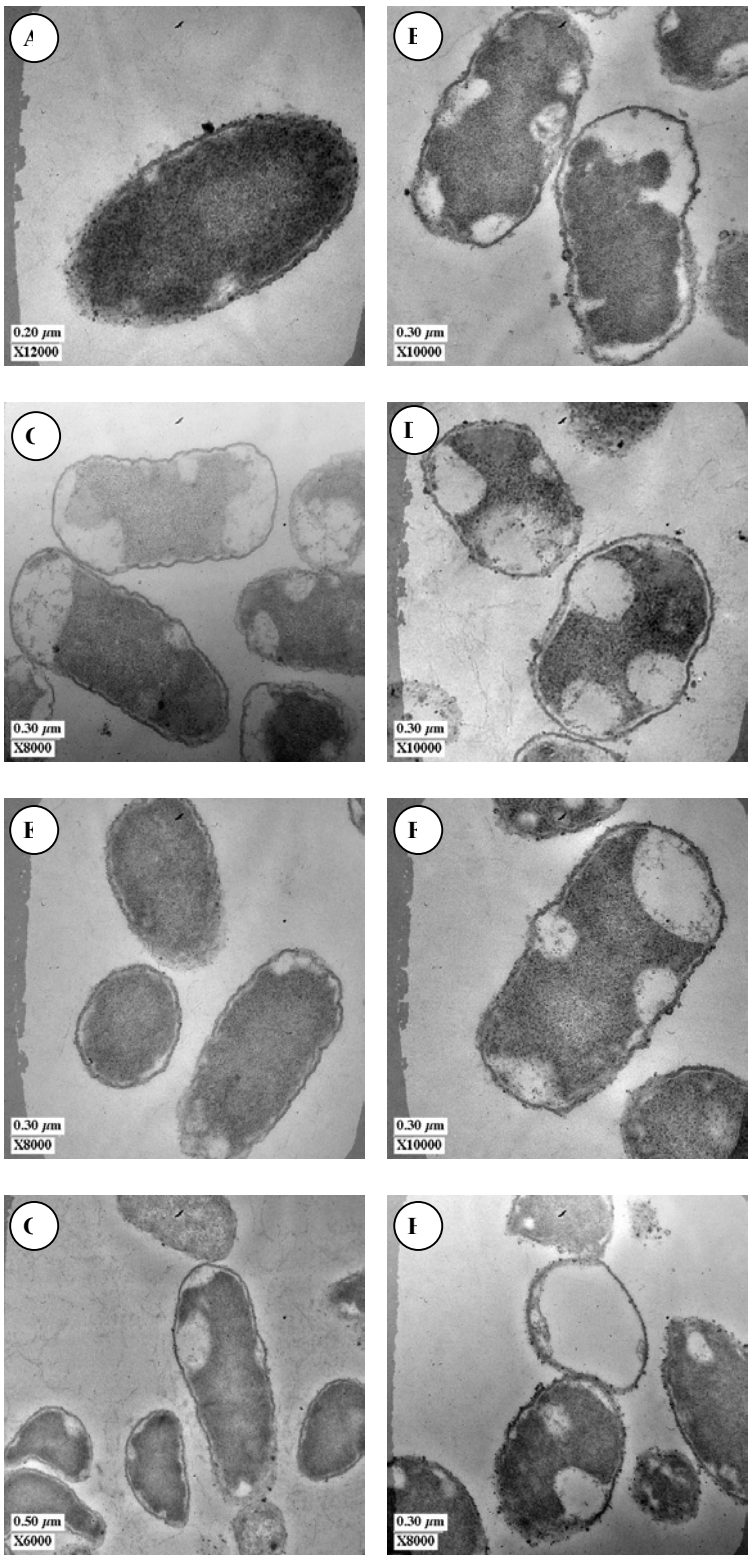


**Fig. 4.3** Transmission electron micrographs of temperature-stressed *S. typhimurium* treated with 0.05% (w/v) CPC and 100 μgml$^{-1}$ nisin. (A) Untreated control; (B) Control treated with CPC-nisin; (C) Control heat stressed; (D) Heat stressed cells treated with CPC-nisin; (E) Chill stressed control; (F) Chill stressed cells treated with CPC-nisin; (G) Freeze stressed control; (H) Freeze stressed cells treated with CPC-nisin.

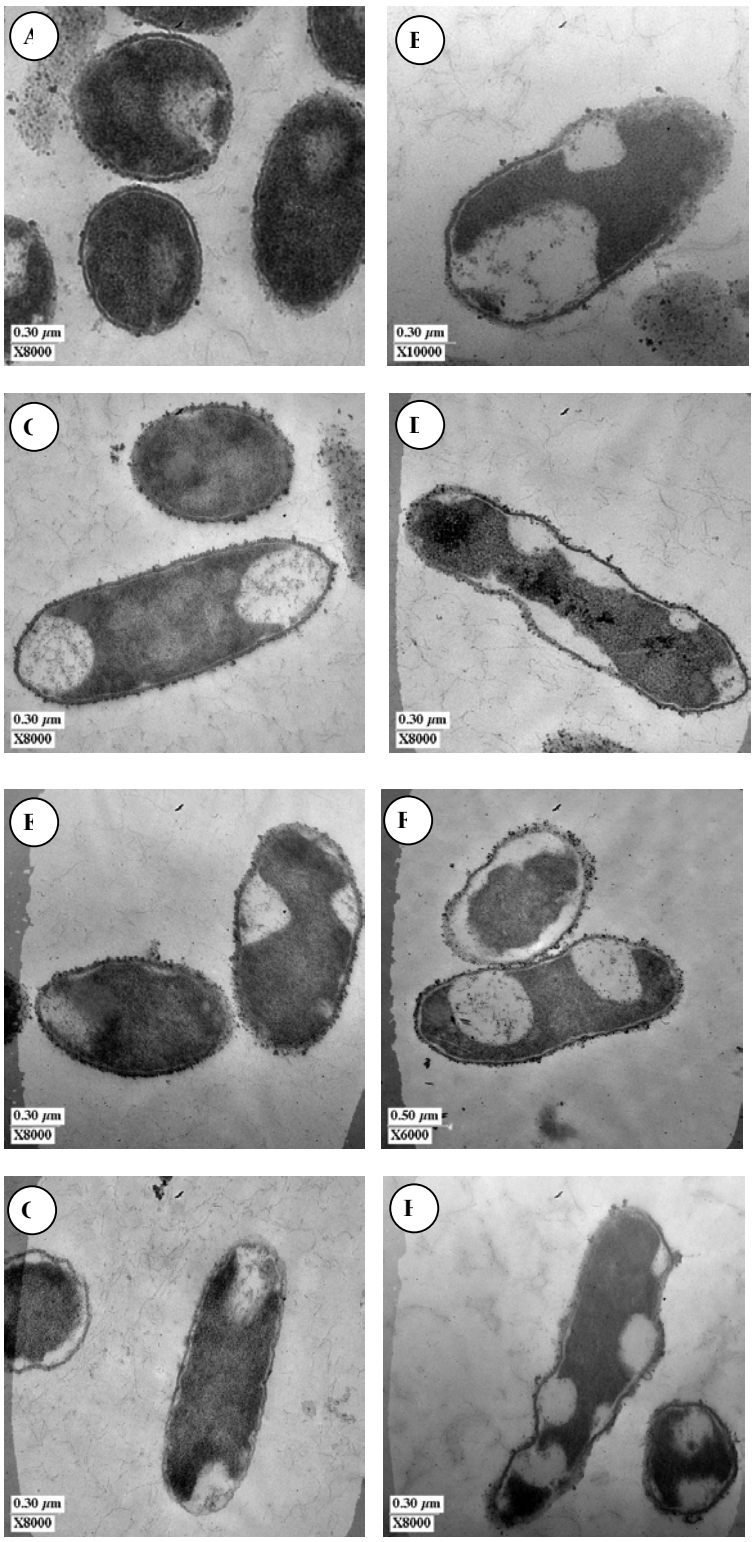


**Fig. 4.4** Transmission electron micrographs of pH-stressed cells treated with 0.05% (w/v) CPC and 100 μgml$^{-1}$ nisin. (A) Control (pH 7.4) cells; (B) Cells treated at pH 7.4 and then with CPC-nisin; (C) Cells treated at pH 6.0; (D) Cells treated at pH 6.0 and then with CPC-nisin; (E) Cells treated at pH 5.0; (F) Cells treated at pH 5.0 and then with CPC-nisin; (G) Cells treated at pH 4.5; (H) Cells treated at pH 4.5 and then with CPC-nisin.

in Fig.4.4G, present surface roughness of cell wall and a larger gap between cytoplasmic and outer membrane than the control cells (Fig. 4.4A). Fig. 4H show the effect of CPC-nisin on cells treated at pH 4.5, with irregularly shaped cells, several cytoplasm-sparse cells and membrane blebs. This micrograph shows the cell was considerably longer than cells treated at pH 4.5 in Fig. 4.4G.

Transmission electron micrographs clearly demonstrated the effect of CPC and nisin on temperature and pH stressed cells. Temperature stresses, particularly freeze and heat stress, and pH stresses at pH 4.5 and 5.0, had a greater effect on morphological changes of the cells at the cell wall and plasma membranes. These results did correlate with scanning electron micrographs (Figs. 4.1 and 4.2). The results in damage of OM could allow CPC and nisin to pass the cytoplasmic membrane and cause pore formation with loss of membrane potential and leakage of intracellular metabolites resulting in cell death (Moll et al., 1996; Breukink and Kruijff, 1999; Long and Phillips, 2003) and also cause of morphological changes in *Salmonella*.

### 4.4.4 Imaging of bacterial cells stained with BacLight nucleic acid stain

The BacLight stain is based on a dual staining methodology which assesses membrane integrity by selective stain exclusion: Viable cells fluoresce in green whilst nonviable ones appear red (Haugland, 1999). Epifluorescent micrographs of BacLight staining demonstrate accurate distinction between live and dead cells.

