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The Effect Of Curcumin (Curcuma Longa) On Biofilm Formation And Surface Proteins Of Listeria Monocytogenes

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**THE EFFECT OF CURCUMIN (*CURCUMA LONGA*) ON BIOFILM
FORMATION AND SURFACE PROTEINS OF *LISTERIA MONOCYTOGENES***

A Thesis Presented

By

SONGSIRIN RUENGWISESH

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

May 2012
Food Science

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DEDICATION

I would like to dedicate this thesis to my parents and my family members in Thailand. Without their great love and support, this work would not be successful.

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I would like to gratefully and sincerely thank Dr. Lynne A. McLandsborough for her guidance, invaluable knowledge she has given me, patience, and more importantly, her kindness during my studies at UMass Amherst. Without an opportunity from her, this work would not be possible.

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ABSTRACT

THE EFFECT OF CURCUMIN (*CURCUMA LONGA*) ON BIOFILM FORMATION AND SURFACE PROTEINS OF *LISTERIA MONOCYTOGENES*

MAY 2012

B.S., CHULALONGKORN UNIVERSITY

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Dr. Lynne A. McLandsborough

The food-borne pathogen *Listeria monocytogenes* can attach to the environmental surfaces and develop biofilm which can cause food contamination in the food industries. Sortase A and surface proteins are involved in biofilm and virulence of *L. monocytogenes*. Curcumin was reported to inhibit sortase A and biofilm in gram positive bacteria. The overall objective of this study was to observe the effect of curcumin (*Curcuma longa*) on the biofilm formation and surface proteins of *L. monocytogenes*.

The antibiofilm effect of curcumin against the strain LM21 (wild type) and s22-11G (sortase A defective mutant) was studied using the microtiter plate assay. No significant differences between the growth of the wild type and the sortase A defective mutant were observed at sub-inhibitory concentrations of curcumin. However, a greater biofilm reduction was observed in the strain s22-11G. The effect of curcumin from two different manufacturers on the wild type was also compared by the microtiter plate assay. Both curcumin did not exhibit statistically different effect on the growth of the wild type. However, a greater biofilm inhibitory effect was observed in one curcumin. The HPLC results suggested that curcumin with the greater antibiofilm activity contained higher amount of curcumin which was reported to be the most potent curcuminoid compound in

curcumin.

Three different protein extraction methods were evaluated and the most efficient method was used for 2D-GE. When cells were grown in the presence of curcumin, 5 proteins, 16 proteins and 4 proteins were up-regulated, down-regulated and absent, respectively in *L. monocytogenes* LM21. The influence of the enzyme sortase A upon surface protein expression was evaluated by comparing proteins expressed by wildtype *L. monocytogenes* LM21 to that of the sortase A mutant, s22-11G. In strain s22-11G, 2 proteins, 8 proteins and 3 proteins were up-regulated, down-regulated and absent in comparison to wildtype LM21. The exact information of these differentially expressed proteins still need to be identified by mass spectrometry.

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CHAPTER 1

LITERATURE REVIEW

1.1 *Listeria monocytogenes*

The food-borne pathogen *Listeria monocytogenes* is a facultative anaerobic, rod shaped, non-spore forming, gram positive bacterium that grows optimally at 37°C at $a_w \geq 0.97$. It is ubiquitous in the environment and can normally be found in waters, soil, rotting parts of plants animal feces and wastewaters (22, 36, 40). It can tolerate high salt concentration (up to 15%), wide range of pH (from 4.5 to 9.6) and temperatures (from 0 to 45°C), and low water activity (a_w down to 0.90-0.93) (18, 40). This bacterium belongs to the genus *Listeria* which consists of six species: *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, and *L. grayi* (3).

L. monocytogenes has the ability to penetrate the eukaryotic cells, grow inside the cells and spread to nearby cells. It primarily causes listeriosis in high risk groups such as pregnant women, neonates and immunocompromised adults (48). Normally, listeriosis lasts 7-10 days and the most common symptoms are fever, muscle aches and vomiting. Nausea and diarrhea are less common symptoms. When the infection spreads to the nervous system it can cause meningitis, an infection that includes the brain and spinal cord. Listeriosis can also lead to other serious problems such as abortion, endocarditis, hepatitis, localized abscesses (*e.g.* in the brain) and muscular, skeletal and skin infections (36, 48). Almost 2000 cases are reported with listeriosis annually and mortality rate is 20% - 30% (22).