Fig. 4.5 shows eplifluorescent micrographs of control cells under untreated control conditions, all cells reveal the green fluorescence of the SYTO9 stain, which represents live or intact cells (Fig. 4.5A). On the other hand, Fig. 4.5B shows both

green (live) and red (dead) fluorescent cells after CPC-nisin treatment. Untreated freeze stressed (Fig. 4.6A) and CPC-nisin treated freeze stressed (Fig. 4.6B) cells show some red cells when compared with the untreated control cells (Fig. 4.5A) while CPC-nisin treatment after freeze stressing (Fig. 4.6B) showed a majority of red (dead) cells probably due to the alteration of OM permeability by ice crystal formation. Subsequently, the larger molecules of PI can gain access to ruptured membranes and stain cells in fluorescent red. The red fluorescence of PI indicates that there is an increase in permeability of CPC-nisin treatments, which is in agreement with several reports (Ananta and Knorr, 2004; Boulos, Pre′vost, Barbeau, Coallier and Desjardins, 1999; Defives, Guyard, Oular′e, Mary and Hornez, 1999; Haugland, 1996; Que′ric, Soltwedel, and Arntz, 2004; Ram′irez et al., 2000). Nevertheless, greater PI uptake was observed in temperature and pH stressed cells treated with CPC-nisin (similar to Figs. 4.5B and 4.6B) than in untreated control of stressed cells. Overall the results of the BacLight nucleic acid stain correlate with viability loss and bioluminescence output.

## 4.5 Conclusions

A variety of stresses (temperature and pH stresses) 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

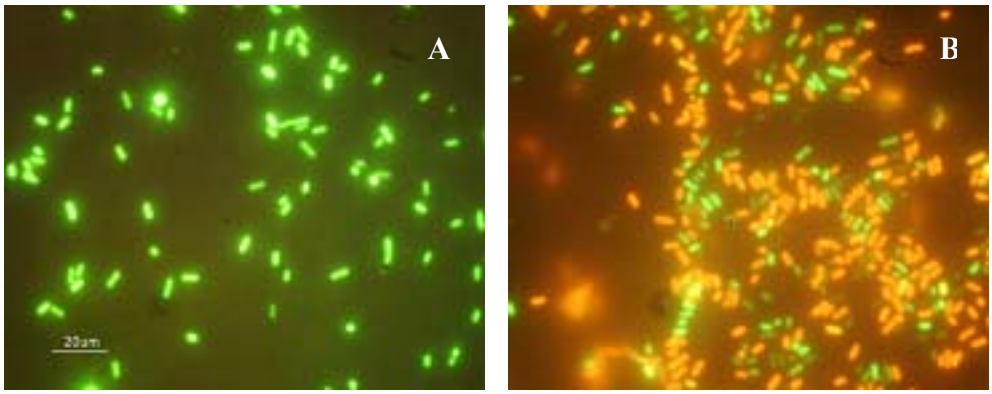


**Fig**. **4.5** Epifluorescent micrographs of live and dead cells of control *S. typhimurium* S36 stained with BacLight nucleic acid stain. (A) The untreated control cells (B) CPC-nisin treated cells

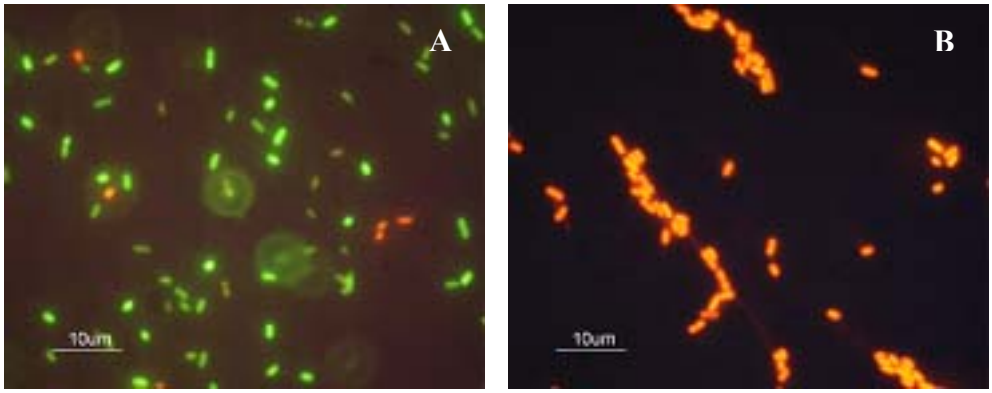


**Fig. 4.6** Epifluorescent micrographs of live and dead cells of freeze stressed *S. typhimurium* S36 stained with BacLight nucleic acid stain. (A) The untreated control of freeze stressed cells (B) CPC-nisin treated cells of freeze stressed cells.

formation resulting in leakage of cellular materials. The combination of CPC-nisin treatment in food processing treatments that impose sublethal stress on Gram-negative bacteria could increase the lethality of the process, enhancing microbiological food safety.

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# CHAPTER V

# EFFECT OF CETYLPYRIDINIUM CHLORIDE AND NISIN ON BIOLUMINESCENT *S. typhimurium* CONTAMINATING ON CHICKEN CARCASSES

## 5.1 Abstract

The effect of cetylpyridinium chloride (CPC) and nisin on the inhibition of viable bioluminescent *Salmonella typhimurium* S36 cells contaminated on chicken carcasses during refrigerated storage at 4$^{o}$C and 10$^{o}$C were investigated. The chicken drumstick sections (5 by 5 cm), inoculated with *S. typhimurium* S36, were treated with CPC and further treated with nisin. It was found that the use of 0.5%(w/v) CPC for 120 sec and 100 μgml$^{-1}$ nisin for 30 min decreased significantly ($p \leq 0.05$) cell viability with minimal effect on the appearance of chicken carcasses. The CPC-nisin treatment reduced the number of *S. typhimurium* S36 contaminated on chicken drumstick skin during storage at 4$^{o}$C for 6 days, and caused a slight difference in chicken skin color. These results also demonstrated the synergistic activity of CPC and nisin against *S. typhimurium* S36 and can be expected to enhance the safety of chicken carcasses and other chicken products.

## 5.2 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 (Henderson, Bounus and Lee, 1999; Slavik, Kim and Walker, 1995, Uyttendaele, Debever, Lips and Neyts, 1998; Vadhanasin, Bangtrakulnonth and Chidkrau, 2004). During the slaughtering and processing steps such as scalding, picking and chilling, poultry skin surfaces are contaminated with *Salmonella* (Natrajan and Sheldon, 2000; Medina, 2004). Various methods were considered to reduce or eliminate of bacteria attached to poultry carcasses, for examples thermal treatments, chemical dipping and spraying and irradiation (Garman, Sofos, Morgan, Schmidt and Smith, 1995; Kim and Slavik, 1996; Lillard, 1994; Shamszzaman, Chuaqui Offermanns, Licht, McDougall and Borsa, 1992; Slavik et al., 1995). 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 poultry carcasses at the end of the evisceration line to reduce the numbers of *Salmonella*, but with limited effectiveness (James, Brewer, Prucha, Williams and Parham, 1992; Yang, Li and Johnson, 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. Ray (1992) and Delves-Broughton and Gasson, (1994) reported that nisin, a heat-stable bacteriocin produced by *Lactococcus lactis* ssp. *lactis* inhibited gram-positive bacteria (*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, ATP, and amino acids resulting in the dissipation of the proton motive force and eventually cell death (Abee, Rombouts, Hugenholtz, Guihard and Letellier, 1994; Breukink, van Kraaij, Demel, Siezen, Kuipers and de Kruijff, 1997; Breukink, Weidemann, van Kraaij, Kuipers, Sahl and de Kruijff, 1999; Bruno and Montville, 1993; Crandall and Montville, 1998; Delves-Broughton and Gasson, 1994; Gao, Abee and Konings, 1991; Harris, Fleming and Klaenhammen, 1992; Ray, 1992). Gram-negative bacteria can also become more sensitive to nisin whenever the OM is penetrated by chelating agents including EDTA, or pretreated with cell membrane disrupters (Carneiro de Melo, Cassar and Miles, 1998; Cutter and Siragusa, 1995; Hauben, Barlett, Soontjens, Cornelis, Wuytack and Michiels, 1997; Sheffet, Sheldon and Klaenhammer, 1995). 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; Frost and Harris, 1994). 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). Pohlman, Stivarius, McElyea and Waldroup (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* cell attachment on chicken carcasses was also studied. Furthermore, the effects of appropriate concentrations and exposure times of CPC and nisin on bioluminescent *S. typhimurium* contaminated on chicken drumstick skin at various storage temperatures were also determined.

## 5.3 Materials and methods

### 5.3.1 Bacterial strain and culture preparation

Bioluminescent *S. typhimurium* S36 was constructed from a slaughter house isolated by the Tn5 mini-transposon methods as described in Chapter III. Suspensions of *S. typhimurium* S36 were prepared from overnight cultures grown aerobically at 37$^{o}$C in BHI broth supplemented with kanamycin (30 μgml$^{-1}$). The cells were harvested by centrifugation (4,000 x g for 10 min at 4$^{o}$C) and washed twice in sterile

maximum recovery diluent (MRD, Oxoid). The cell pellet was finally resuspended in MRD to yield a suspension containing ca. $10^7$ CFUml$^{-1}$ ($A_{600}$ 0.2).

### 5.3.2 Cetylpyridinium chloride (CPC) and nisin

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

### 5.3.3 Sample inoculation and treatment application

#### 5.3.3.1. Inoculation of chicken drumstick sections

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 of approximately 5 by 5 cm, with a flesh thickness of about 1.5 cm. The samples were submerged in *S. typhimurium* S36 ($10^7$ CFUml$^{-1}$; Polman, Stivarius, McElyea and Waldroup, 2002) suspensions for 5 min and then were held at room temperature (~22-24$^o$C) for 20 min to allow the attachment of bacteria.

#### 5.3.3.2. Effect of various CPC and nisin concentration

Inoculated chicken drumstick sections as described above, 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 μgml$^{-1}$ nisin at room temperature. Bacterial enumeration was performed.

#### 5.3.3.3. Effect of various exposure times of CPC and nisin

The inoculated chicken drumstick sections were dipped in a CPC solution with an optimum concentration determined from the previous experiment (from 5.3.3.2) for 15, 30, 60 and 120 sec and then treated with a nisin solution of optimum concentration determined from the previous trial (from 5.3.3.2) for 15, 30, 45 and 60 min at ambient temperature. Surviving cells were enumerated.

### 5.3.4 Bioluminescence images

Bioluminescence images were performed in a Night-Owl luminometer analyzer in conjunction with the manufacturer computer software (E. G. & G., Berthold, Munich, Germany) after chicken drumstick sections had been inoculated and also following treatments with CPC and nisin to demonstrate the distribution of cells attachment on chicken carcass.

### 5.3.5 Detection of *S. typhimurium* contaminating on chicken skin using digoxigenin (DIG)-labeled oligonucleotide probe

#### 5.3.5.1 Preparation of chicken skin sections for hybridization

In *situ* hybridization using digoxigenin (DIG)-labeled with the oligonucleotide of *Salmonella* was used in this study. The 23S rRNA of *Salmonella* (5′-AATCACTTCACCTACGTG-3′) was labeled with DIG at the 5′ end (Sigma Genosys Ltd, Cambridge, UK). The DIG probe was suspended in Tris buffer (10mM Tris buffer (pH 7.4), 1mM EDTA), dispensed into small volumes of 50-100 µl in

Eppendorf tubes and stored at -20$^{o}$C. One tube was removed and left to defrost on ice prior use.

The inoculated skin samples were cut (1 by 1 cm) using a sterile scissors, submerged in liquid nitrogen and sliced (6-12 μm) using a cryomicrotome (Bright starlet cryostat, UK), and transferred to Eppendorf tube. The samples were fixed with 4% (w/v) paraformaldehyde solution in PBS at room temperature for 30 min, and washed once with PBS. Fixed skin samples were then dehydrated by soaking the samples for 3 min in progressively higher concentrations of ethanol (50%, 80% and 100% (v/v)).

**5.3.5.2 Detection of DIG-labeled oligonucleotide with enzyme-conjugated anti-DIG $F_{ab}$-fragments**

Alkaline-phosphatase conjugated anti-dig $F_{ab}$-fragments were purchased from Roche, UK. An aliquot of 90 μl of hybridization buffer [25 mM maleic acid, 100 mM Tris (pH 7.4), 300 mM $MgCl_2$, 0.01% (w/v) SDS, 0.5% (v/v) blocking reagent (Roche, UK)] and 10 μl of DIG probe solution (50 ngμl$^{-1}$oligonucleotide) were placed on the sample. Hybridization was then performed at 55$^{o}$C for 30 min in an isotonically equilibrated humid chamber. After an additional washing step in 100 mM Tris-HCl (pH 7.5) to remove non-hybridized probes and a washing with SDW, the samples were incubated with 10μl portions of anti-DIG-$F_{ab}$ fragment (Roche, UK) diluted 1:30 in Maleic acid buffer (150 mM NaCl, 100 mM maleic acid, 0.5 % blocking reagent) at 27$^{o}$C for 1h in humid chamber. Then the samples were washed in a wash solution (100 mM Tris-HCl, 150mM NaCl (pH 7.5), 0.01% SDS) and briefly rinsed with SDW.

Alkaline phosphatase was visualized by formation of a color precipitate. The substrate was a dilution of 45 µl nitroblue tetrazolium (75 $mgml^{-1}$ in 70% (v/v), dimethylformamide) and 35 µl 5-bromo-4-chloro-3-indolylphosphate (50 $mgml^{-1}$ in dimethylformamide) in detection buffer (100 mM-Tris/HCl, 100 mM NaCl, 50 mM $MgCl_2$ (pH 9.5)) as described by Leary, Brigati and Ward (1983). An aliquot of 50 µl of the described mixture was applied to the sample and incubated at room temperature in a humid chamber for 1 to 12 h. The reaction product was purple and water insoluble, and was visualized by conventional microscopy in bright field or phase contrast configuration.

**5.3.6 Scanning electron microscopic study**.

*S. typhimurium* S36 contaminated chicken drumstick skins (1 by 1cm) were removed with sterile scissors and forceps. They were then treated with appropriate CPC and nisin concentration (from 5.3.3.2) and their appropriate exposure time (from 5.3.3.3). The samples were cut to small pieces with a sterile surgical blade and further processed for scanning electron microscopy (SEM). Chicken skin samples were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), and post-fixed in 1% $OsO_4$. Specimens were taken through a graded series of ethanol and then dried in critical point dryer, and mounted on aluminum stubs. Samples were sputter coated with gold and examined under a JSM-6400 scanning electron microscope (JEOL, Japan) at 15 kV.

### 5.3.7 Imaging of *Salmonella* cells on chicken drumstick skin

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, Leiden, The Netherlands) were spotted on the chicken skin surface 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. *Salmonella* cells attached to the sample 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.

### 5.3.8 Effect of CPC and nisin on bioluminescent *S. typhimurium* contaminating on chicken skin storaged at $4^{o}$C and $10^{o}$C

#### 5.3.8.1 Chicken preparation and treatments

Eighty chicken drumsticks were purchased from a local supermarket. Their average weight was 50 g per drumstick. They were washed in sterile reverse osmosis water to remove surface dirt and debris. Cleaned chicken drumsticks were immersed for 5 min in suspensions of *S. typhimurium* S36 ($10^{7}$ CFUml$^{-1}$) at room temperature (~$22^{o}$C). After inoculation, drumstick samples were kept for 20 min at room temperature to permit bacterial attachment.

Forty inoculated samples were dipped in 0.5% (w/v) CPC for 120 sec and then in 100 $\mu gml^{-1}$ nisin for 30 min. After immersion, these CPC-nisin treated drumsticks samples were kept at room temperature for 15 min. Twenty eight of CPC-nisin treated drumsticks and twenty eight untreated control drumsticks were placed in a commercial foam tray pack (2 drumsticks per pack) and were overwrapped with polyvinyl chloride film. Samples were stored at $4^oC$ (for refrigerator condition) and $10^oC$ (for open refrigerator units) to simulate grocery store conditions. Viable count determination was then performed.

**5.3.8.2 Viability cells of *S. typhimurium* contaminating on chicken drumstick skin treated with CPC and nisin storaged at $4^oC$ and $10^oC$**

The samples were analyzed everyday after 0 to 6 days of storage. Day 0 samples were tested immediately after treatment was completed. For whole chicken drumsticks, samples were prepared by excising 10 g of skin (Kotula and Davis, 1999) with a sterile scissors immediately before microbiological examination. Skin sample were placed in a stomacher bag containing 90 ml of sterile MRD and were homogenized with stomacher 400 blender for 2 min. Decimal dilutions in sterile MRD were prepared from the homogenized sample, and 0.1 ml aliquots were placed in duplicate onto BHI agar. Plates were incubated at $37^oC$ for 24 h and colonies were counted. Duplicate experiments were performed on different days.

**5.3.8.3 Bioluminescent images of bioluminescent *S. typhimurium* contaminating on chicken drumstick skin treated with CPC and nisin storaged at $4^oC$ and $10^oC$**

Bioluminescence images were performed in a Night-Owl image analyzer after chicken drumsticks had been inoculated and also following treatment with CPC-nisin. For whole drumsticks, six untreated control skin samples and six CPC-nisin treated samples skin samples were removed using sterile scissors and were aseptically transferred to sterile petri dishes. The samples were stored at $4^oC$ and $10^oC$ for a maximum period of 6 days. Images of luminescent *S. typhimurium* S36 were taken every day during 6 days of storage.

**5.3.8.4 Color evaluation**

Six untreated control drumstick skins and six CPC-nisin treated drumstick skins were removed from whole drumsticks using sterile scissors and were aseptically transferred to sterile plates. The samples were overwrapped with polyvinyl chloride film and were stored at $4^oC$ and $10^oC$. The color of untreated control drumstick skins and CPC-nisin treated drumstick skins were evaluated everyday during storage at $4^oC$ and $10^oC$ for up to 6 days. Hunter L (lightness), a (redness), and b (yellowness) color values were measured using a Hunterlab ColorQuest Sphere Spectocolorimeter SnC5330 (Virginia, USA) and standardized to a black and white tile. Color measurements were averaged from four readings taken at different locations on each drumstick skin.

### 5.3.9 Microbiological analysis

For the counting of *S. typhimurium* S36, the samples were placed in a stomacher bag containing MRD and then homogenized by a Seward Lab-Blender 400 for 2 min. Serial dilutions from sample homogenates were prepared in MRD for spread plating. The aliquot (100 μl) was surface plated in duplicate onto BHIA-kanamycin. The plates were incubated at 37$^{o}$C for 16-18 h prior to counting. Confirmation of the presence of *S. typhimurium* S36 was accomplished by visualizing bioluminescent colonies with a Night-Owl image analyzer as described in Chapter III.

### 5.3.10 Statistical analysis

Microbiological counts were transformed into $\log_{10}$ data before statistical analysis. All experiments were performed in triplicates. Statistical analysis was evaluated by Factorial in Completely Randomized Design (CRD) with Statistical Analysis System (SAS Institute, Inc., 1995). Analysis of variance (ANOVA) was analyzed and means comparison was conducted using Duncan's New Multiple Range Test (DMRT).

## 5.4 Results and discussions

### 5.4.1 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 5.1. The most effective ($p \leq 0.05$) decontamination of *S. typhimurium* S36 on chicken samples was observed at 0.5% (w/v) CPC and 100 $\mu g ml^{-1}$nisin. The concentration of CPC required to kill attached cells was higher than that for

**Table 5.1** Viable cells of *S. typhimurium* S36 on chicken drumstick sections treated with 0, 0.1, 0.3 and 0.5% (w/v) CPC and 0, 20, 60 and 100 μgml$^{-1}$ nisin

| CPC%(w/v) | $Log_{10}$ CFU.g$^{-1}$* | | | |
|---|---|---|---|---|
| | nisin (μgml$^{-1}$) | | | |
| | 0 | 20 | 60 | 100 |
| 0 | 4.54$^{a}$ ± 0.00 | 4.32$^{abc}$ ± 0.13 | 4.10$^{cde}$ ± 0.09 | 4.11$^{cde}$ ± 0.08 |
| 0.1 | 4.01$^{de}$ ± 0.00 | 3.97$^{de}$ ± 0.08 | 4.05$^{de}$ ± 0.13 | 3.99$^{de}$ ± 0.16 |
| 0.3 | 4.44$^{ab}$ ± 0.00 | 4.37$^{ab}$ ± 0.08 | 4.22$^{bcd}$ ± 0.23 | 3.86$^{ef}$ ± 0.19 |
| 0.5 | 3.99$^{de}$ ± 0.16 | 3.87$^{ef}$ ± 0.01 | 3.70$^{f}$ ± 0.03 | 3.16$^{g}$ ± 0.04 |

*Means with different letters are significantly different ($p \leq 0.05$).

suspension cells. Attached cells of *S. typhimurium* S36 appeared less susceptible to nisin than did suspended cells (Table 3.2 in Chapter III). 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 (Delves-Broughton, 1990) and poultry skin. 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.