Listeriosis has become a major foodborne disease over the past 25 years. Some ready-to-eat foods (*e.g.* hot dogs, soft cheese, ice cream, delicatessen meats and poultry

products) have been found to be sources of *L. monocytogenes* since this bacterium is a psychrotroph which can grow at refrigeration temperature. Raw milk is also a source of *L. monocytogenes*; nevertheless, pasteurization is considered a sufficiently safe process to reduce the number of *L. monocytogenes* to levels that do not pose risk to human health. Compared to other meats, *L. monocytogenes* can grow more efficiently in poultry (47). In the United States, the annual cost of acute foodborne disease owing to *L. monocytogenes* is approximately 2.3 billion dollars. According to this, public health and regulatory agencies in the United States have established zero tolerance policy for *L. monocytogenes* (40).

1.2 Biofilm formation

Microorganisms can grow on surfaces and develop biofilms, which are complex microbial communities embedded in extracellular matrix or exopolymeric substances including polysaccharides, proteins and nucleic acids (47). Biofilms improve survival and growth of microorganisms due to many reasons. First, biofilms serve as a protective shelter for microorganisms as they can resist physical forces that could remove unattached cells, phagocytosis by immune cells, or penetration of toxic chemicals. Second, biofilms allow microbial cells to remain in a favorable niche as they can fix microorganisms to nutrient-abundant surfaces. Third, biofilm formation allows microorganisms to live in close proximity with each other. This provides better opportunities for quorum sensing which is a process of bacterial cell-to-cell communication involving the production and detection of extracellular signaling molecules called autoinducers. Also, genetic exchange improves when cells are in close

association. Biofilms are the typical mode of growth that bacteria grow in nature when nutrients are not as rich as culture media. (25, 53)

Bacterial biofilm formation and propagation occurs in five stages. In the first step, microorganisms move to surfaces by bacterial motility, diffusion through the environment or natural forces in the system (26). It was reported that in the static condition, flagella-based motility was necessary for *L. monocytogenes* in order to propel cells to the surface. On the other hand, under continuous flowing system, loss of flagellar motility resulted in lower initial attachment but greater biofilm formation (1). In stage 1, bacteria reversibly attach to surfaces due to physical forces known Van der Waals interactions (> 50nm from the surface), repulsive or attractive electrostatic interactions (2–10 nm from the surface), and hydrophobic interactions (0.5–2 nm from the surface) (17). In stage 2, irreversible cell attachment occurs since microbial cells anchor themselves more permanently by using cell adhesion structures such as pili as well as producing exopolymeric material which is a stronger adhesive compound (45, 47). Also, it has been suggested that proximity to neighboring cells might govern the conversion to permanent attachment (38). Step 3 involves microcolony formation and maturation of biofilms. In the fourth step, more maturation occurs and biofilms develop into a three-dimensional structure containing clusters of cells with channels between them. These channels facilitate water and nutrients delivery to cells as well as waste removal from cells. In mature biofilms, cell division does not really occur and most energy mainly utilized for exopolysaccharide production (30). The last step in biofilm formation is cell dispersion which microbial cells are dispersed from biofilms into the environment (47).