### 5.4.2 Effect of exposure times to CPC and nisin

The cell viability was obviously decreased by treatment with 0.5% (w/v) CPC followed by 100 μgml$^{-1}$ nisin as shown in Table 5.2. The most marked effects were observed with 0.5% (w/v) CPC for 60 sec and 100 μgml$^{-1}$ nisin for 60 min, 0.5% (w/v) CPC for 120 sec and 100 μgml$^{-1}$ 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 μgml$^{-1}$ 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 bloated. This result can be supported by Thomas and McMeekin (1982), who reported that

**Table 5.2** Viable cells of *S. typhimurium* S36 on chicken drumstick sections treated with 0.5% (w/v) CPC for 15, 30, 60 and 120 sec and 100μgml$^{-1}$ nisin for 15, 30, 45 and 60 min.

| 0.05% (w/v) CPC (sec) | $Log_{10}$ CFUg$^{-1}$* | | | |
|---|---|---|---|---|
| | 100 μgml$^{-1}$ nisin (min) | | | |
| | 15 | 30 | 45 | 60 |
| 15 | 4.43$^{a}$ ± 0.03 | 4.01$^{bcd}$ ± 0.04 | 3.89$^{de}$ ± 0.03 | 3.95$^{cd}$ ± 0.01 |
| 30 | 4.03$^{bc}$ ± 0.03 | 3.96$^{bcd}$ ± 0.08 | 3.94$^{cd}$ ± 0.01 | 3.98$^{bcd}$ ± 0.03 |
| 60 | 4.07$^{b}$ ± 0.04 | 3.94$^{cd}$ ± 0.03 | 3.83$^{e}$ ± 0.01 | 3.62$^{f}$ ± 0.11 |
| 120 | 3.81$^{e}$ ± 0.06 | 3.70$^{f}$ ± 0.16 | 3.70$^{f}$ ± 0.04 | 3.65$^{f}$ ± 0.03 |

*Means with different letters are significantly different ($p\leq0.05$).

water-immersion cleaning and chilling of poultry carcasses caused the skin to 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 $\mu gml^{-1}$ nisin for 30 min, which do not cause undesirable carcass characteristics such as bleaching and swelling of the skin.

### 5.4.3 Bioluminescent *S. typhimurium* on chicken carcasses

Bioluminescence can be a valuable mean to identify directly the distribution of bacterial cells *in situ*. *S. typhimurium* S36 was inoculated onto chicken drumstick cuts (Fig. 5.1A). *Salmonella lux* expressing the *lux* operon could be readily 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 (Figs.5.1B and 5.1C). When the same samples were treated with 0.5%(w/v) CPC for 15 sec, followed by 60 $\mu gml^{-1}$ nisin for 30 min (Fig. 5.1D), a low intensity luminescence of *S. typhimurium* S36 cells attached on chicken skins (Figs.5.1E and 5.1F) 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, Nawotka, Spilman, Contag and Contag, 1999). A comparison of the light intensities before and after treatment with CPC and nisin showed that *S. typhimurium* S36 cells preferentially adhered to the untreated control 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 quantities of viable cells on

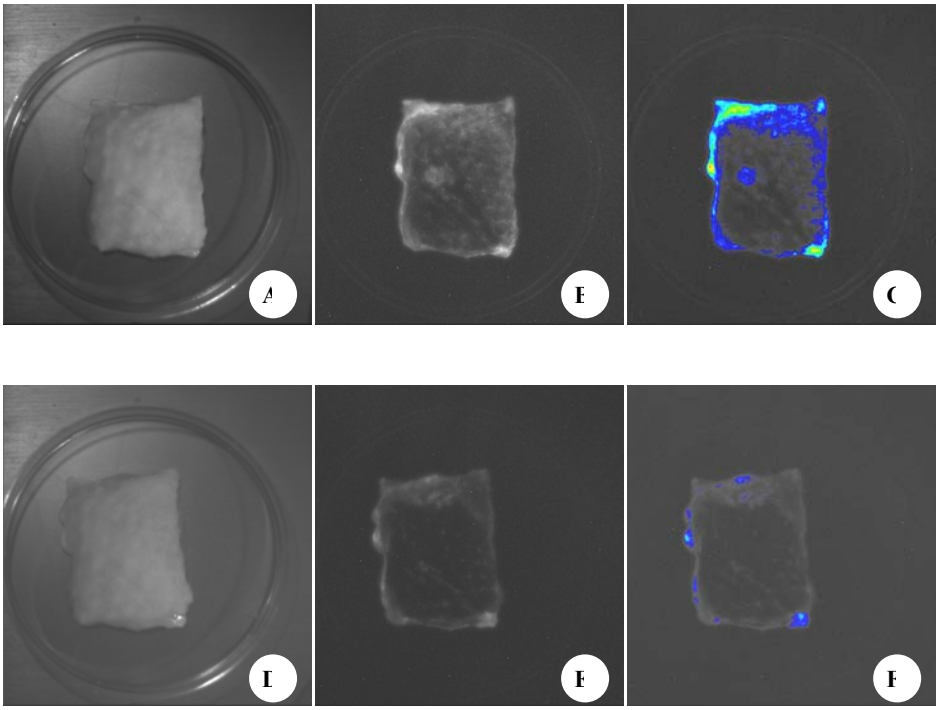

**Fig. 5.1** Bioluminescence images of luminescent *S. typhimurium* S36 distribution on chicken drumstick sections derived from untreated control (A-C) and CPC-nisin treated chicken drumstick sections (D-F). Chicken drumstick sections were inoculated with *S. typhimurium* S36 and images taken under illumination (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.

chicken sample, hence, the “real time” response was a decrease in light output. 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.

### 5.4.4. Detection of *S. typhimurium* contaminating on chicken skin using hybridization with digoxigenin (DIG)-labeled oligonucleotide probe

Until recently, little was known about the mechanism by which bacteria become attached to the surfaces of food products. It is generally accepted that bacterial attachment involves two stages. The first stage consists of a loose reversible sorption that may be related to physiochemical factors. The second stage consists of an irreversible attachment to surfaces by extracellular polymers (glycocalyx) (Jackson, Acuff and Dickson, 1997). Moreover, bacteria may be trapped mechanically in ridges, crevices, capillary-sized channels (Thomas and McMeekin, 1982), and feather follicles (Ellerbroek, Okolocha and Weise, 1997; Kim, Frank and Craven, 1996). Chemical compounds may not be able to gain access to the bacteria that are more firmly attached in these sites, thus providing physical protection and making their removal more difficult. Korber, Choi, Wolfaardt, Ingham and Caldwell (1997) reported crevices and roughness still provided the best protection against trisodium phosphate in terms of the number of surviving bacteria. The loss of epidermis during scalding could also facilitate the firm attachment of bacteria to chicken skin (Slavik et al., 1995).

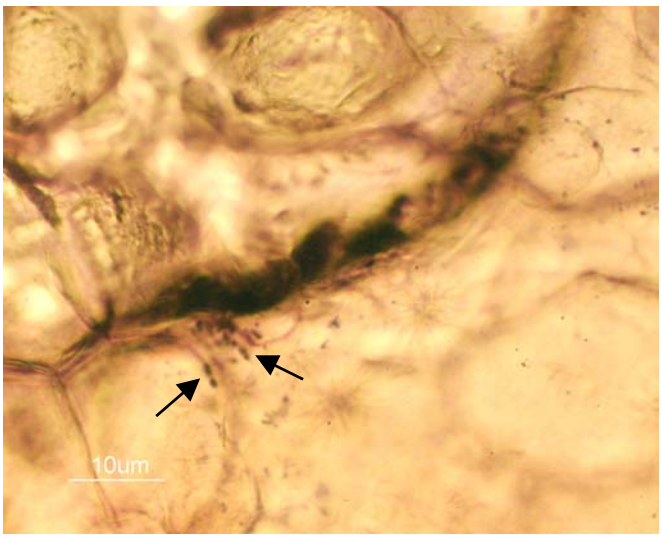


**Fig. 5.2** Bright-field micrograph of chicken skin contaminated with *S. typhimurium* S36 and hybridized with DIG labeled salmonella probe demonstrate that rod-shape *Salmonella* cells (black arrows) attached on the chicken skin (x 400).

Furthermore, digoxigenin-labeled 23S rRNA *Salmonella* probe in combination with antibody-alkaline phosphatase detection was used to detect *Salmonella* attached on chicken skin. Hybridization performed with DIG labeled probe proved more successful. Possible reason for this is that by using bright field the autofluorescence derived from the sample was minimized. In addition the use of an alkaline phosphatase conjugate greatly amplifies the hybridization event by forming a relatively large quantity of colored precipitate (i.e. with fluorescent probes no amplification is achieved). On Fig. 5.2, the salmonella rod shape cells can be seen present on the surface of chicken skin. It should be noted that the cells appear purple through use mixture of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate substrate and turn on the light to increase clearly appearance of chicken skin. *Salmonella* cells adhering to the skin were located primary on rough

areas of the chicken skin in crevices, wrinkles or entrapped inside feather follicles with water.

### 5.4.5 *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 (Fig. 5.3). Scanning electron micrograph of *S. typhimurium* S36 contaminated on chicken drumstick skin untreated control (Figs. 5.3A and 5.3B) indicated *Salmonella* cells adhered on the flat portion of the skin surface area (Fig. 5.3A) and were mostly located in crevices or surface grooves (Fig. 5.3B). After treatment with CPC and nisin, *Salmonella* cells remained on the poultry skin surface (Fig. 5.3C), but the cells lost their original shapes and showed indentations and fibril on the surface (Fig. 5.3D). In addition, the SEM method showed that CPC and nisin do not detach cells from the chicken skin. Moreover, CPC was reported to enhance microbial attachment to hydrophobic surface by diminishing surface charge and increasing cell surface hydrophobicity (Goldberg, Konis and Rosenberg, 1990). Kim and Slavik (1996) reported that CPC may kill salmonellae cells by causing metabolic damage, with little effect on the morphological structure. On Fig. 5.3D also, unlike the control (Fig. 5.3B), 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 the exopolysaccharide on the outer membrane of the cells were untangled and released during CPC-nisin treatment.

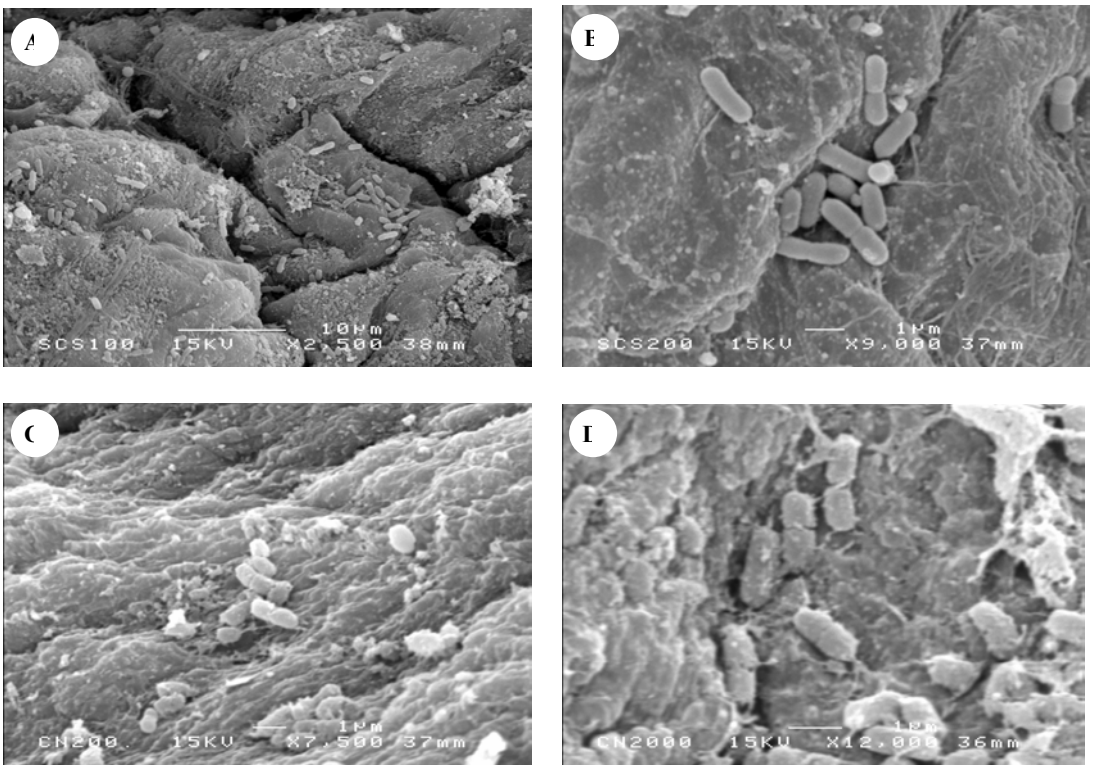


**Fig. 5.3** Scanning electron micrographs of *S. typhimurium* S36 contaminating on chicken drumstick skin untreated control (A and B) and treated with CPC and nisin (C and D). Intact cells attached on the chicken skin surface (A, x 2,500). Cells entrapped in crevices or wrinkles on chicken skin (B, x 9,000). CPC-nisin treated cells remained to attach on chicken skin surface (C, x 7,500). Indentations and fibril of CPC-nisin treated cells appeared on their cell surfaces (D, x 12,000).

### 5.4.6 Visualization of *S. typhimurium* on chicken drumstick skin

On the screen of the SEM (Fig. 5.3), 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, Choi, Wolfaardt and Caldwell, 1996). Epifluorescent micrograph (Fig 5.4A) shows the majority of live 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 PI (Boulos, Pre′vost, Barbeau, Coallier and Desjardins, 1999), Since PI is membrane impermeable and can only penetrate into damaged membrane cells (Haugland, 1999). Thus, BacLight stain produced a clear color difference to distinguish dead cells from live cells. With this technique, CPC-nisin treated chicken skin (Fig. 5.4B) appears with a mixture of live and dead cells attached to its surface.

These results 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 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 (Finlay, Heffron and Falkow, 1989; Lillard, 1990). Thus, cells can become very firmly attached to poultry skin. Those cells that do not attach can be washed off easily, while those cells that 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, Compadre, Fifer, Salari, Serbus and Lattin, 1995). However, these studies demonstrate that CPC and nisin were more effective to inhibit or kill viable *S.*

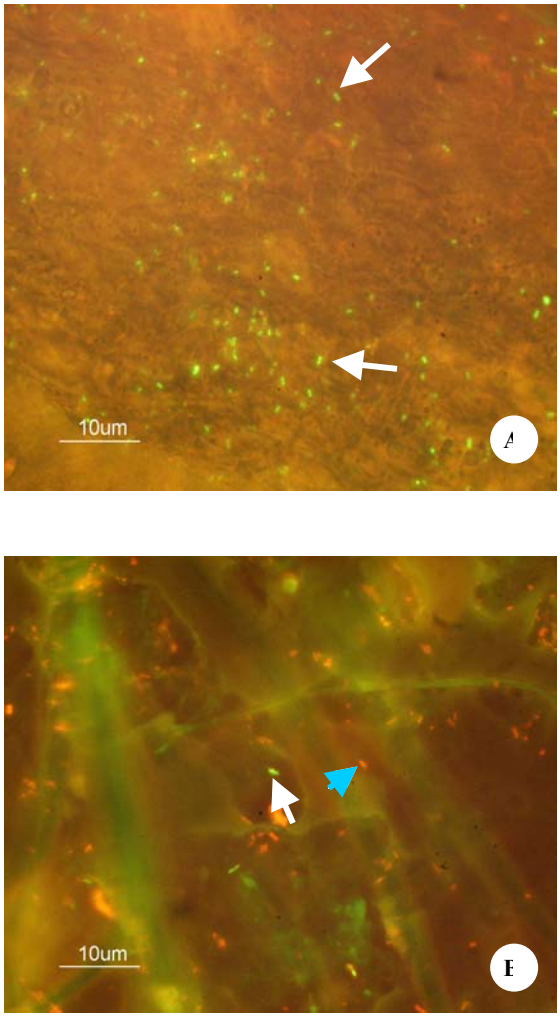


**Fig. 5.4** Epifluorescent micrographs showing attachment of *S. typhimurium* S36 on chicken drumstick skin. Bacteria were stained using the BacLight stain. (A) Untreated control, arrows indicate viable cells (green) of *S. typhimurium* S36 adhesion onto chicken skin. (B) White arrow shows a viable cell (green), blue arrow shows a dead cell (red) on chicken drumstick skin after treated with CPC-nisin (x 400).

*typhimurium* S36 cells attached on chicken skin as seen with the numbers of red cells in Fig. 5.4B. 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.

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 100 $\mu gml^{-1}$ 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.

### 5.4.7 Effect of cetylpyridinium chloride and nisin on bioluminescent *S. typhimurium* contaminating on chicken carcasses during storaged at 4°C and 10°C.

#### 5.4.7.1 Viable cells of bioluminescent *S. typhimurium* contaminating on chicken drumstick skin treated with CPC and nisin and stored at 4°C and 10°C

Viable cells of *S. typhimurium* S36 for the CPC-nisin treated chicken drumstick skin during refrigerated storage at 4°C and 10°C are shown in Fig. 5.5. Compared to untreated control chicken drumstick skin, population reduction of *S.*

*typhimurium* S36 storage at 4°C and 10°C were 1.96 and 1.80 $\log_{10}$ $CFUg^{-1}$ respectively on day 0. In the case of refrigerated storage at 10°C, on day 3, the log reduction of *Salmonella* cells was 2.15 $\log_{10}$ $CFUg^{-1}$. On day 4 both control and CPC-nisin treated chicken drumstick skin had the appearance of spoiled chicken skin. Additionally, control chicken drumstick skin also spoiled on day 4 of storage at 4°C, whereas CPC-nisin treated chicken drumstick skin on day 6 of storage at 4°C demonstrated larger log reduction (2.93 $\log_{10}$ $CFUg^{-1}$). Normally, spoilage of poultry occurs when microbial count exceed $10^7$ $CFUg^{-1}$(Cox, Russell and Bailey, 1998). Although the viable cells of all samples in this experiment were lower than $10^7$ $CFUg^{-1}$, spoiled characteristics appeared on day 4 for the CPC-nisin treated and untreated control chicken skin stored at 10°C. It may be due to the storage temperature at 10°C, a mildly abusive temperature during distribution, retail or consume storage, which can not inhibit the growth of survivor *Salmonella* cells and of the native microflora of broiler carcasses, especially psychrotrophic bacteria that survive processing and may multiply during refrigerated storage. These can competitively grow with *Salmonella* at low temperature and cause spoilage of fresh poultry (Hinton, Cason and Ingram, 2002; Russell, Fletcher and Cox, 1996). At refrigeration temperature (4°C), untreated control chicken drumstick skin revealed spoiled characteristics on day 5 of the storage period. This was because during refrigerated storage, there was an increase in the population of psychrotrophs on the carcasses. Bacterial genera recovered from carcasses undergoing processing and from carcasses stored at refrigeration temperatures included *Aeromonas*, *Chromobacterium* and *Pseudomonas* (Gallo, Schmitt and Schmidt-Lorenz, 1988). In particular, *Pseudomonas* spp. was the predominant bacteria recovered from poultry carcasses

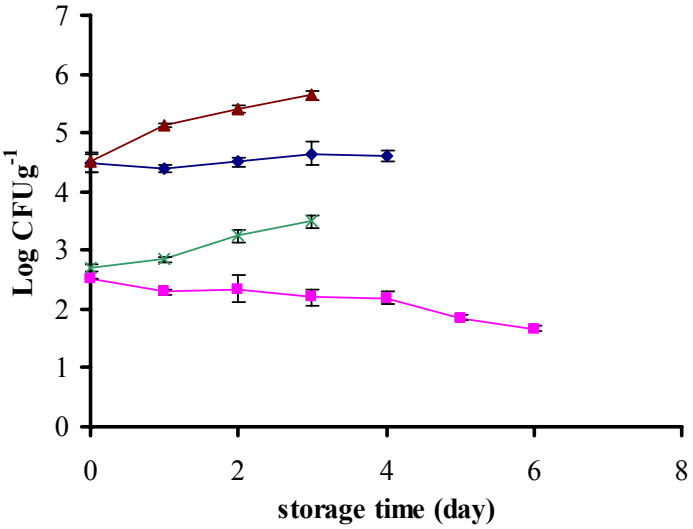


**Fig. 5.5** Viable cells of *S. typhimurium* S36 contaminating on chicken carcasses treated with CPC and nisin stored at 4°C and 10°C for 6 days. Control sample stored at 4°C (♦), CPC-nisin sample stored at 4°C (■), control sample stored at 10°C (▲), and CPC-nisin sample stored at 10°C (×)

(Hinton, Cason and Ingram, 2004). Some pseudomonads are resistant to the chlorination levels used in immersion chiller water (Mead, Adams and Parry, 1975), therefore, low levels of these psychrotrophs may contaminate carcasses during chilling then proliferate on the carcasses during refrigerated storage. As the *Pseudomonas* population increased, it may have overgrown bacteria. Thus, most cross-contamination by pseudomonas might be evident in the refrigerated carcasses. However, CPC-nisin treatment indicates the efficacy in reducing viable *Salmonella* cells attached on chicken skins when stored at 4°C, including the extension of shelf life of samples longer than the untreated control.