1.3 Biofilms in food industry and control of biofilms

Biofilms are undesirable in the food industry since they serve as a source of product contamination and also a reservoir for pathogenic or spoilage microorganisms. It was found that moist surfaces facilitate formation of biofilms. The common sites in food processing plants where biofilms exist include filling or packaging equipment, floor drains, walls, cooling pipes, conveyors, collators used for assembling product for packaging, racks for transporting products, hand tools or gloves, and freezers (13). Compared to planktonic microorganisms, biofilms are more resistance to antimicrobial agents due to the impenetrable character of biofilms, the slow growth rate of microorganisms and the induction of resistance mechanisms (1). Therefore, effective methods to eliminate biofilm from food processing sites are required. Some studies showed effective strategies to decrease bacterial biofilms in food processing environment. According to Norwood and Gilmour (2000). *Listeria monocytogenes* biofilms reduce by two log-cycle after exposure to 100 ppm chlorine for 20 minutes, while planktonic cells of *L. monocytogenes*, *Pseudomonas fragi* and *Staphylococcus xylosus* were eliminated by an exposure to 10 ppm free chlorine for 30 seconds (31). The study by Chmielewski and Frank (2004) showed that with appropriate time and temperature, hot water sanitation could be an efficient way to eliminate *L. monocytogenes* biofilms from stainless steel surfaces (12). Zhao *et al.* (2004) studied the competitive-exclusion of *L. monocytogenes* by microorganisms isolated from biofilms in drains of food processing facilities. The organisms with anti-listerial activity isolated were tested further for their effectiveness to eliminate *L. monocytogenes* biofilms on stainless steel coupons. *Enterococcus durans* and *Lactococcus lactis* were the two isolates that caused a

reduction of more than 5 log CFU/cm² of *L. monocytogenes*/cm² (54). These sanitation methods might be helpful for biofilm elimination in the food industry.

1.4 Surface proteins of *Listeria monocytogenes*

Surface proteins play a critical role in virulence and pathogenicity of *L. monocytogenes* (8, 28). They are characterized by specific structural features into 3 groups which are 1. proteins covalently linked to murein through their C-terminal domain (proteins with LPXTG sequence motif), 2. proteins non-covalently bound by their C-terminal domain (GW proteins, P60-like proteins, and hydrophobic tail proteins,) and 3. proteins linked to cell wall structures via their amino-terminal region (lipoproteins) (10).

1.4.1 Proteins covalently linked to the cell wall

1.4.1.1 Proteins containing the LPXTG motif

The covalent linkage of surface proteins to the cell wall of Gram positive bacteria requires a specific carboxy-terminal sorting signal which consists of a conserved LPXTG (leucine, proline, X, threonine and glycine, where X is any amino acid) sequence motif followed by a hydrophobic domain comprising approximately 20 amino acids and a tail of positively charged amino acids (10, 33). LPXTG sorting signal is the substrate of sortase A, a membrane-bound transpeptidase that cleaves the LPXTG motif between the threonine and glycine residues and catalyzes the formation of an amide link between the carboxyl group of the threonine and the meso-diaminopimelic acid (m-Dpm) in cell wall precursor. Among all gram-positive bacteria that surface proteins have been studied, *L. monocytogenes* contains highest number of LPXTG proteins (5, 10).

1.4.1.2 InlA and LRR-containing proteins

The most studied LPXTG protein in *L. monocytogenes* is Internalin A (InlA) which consists of 800 amino acids and promotes bacterial entry into epithelial cells by binding to the E-cadherin host cell receptor (8, 36). The N-terminal part of the InlA contains a leucine-rich repeat (LRR) domain, followed by the inter-repeat (IR) region, but its C-terminus contains two and a half repeats of 75 amino acids, followed by a region that contains the LPXTG motif. Amino acids from 36 to 78 form a domain composed of three α -helices -a cap domain. The LRR domain contains 15 and a half repeats of a 22 amino acid sequence, followed by a Ig-like domain between 415 and 495 amino acid (10, 43). *L. monocytogenes* also encodes proteins containing the LRR domain without the LPXTG motif. One of these proteins which is well-studied is InlB. The LRR region at the N-terminus of InlB harbors 213 amino acids, from 36 to 242. Amino acids 1-35 form a signal sequence that is cleaved off, so in the mature protein the LRR domain takes up the whole N-terminus. This region contains a hydrophilic cap composed of two β - and three α -helices and eight LRRs. Like many other internalin proteins, InlB also contains a B repeat (44). InlB is also involved in invasion into epithelial cells like InlA but it binds to different mammalian receptor named Met.

Four internalin-like proteins (InlE, InlF, InlG and InlH) were identified. These proteins belongs to “internalin multigene family” which contains an amino-terminal LRR (leucine-rich repeat) domain, followed by a conserved IR (inter-repeat) region, several other repeats and the LPXTG sorting signal (10, 36). Unlike InlA, these four internalin-like proteins (InlE, InlF, InlG and InlH) do not involve in invasion but play an important role in colonization of host tissues *in vivo* (43).