**5.4.7.2 Bioluminescence profiles of bioluminescent *S. typhimurium* contaminating on chicken carcasses treated with CPC and nisin storaged at 4°C and 10°C during 6 days.**

*S. typhimurium* S36 expressing the *lux* operon could be readily visualized macroscopically directly on whole chicken drumstick (Fig.5.6). Emitted photons were detected immediately over the entire inoculated chicken drumstick. After a 5 min integration of the signal, it became apparent that there were different signal intensities in different areas of chicken drumstick skin (Figs. 5.6B and 5.6C). The differences appeared to be associated with topographic features, which suggested that there was differential adherence of bacterial cells on skin surface. In particular, feather follicles on chicken drumstick skin displayed greater bioluminescence intensity, which demonstrated that there were large amounts of viable *S. typhimurium* S36 cells on that area (Figs. 5.6 B and 5.6C). Using luminescent *S. typhimurium* S36 also revealed the efficacy of CPC and nisin on *S. typhimurium* S36. Fig. 5.7 shows the luminescent images of *S. typhimurium* S36 on contaminated chicken drumstick skins treated with CPC-nisin and then stored at 4°C and 10°C for 6 days. At 4°C of storage temperature, CPC-nisin chicken skin did not show luminescent images for all 6 days (Fig. 5.7B), whilst untreated control chicken skin revealed slightly luminescent intensities since day 0 to day 4 (Fig. 5.7A). In addition, at 10°C storage temperature, the luminescent image of chicken drumstick skin treated with CPC-nisin (Fig. 5.7D) showed low luminescent intensity on day 3 of the storage period, whereas the luminescent image of untreated control chicken skin (Fig. 5.7C) showed high luminescent intensity on day 3. As well, these results agree with the result of viable cells from Fig. 5.5.

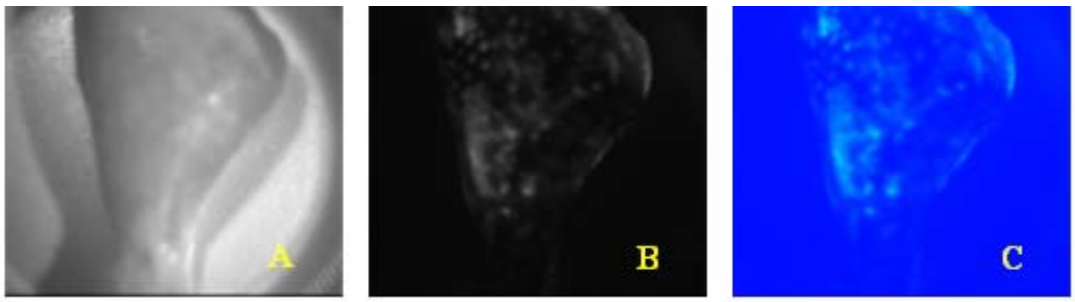


**Fig. 5.6** Chicken drumstick was inoculated with *S. typhimurium* S36 and images taken under illumination (A) and in the dark (B and C): (A) is a visible appearance of whole chicken drumstick, (B) bioluminescence on grayscale image and (C) bioluminescence pseudocolor image of the same drumstick in (A).

**5.4.7.3 Hunterlab analysis of chicken carcasses contaminated with bioluminescent *S. typhimurium,* treated with CPC and nisin, and stored at 4°C and 10°C during 6 days.**

Changes in Hunter color *L*, *a*, and *b* values of chicken drumstick skin treated with CPC-nisin and stored at 4°C and 10°C for 6 days are shown in Figs. 5.8A, 5.8B, and 5.8C respectively. Chicken drumstick skin treated with CPC-nisin and stored at 4°C was initially lighter in color (i.e. higher Hunter *L* value) than the untreated control stored at 4°C. Drumstick skin treated with CPC-nisin and stored at 10°C that was initially slightly lighter in color than the untreated control stored at 10°C (Fig. 5.8A). However, during storage, Hunter *L* values of all samples tended to slightly decrease. Chicken drumstick skins treated with CPC-nisin and stored at 4°C had lower Hunter *a* values (less red) than the untreated control after storage, whereas chicken drumstick skin treated with CPC-nisin and stored at 10°C had significantly higher Hunter *a* values than the untreated control initially and after storage (Fig. 5.8B).

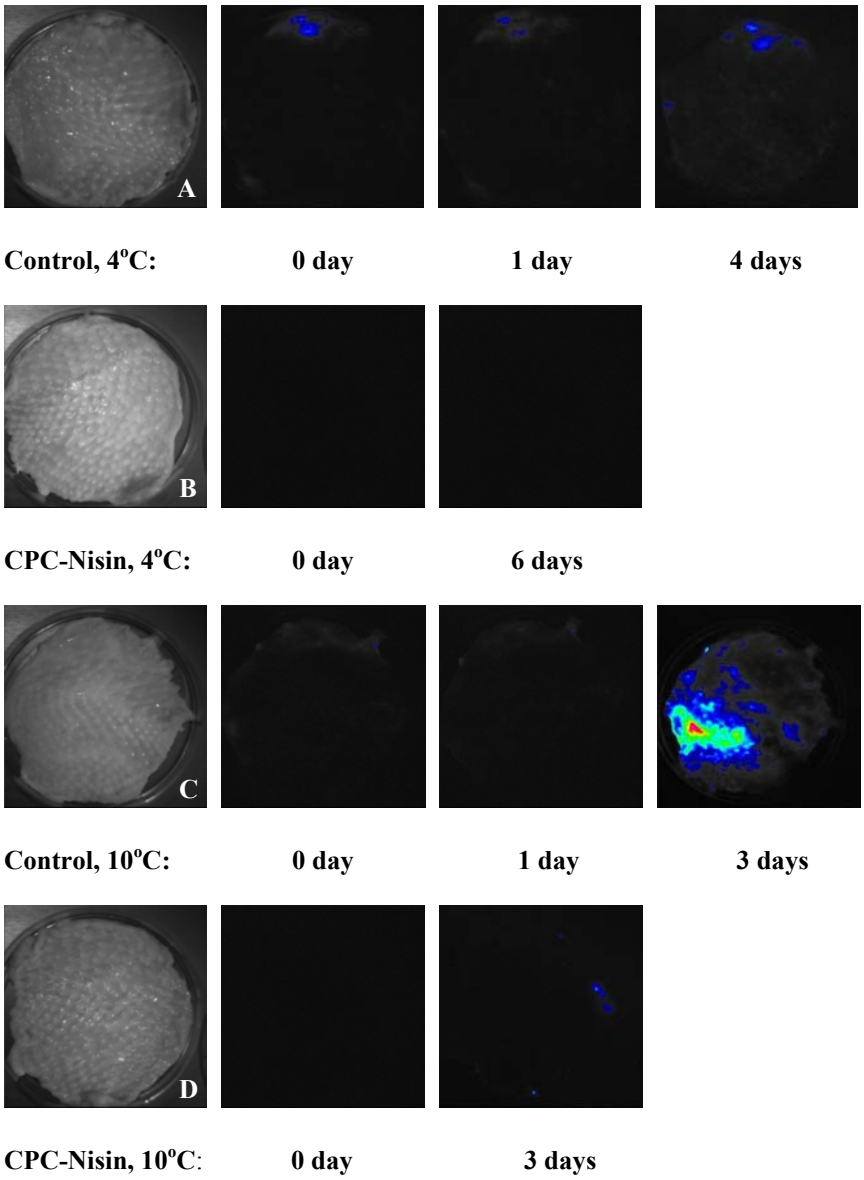


**Fig. 5.7** Luminescent images of *S. typhimurium* S36 contaminated chicken skins treated with CPC and nisin stored at 4°C and 10°C for 6 days. Untreated control chicken skin stored at 4°C and 10°C respectively (A and C) and CPC-nisin chicken skin stored at 4°C and 10°C respectively (B and D).

Hunter *b* values of chicken drumstick skins treated with CPC-nisin and stored at 4°C were initially lower than the untreated control at 4°C and remained low at the end of the storage period indicating a slight yellowness. Hunter *b* values of CPC-nisin treated skins stored at 10°C were initially higher than the untreated control (Fig. 5.8C) During storage, Hunter *b* values, i.e. yellowness, of untreated control and CPC-nisin treated chicken drumstick skins tended to increase up to the end of the storage period (day 3) and thereafter spoiled.

The results of this study demonstrate that in the case of storage at 4°C, CPC-nisin treated chicken drumstick skins were lighter, less red and less yellow than the untreated control skin stored for 6 days. Whereas, chicken skins treated with CPC-nisin and stored at 10°C were darker, redder and more yellow than untreated control skins stored for 3 days. Discoloration of chicken skin may be caused by high concentration of organic acid and other chemical treatments such as TSP. However, Xiong, Li, Slavik and Walker (1998) reported that CPC has neutral pH values and did not cause discoloration of chicken skin. Therefore, an explanation may be that the different external color appearance of chicken skins was caused by the overgrowth of survivors of *Pseudomonas* and other psychrotrophic bacteria, as shown by spoilage characteristics such as sliminess.

## 5.5 Conclusions

Results of this investigation demonstrated that a treatment of 0.5% (w/v) CPC for 120 sec and 100 $\mu g ml^{-1}$ nisin for 30 min was the most effective in suppressing *S. typhimurium* S36 attachment on chicken skin carcasses. Shelf life of CPC-nisin treated chicken carcasses stored at 4°C could be extended. Moreover, CPC and nisin

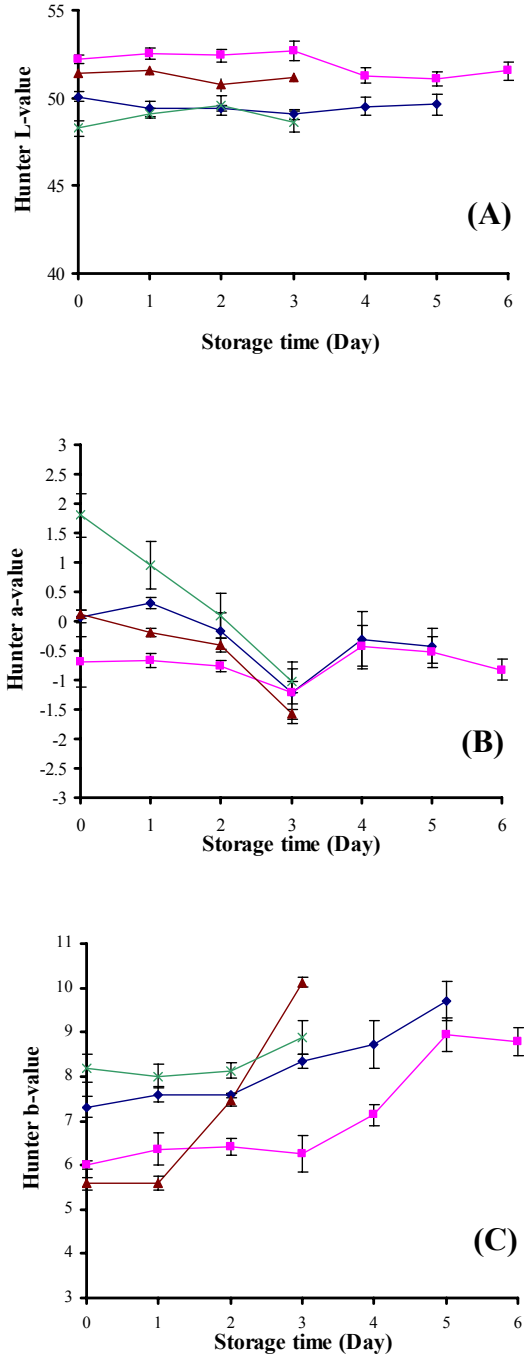


**Fig. 5.8** Hunter L-value (A), a-value (B) and b-value (C) of *S. typhimurium* S36 contaminated chicken skins treated with CPC and nisin stored at 4°C and 10°C for 6 days. Control sample stored at 4°C (♦), CPC-nisin sample stored at 4°C (■), control sample stored at 10°C (▲), and CPC-nisin sample stored at 10°C (×)

use in chilling tanks or as post-cutting dips or sprays may be benefit to reduce contamination of microorganisms during poultry processing and to produce safer products with an extended shelf life.

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# CHAPTER VI

# SUMMARY

*Salmonella typhimurium* S36 was constructed by transformation with the mini-Tn5 plasmid containing the *lux CDABE* cassette for monitoring the sensitivity of bacteria to antimicrobial agents and other treatments in real time. Their abilities to inhibit *S. typhimurium* S36 were performed by determining viability and bioluminescence output following exposure to trisodium phosphate (TSP), cetylpyridinium chloride (CPC), sodium lactate (SL) and nisin treatments at mid-exponential and stationary phase of bioluminescent *S. typhimurium*. The stationary phase of *S. typhimurium lux* is more resistant to the treatments than the mid-exponential phase cells. CPC-nisin is, apparently, the most effective treatment to inhibit *S. typhimurium* S36 suspension. Therefore, CPC and nisin were selected to use in further experiment.

On the other hand, because the outer membrane (OM) of Gram-negative bacteria act as a permeability barrier, preventing nisin from gaining access to the cytoplasmic membrane. Alteration of the OM permeability can increase nisin sensitivity of Gram-negative bacteria including *Salmonella*. Physical stresses: temperature stress (heating, chilling and freezing) and pH stress (pH 4.5, 5.0, 6.0) and treatment with CPC solution were able to alter the OM permeability of *S. typhimurium* S36, producing a loss of barrier function and reducing resistance to nisin. Temperature and pH stressed cells, untreated and treated with CPC-nisin, had

perturbed cell morphology, including apparent indentations, craters in the cell surfaces and collapsed amorphous bodies, which were examined using scanning electron microscopy (SEM). Moreover, blebs, cytoplasm-sparse and an abnormal shape of cells were observed under transmission electron microscopy (TEM). Notably, BacLight nucleic acid stain confirmed the morphological changes of cell wall and membrane of ruptured *Salmonella* cells. The inclusion of CPC-nisin treatment as a lethal process for Gram-negative bacteria could enhance food safety during food processing.

Furthermore, the use of a bioluminescent strain of *S. typhimurium* S36 showed that cells adhered preferentially to convoluted areas of chicken skin surface. The effect of CPC and nisin on the inhibition of viable *S. typhimurium* cells contaminated on chicken carcasses was investigated. Chicken drumstick sections (5 by 5 cm), inoculated with *S. typhimurium* S36, were treated with CPC and further treated with nisin. It was found that the use of 0.5%(w/v) CPC for 120 sec and 100 ม $gml^{-1}$ nisin for 30 min increased ($p \leq 0.05$) the loss of cell viability with minimal effect on the appearance of chicken carcasses. Following the CPC-nisin treatment, the live and dead attached cells on chicken skin were revealed by staining with BacLight stain, using eplifluorescent microscopy. The most effective of CPC-nisin treatment in reducing *S. typhimurium* S36 contaminated on chicken drumstick skin during refrigerated storage was at $4^{o}C$ for 6 days with a slight effect on chicken skin color characteristic. These results also demonstrated the synergistic activity of CPC and nisin treatment against *Salmonella* and could be expected to enhance the safety of chicken carcasses and other chicken products.

# APPENDIX

# APPENDIX A

## QIAGEN Plasmid Maxi Protocol

1. Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 2 ml LB medium containing 30 $mgml^{-1}$kanamycin. Incubate for ~8h at 37$^{o}$C with vigorous shaking (~200 rpm).
2. Dilute the starter culture 1/500 into selective LB medium. Inoculate 500 ml medium and grow at 37$^{o}$C for 12-16 h with vigorous shaking (~200 rpm).
3. Harvest the bacterial cells by centrifugation at 6000 x g for 15 min at 4$^{o}$C.
4. Resuspend the bacterial pellet in 10 ml of Buffer P1.
5. Add 10 ml of Buffer P2, mix gently but thoroughly by inverting 4-6 times, and incubate at room temperature for 5 min.
6. Add 10 ml of chilled Buffer P3, mix immediately but gently by inverting 4-6 times, and incubate on ice for 15 min or 20 min.
7. Centrifuge at $\geq$ 20,000 x g for 30 min at 4$^{o}$C. Remove supernatant containing plasmid DNA promptly.
8. Re-centrifuge the supernatant at $\geq$ 20,000 x g for 15 min at 4$^{o}$C. Remove supernatant containing plasmid DNA promptly.

9. Equilibrate a QIAGEN-tip 500 by applying 10 ml Buffer QBT, and allow the column to empty by gravity flow.
10. Apply the supernatant from step 8 to the QIAGEN-tip and allow it to enter the resin by gravity flow.
11. Wash the QIAGEN-tip with 2x30 ml Buffer QC.
12. Elute DNA with 15 ml Buffer QF.
13. Precipitate DNA by adding 10.5 ml room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at $\geq$ 15,000 x g for 30 min at 4$^{o}$C. Carefully decant the supernatant.
14. Wash DNA pellet with 5 ml of room-temperature 70% ethanol, and centrifuge at $\geq$ 15,000 x g for 10 min. Carefully decant the supernatant without disturbing the pellet.
15. Air-dry the pellet for 5-1 min, and redissolve the DNA in a suitable volume of TE buffer pH 8.0.

**Note:** Composition of buffer

| Buffer | Composition | Storage |
|---|---|---|
| Buffer P1 | 50mM Tris.Cl, pH 8.0; 10 mM EDTA; | 2-8$^{o}$C, after addition of |
| (Resuspension Buffer) | 100 µgml$^{-1}$ Rnase A | RNase A |
| Buffer P2 (Lysis Buffer) | 200 mM NaOH, 1% (w/v) SDS | room temperature |
| Buffer P3 | 3.0 M potassium acetate, pH 5.5 | room temperature |
| (Neutralization Buffer) | | |
| Buffer QBT | 750 mM NaCl; 50 mM MOPS, pH 7.0; | room temperature |
| (Equilibration Buffer) | 15% (v/v) isopropanol; 0.15%(v/v) Triton X-100 | |
| Buffer QC | 1.0 M NaCl; 50 mM MOPS, pH 7.0; | room temperature |
| (Wash Buffer) | 15% (v/v) isopopanol | |
| Buffer QF | 1.25 M NaCl; 50 mM Tris.Cl, pH 8.5; | room temperature |
| (Elution Buffer) | 15% (v/v) isopropanol | |
| TE | 10 mM Tris.Cl, pH 8.0; 1 mM EDTA | room temperature |

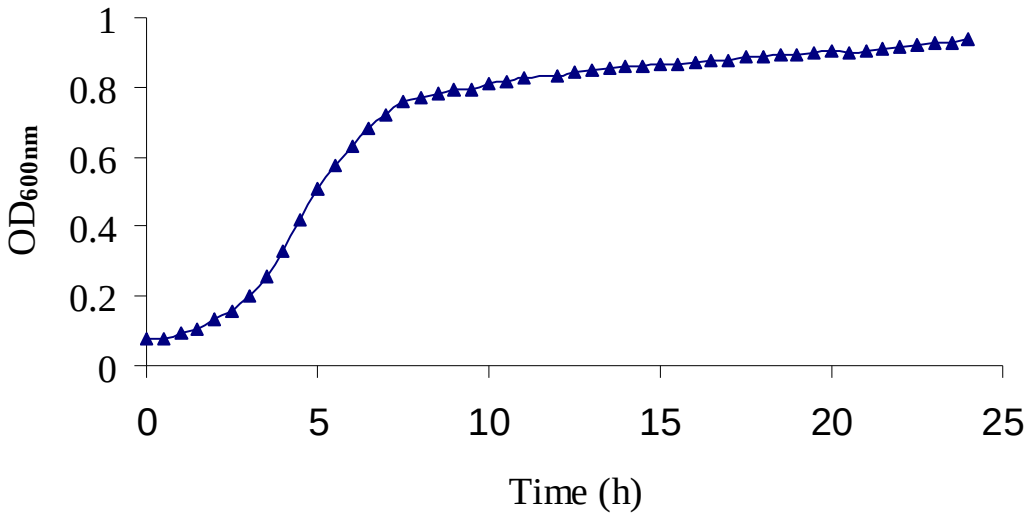


**Fig. 7.1** Growth curve of *Salmonella typhimurium* S36

# APPENDIX B


**Fig. 7.2** Instruments for construction bioluminescent *S. typhimurium* (A) Lucy1 Photoluminometer, (B) the Bio-Rad Gene pulsar™ electroporator, (C) Turner TD20e luminometer