1.4.1.3 Proteins with the RGD motif

Besides the LPXTG motif and 10 PKD repeats, the *L. monocytogenes* protein Lmo1666 contains a RGD (Arg-Gly-Asp) motif which has been found in proteins participating in adhesion to eukaryotic cells. Also this motif has been shown to be the core recognition sequence for many integrins. They are present in a variety of integrin ligands, including collagen, fibronectin and pathogen surface proteins from *Leishmania* and *Bordetella pertussis*. Thus protein Lmo1666 may be involved in the invasion of the host cells. Apart from protein Lmo1666, the RGD motif has also been found in surface proteins ActA and in Lmo0460 which is a lipoprotein with unknown function in *L. monocytogenes* (2, 10).

1.4.2 Proteins non-covalently linked to the cell wall

1.4.2.1 GW proteins

A GW module contains about 80 amino acids with a highly conserved glycine-tryptophan dipeptide. It usually exists in multicopy which enhances the attachment to cell wall. GW modules interact with lipoteichoic acid of the cell wall and results in anchoring and surface exposure of proteins (51).

InlB, which has been described in detail in section 1.4.1 is the most studied GW protein in *L. monocytogenes*. GW modules in InlB non-covalently link the protein with lipoteichoic acid (LTA), a membrane-anchored polymer present on the surface of Gram-positive bacteria (4). Also, GW residues interact with glycosaminoglycans on mammalian cells. It has been reported that Ami, surface associated proteins of *L. monocytogenes* relating to adhesion to eukaryotic cells, possesses eight GW modules. Compared to InlB,

the greater number of GW residues in Ami might cause stronger binding to bacterial cell surfaces (10). Seven other proteins in *L. monocytogenes* containing the GW motif were identified. Six of them, like Ami (Lmo2558), contain the amidase domain (Lmo1215, Lmo1216, Lmo2203, Lmo1521, Lmo2591, lmo1076). InlB is the only protein of this group that harbors both GW modules and an LRR domain (36). In *Staphylococcus*, GW modules enhances cell surface binding of several surface autolysins (eg, AtlC from *Staphylococcus caprae*, AtlE from *Staphylococcus epidermidis*, and Aas from *Staphylococcus saprophyticus*) (5).

1.4.2.2 P60-like proteins

The P60 (also known as Cwha or Iap) is a 60-kDa surface protein that is involved in the invasion of nonprofessional phagocytic cells (52). It also has murein hydrolase activity and thus has a role in cell division (10, 52). The P60 protein possesses two LysM domains, a SH3 domain (bacterial Src homology 3) and the C-terminal domain NLPC/P60. The LysM domain is present in many cell wall degrading enzymes and it mainly functions by anchoring to murein. The bacterial SH3 domain (SH3b) is homologous to eukaryotic SH3 domains. It is also found in P60-like proteins in other *Listeria* species (49) as well as in other bacteria, such as *Bacillus subtilis*, *Escherichia coli*, *Chlamydia trachomatis*, *Haemophilus influenzae*, *Helicobacter pylori*, *Staphylococcus aureus* and *Streptococcus pyogenes* (10). However, the function of SH3 domain still remains unclear.

The NLPC/P60 domain contains 100-110 amino acids. It was first characterized in *Listeria* P60 and in the *E. coli* lipoprotein precursor NlpC. The function of this domain is still unknown but it has been found in several other lipoproteins and bacterial surface

proteins. Three other proteins in *L. monocytogenes* were found to contain NLPC/P60 domain. One of them is P45 which possesses murein hydrolyzing activity. The other two proteins with unclear function are Lmo0394 and Lmo1104 (10, 43).

1.4.2.3 Proteins with hydrophobic tail

Eleven proteins of *L. monocytogenes* contain a carboxyl terminus consisting of a hydrophobic domain, followed by positively charged amino acids. This tail serves to attach the proteins to the bacterial cell surface. Among these proteins, ActA is the best-known protein which is responsible for actin-based bacterial motility (14). The protein ActA consists of three functional regions which are the N-terminal region, a central proline-rich region, and a C-terminal region. ActA is anchored to the bacterial cell surface by C-terminal region. N-terminal region and the proline-rich repeat region of ActA are responsible for actin polymerization and movement of *L. monocytogenes* (46). Apart from ActA, other 9 proteins of *L. monocytogenes* were found to contain C-terminal hydrophobic region. These proteins are (Lmo0058, Lmo0082, Lmo0528, Lmo0552, Lmo0586, Lmo0701, Lmo0821, Lmo2061 and Lmo2186) (36).

1.4.3 Proteins linked to cell wall structures via their amino-terminal region (lipoproteins)

Bacterial lipoproteins are characterized by a specific signal peptide. The lipoprotein signal peptides are usually shorter than classical signal peptides. They have more hydrophobic amino acids in their central region, which are followed by cysteine residues. Lipoproteins are synthesized in the form of a prolipoprotein. They are then cleaved by a lipoprotein-specific peptidase (prolipoprotein peptidase or peptidase II), to

produce mature lipoproteins which are anchored to cytoplasmic membrane by their fatty acids (36).

Bacterial lipoproteins were found to be efficient proinflammatory molecules that initiate both the innate and adaptive immune response in mammals. *L. monocytogenes* contains 68 genes (2.5% of all *L. monocytogenes* genes) which is the highest number of genes coding lipoproteins compared to other gram-positive bacteria. A minority of *L. monocytogenes* lipoproteins have been studied. One of them is TcsA which is presented by MHC class II molecules and mediate CD4⁺ T-cell activation. Lipoproteins Lmo1847 and Lmo1800 are found to participate in host-pathogen interactions. The function of Lmo1847 remains unclear while Lmo1800 might function as tyrosine phosphatase (10, 36).

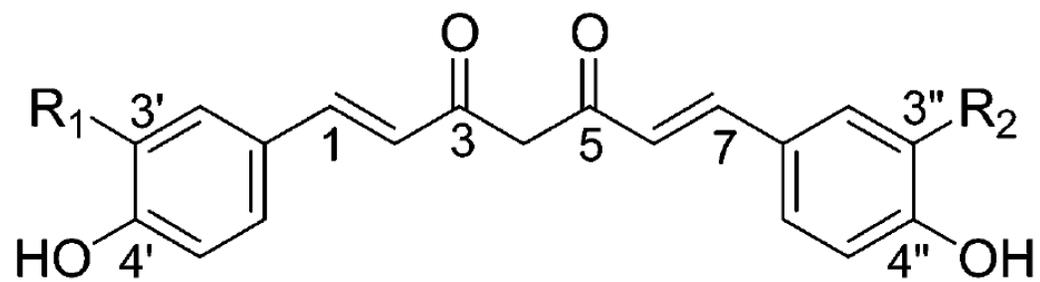
1.5 Curcumin

Curcumin (*Curcuma longa*) is a polyphenolic compound which is a member of the ginger family (Zingiberaceae). It has been used as a yellow coloring agent and spice in foods. It has also been used as an essential ingredient in medicine as a carminative, anthelmintic, laxative and as a cure for liver ailments. The use of turmeric as an insect repellent has also been known (9, 29). Curcumin has been reported to have a wide spectrum of biological actions such as anti-inflammatory, antioxidant, anticancer, antidiabetic, antiallergic, antiviral, antiprotozoal and antifungal activities. Furthermore, antibacterial activity of curcumin has widely been reported (41). The mechanism of action of phenolic compounds is involved in the interaction of their hydroxyl groups with the cell membrane resulting in cell leakage, alteration of fatty acids and

phospholipid profiles and a damage of the energy metabolism and synthesis of genetic materials (15).

Besides the biological effects of curcumin described above, curcumin also possesses antibiofilm and anti-sortase activities. Pattiyathane *et al* (2009). reported that sub-inhibitory concentrations of curcumin inhibited the biofilm formation of *Helicobacter pylori* in a dose dependent manner. However, *H. pylori* could restore biofilm forming ability during a prolonged incubation period (34). Park *et al.* (2005) used curcuminoid compounds (curcumin, demethoxycurcumin and bisdemethoxycurcumin) from dried rhizomes of *C. longa* to inhibit sortase A of *Staphylococcus aureus* ATCC6538P. The result showed that curcumin ($IC_{50} = 13.8 \pm 0.7 \mu\text{g/ml}$) could inhibit sortase A more efficiently than demethoxycurcumin ($IC_{50} = 23.8 \pm 0.6 \mu\text{g/ml}$) and bisdemethoxycurcumin ($IC_{50} = 31.9 \pm 1.2 \mu\text{g/ml}$) (9). This result suggested that curcumin can be used as a potent sortase A inhibitor.