# APPENDIX C

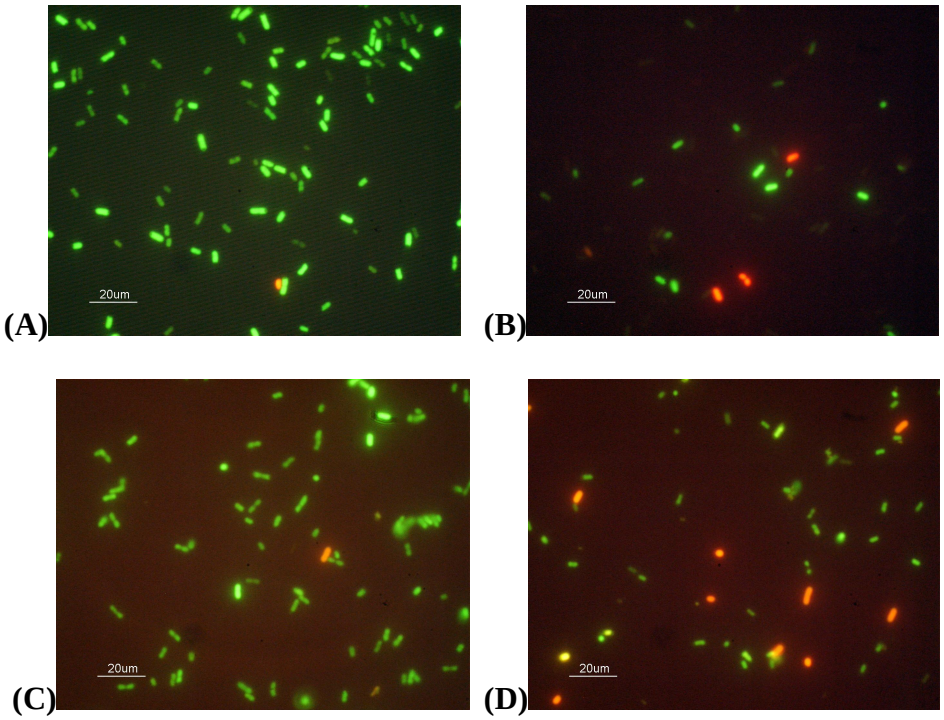


**Fig. 7.3** Epifluorescent micrographs of stationay phase *S. typhimurium* S36 treated with TSP, SL and nisin. Cells treated with SL (A) and treated with SL-N (B), Cells treated with TSP (C) and treated with TSP-N (D).

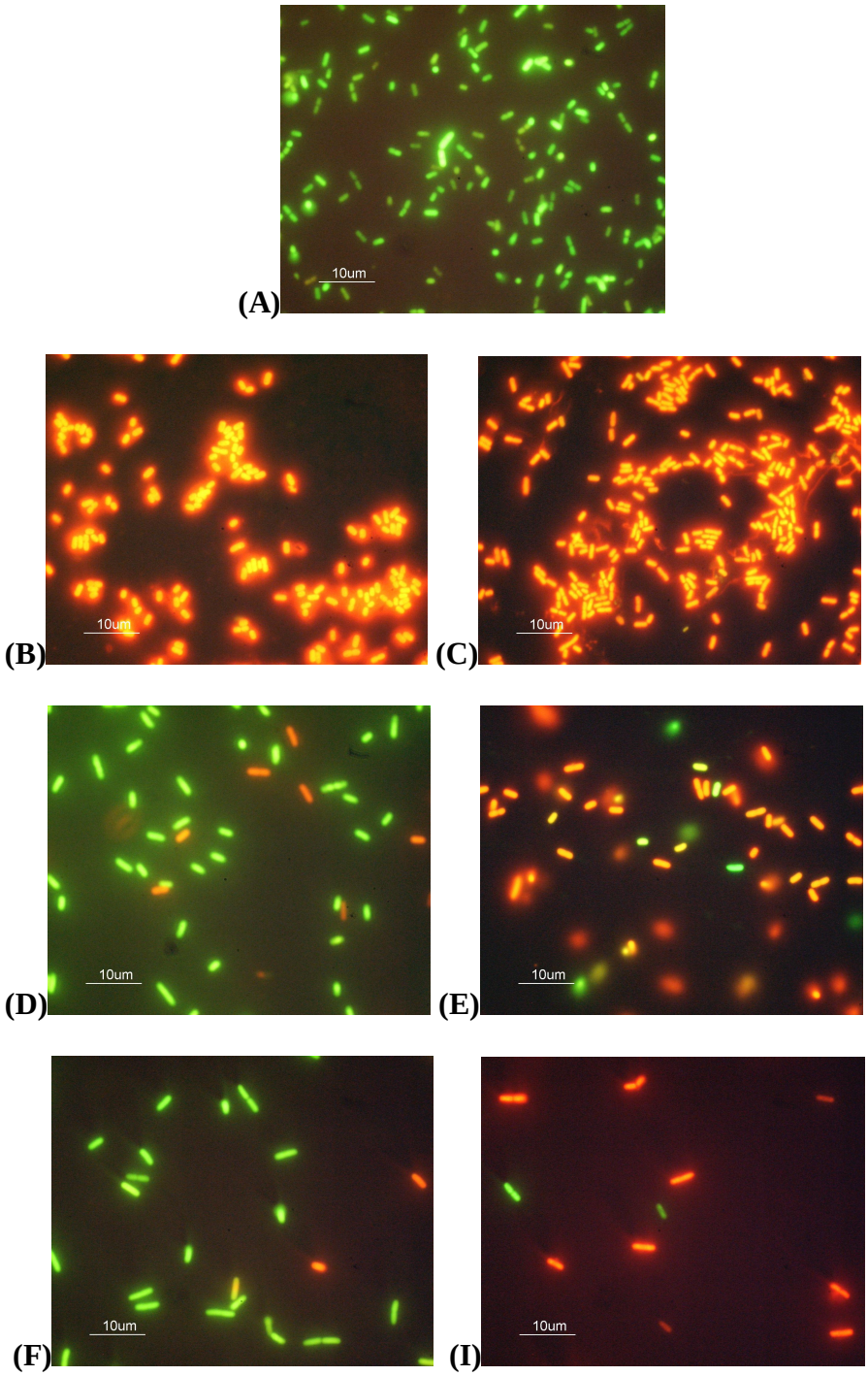


**Fig. 7.4** Epifluorescent micrographs of mid-log phase *S. typhimurium* S36 treated with TSP, CPC, SL and nisin. Control cells (A), Cells treated with CPC (B) and with CPC-nisin (C), Cells treated with TSP (D) and with TSP-N (E), Cells treated with SL (F) and with SL-N (I).

# APPENDIX D

## Bacterial media

Unless stated otherwise all midia were brought to volume with reverse osmosis (RO) water and autoclaved at 121$^{o}$C for 15 min. Where required, agar (1.5% (w/v); Oxoid, Basingstoke, UK) was added prior to autoclaving.

### 1. Luria-Bertani (LB) broth and agar

The broth contained 10 g tryptone (difco), 5 g extract (Difco), 4 ml 1M NaOH and 5 g NaCl (Fisher Scientific, UK) made up to 1 litter with RO water. LB was solidified using bacteriogical agar No.1 (Oxoid) (1.5% w/v).

### 2. Brain Heart Infusion (BHI) broth and agar

Dehydrated BHI broth (12.5 $gl^{-1}$ calf brain infusion solids, 5 $gl^{-1}$ sodium chloride and 2.5 $gl^{-1}$ di-sodium phosphate) (Oxoid, UK) was added to RO water at 37 $gl^{-1}$ BHI broth was solidified using bacteriological agar No.1 (Oxoid) (1.5% w/v).

### 3. Xylose Lysine Desoxycholate (XLD) agar

XLD agar base (53 $gl^{-1}$, Oxoid) was suspended in RO water and heated with stirring until the solution boiled. The agar was tempered to 55$^{o}$C and poured into sterile petridishes.

**4. Maximum Recovery Diluent (MRD)**

Maximum recovery diluent (MRD) (9.5 $gl^{-1}$, Oxoid) was suspended in RO water and autoclaved.

**5. Rappaport-Vassiliadis (RV) enrichment broth**

A selective enrichment broth for the isolation of salmonellae, RV (30 $gl^{-1}$, Oxiod) was suspended in RO water. Heat gently until dissolved completely. Dispense 10 ml volumes into screw-capped bottles and sterilize by autoclaving at 115$^{o}$C for 15 min.

# BIOGRAPHY

Bussagon Thongbai was born in Surat-thani and brought up in Pathum-thani, Thailand. She attended Kasetsart University, Thailand and received her Bachelor's degree in Microbiology (1990). In 1995 she received a Master's degree in Food Technology at Chulalongkorn University, Thailand and In 2000 she was granted a scholarship by Consortium Sandwich Ph. D. Program, Office of the Higher Education Commission, Ministry of Education (then Ministry of University Affairs) jointly with School of Food Technology, Suranaree University of Technology and School of Food Sciences, Division of Food Microbiology, University of Nottingham to pursue Ph. D. studies in Thailand and United Kingdom.

## Poster presentation:

1. Bussagon Thongbai, Korawan Ratanachai, Piyawan Gasaluck, William M. Waites. 2004. Temperature, cetylpyridinium chloride and nisin effect on morphological changes of *Salmonella*. 8$^{th}$ Asia-Pacific Conference on Electron Microscopy, June 7-11, 2004, Kanazawa, JAPAN.

2. Bussagon Thongbai and Piyawan gasaluck. 2005. Effect of temperature, cetylpyridinium chloride and nisin on morphological changes of *Salmonella*. 22$^{nd}$ Annual Conference of The Microscopy Society of Thailand, Febuary 2-4, 2005, Chonburi, THAILAND.