Commercially available curcumin consists of a mixture of three curcuminoids, namely curcumin, demethoxycurcumin, and bisdemethoxycurcumin (Figure 1.1).



	R ₁	R ₂
curcumin (1)	OMe	OMe
demethoxycurcumin (2)	OMe	H
bisdemethoxycurcumin (3)	H	H

Figure 1.1: Structures of curcuminoid compounds

CHAPTER 2

OBJECTIVES

The general objective of this research is to observe the effect of curcumin (*Curcuma longa*) on biofilm formation and surface proteins of *Listeria monocytogenes*.

Objective 1: Study the effect of curcumin against biofilm formation of *Listeria monocytogenes* by the microtiter plate assay

1.1 Study the effect of curcumin against biofilm formation of *L. monocytogenes* LM21 (wild type) and s22-11G (sortase A defective mutant)

1.2 Study the effect of curcumin from two different manufacturers against biofilm formation of *L. monocytogenes* LM21

Objective 2: Study the effect of curcumin on surface proteins of *Listeria monocytogenes* by performing two-dimensional gel electrophoresis (2D-GE) and analyzing protein spots by Biorad's PDQuest™ software

CHAPTER 3

MICROTITER PLATE ASSAY FOR ASSESSMENT OF THE EFFECT OF CURCUMIN (*CURCUMA LONGA*) AGAINST BIOFILM FORMATION OF *LISTERIA MONOCYTOGENES* AND ANALYSIS OF CURCUMIN FROM DIFFERENT MANUFACTURERS BY REVERSED-PHASE HPLC

3.1 Introduction

L. monocytogenes is a ubiquitous food borne pathogen that has an ability to produce biofilms in food processing environments (50). It was reported that *L. monocytogenes* attached more strongly to polymers compared to other microorganisms on the surface. Also, attachment strength of most *L. monocytogenes* strains on polymers was higher than on stainless steel (13, 19).

PVC microtiter plate assay is a rapid and simple method to screen differences in biofilm formation between strains or growth conditions prior to performing labor-intensive analyses (13). In this experiment, growth and biofilm formation of *L. monocytogenes* LM21 and s22-11G with and without curcumin (*Curcuma longa*) were assessed. *L. monocytogenes* s22-11G is a sortase A defective mutant which was generated by insertion of mariner-based transposon, pMC38, to lmo0929 gene that has the similar function to the sortase gene (11).

Reversed phase HPLC is a simple, precise and accurate method that uses a non-polar stationary phase and polar mobile phase. Adding more organic solvent will lead to decreased mobile phase polarity. This will reduce hydrophobic interaction between the mobile phase and stationary phase and result in desorption.

This experiment also aimed at determining the differences of curcumin from different manufacturers by performing reversed phase HPLC. Due to the very labile characteristics of curcuminoid compounds, a C18 column was used in this research (23).

3.2 Material and methods

3.2.1 Culture preparation

Listeria monocytogenes LM21 and s22-11G (sortase A defective mutant) were stored in trypticase soy broth-0.6% yeast extract (TSBYE) with 12.5% glycerol at -75°C. Monthly, the working cultures were transferred on TSAYE slants (Difco, Detroit, MI.) and incubated at 32°C for 24 hours. The working cultures were stored at 4°C for 30 days. Prior to every experiment, a loopful of cultures from the slants were transferred to 10 ml of TSBYE and were incubated at 32°C for 18 hours. Erythromycin was added to the growth of *Listeria monocytogenes* s22-11G culture to a final concentration of 10 µg/ml.

3.2.2 Microtiter plate preparation

Before each assay, the 96-well PVC microtiter plates and lids (Becton Dickinson Labware, Franklin Lakes, N.J.) were soaked in 70% ethanol were air dried in a biological safety cabinet overnight.

3.2.3 Curcumin solution

The curcumin powder from Bepfarm Ltd. (Shanghai, China) (kindly provided by Dr. Hang Xiao) and from Acros Organics (New Jersey, USA) were separately dissolved in DMSO (Sigma-Aldrich, St. Louis, MO) to make a stock solution of 25.6 mg/ml

(0.069M).

3.2.4 Microtiter plate assay for assessment of curcumin effect

After 18 hours, 0.1 ml of growth of each strain in TSBYE was transferred into 10 ml of a minimally defined media, MWB. 0.1 ml of growth in TSBYE was also transferred into 10 ml of MWB supplemented with 256 µg/ml curcumin and then vortexed. After vortexing, 100 µl of both inoculation mixtures were transferred into eight microtiter plate wells and curcumin was diluted to final concentration of 128, 64, 32, 16, 8, 4, 2 and 1 µg/ml. Cells numbers of *L. monocytogenes* at each curcumin concentration were the same (approximately 10^7 CFU/ml). The control was made in new plates including curcumin solution diluted with MWB (without *L. monocytogenes*) to the final concentration ranging from 128 to 1 µg/ml. Each plate also included sixteen wells of *L. monocytogenes* without curcumin and sixteen wells of MWB. Plates were covered with lids and incubated at 32°C for 48 hours.

After 48 hours, growth was mixed by pipette and the cell turbidity was measured at an optical density of 570 nm (OD₅₇₀) using Bio-TEK[®] ELX800 microtiter plate reader (Biotek Instruments, Winooski, VT). The average OD of the control plates were subtracted from the sample plates. Growth and medium were removed from microtiter plate wells and the wells were washed three times with sterile distilled water to removed loosely attached cells. Plates were allowed to dry for 2 hours at 55°C and each well was stained with 150 µl of 0.1% v/v crystal violet for 30 minutes at 32°C. After staining, each well was washed with sterile distilled water five times and was destained with 190 µl of 95% ethanol for 1 hour. 150 µl from each well was transferred to new plates and

absorbance was measured at 570 nm.

3.2.5 Microtiter plate assay for assessment of DMSO effect

The microtiter plate assay was also performed to observe the effect of DMSO on growth and biofilm formation of *L. monocytogenes*. 18-hour growth of *L. monocytogenes* LM21 in TSBYE was transferred (0.1 ml) to 10 ml of MWB, a minimal defined media, and 10 ml of MWB supplemented with 1% (v/v) DMSO and vortexed. After vortexing, 100 µl of both inoculation mixtures were transferred into eight microtiter plate wells and DMSO was diluted to final concentration of 0.50%, 0.25%, 0.125%, 0.0625%, 0.0313%, 0.0156%, 0.0078% and 0.0039% v/v. Cells numbers of *L. monocytogenes* at each curcumin concentration were the same (approximately 10^7 CFU/ml). The control was made in new plates including DMSO diluted with MWB (without *L. monocytogenes*) to the final concentration ranging from 0.50% to 0.0039% v/v. Each plate also included sixteen wells of *L. monocytogenes* without curcumin and sixteen wells of MWB. Plates were covered with lids and incubated at 32°C for 48 hours.

After 48 hours, growth was mixed by pipette and the cell turbidity was measured by a microtiter plate reader at an optical density at 570 nm (OD₅₇₀). The average OD of the control plates was subtracted from the sample plates. Growth and medium were removed from microtiter plate wells and the wells were washed three times with sterile distilled water to remove loosely attached cells. Plates were allowed to dry for 2 hours at 65°C and each well was stained with 150 µl of 0.1% v/v crystal violet for 30 minutes at 32°C. After staining, each well was washed with sterile distilled water five times and was destained with 190 µl of 95% ethanol for 1 hour. 150 µl from each well was transferred to

new plates and absorbance was measured at 570 nm.

3.2.6 Sample Preparation for reversed-phase HPLC

Curcumin powder from Bepharma Ltd. (~95.2% pure, from *Curcuma longa*) and Acros Organics (98+% mixture of curcumin, demethoxycurcumin and bisdemethoxycurcumin) were used. 18.6 mg of curcumin powder from each company was dissolved in 1 ml of DMSO and was then 500-fold diluted by methanol. Each sample was analyzed by CoulArray® Multi-Channel EC detector model 6210 (Waters, Milford, MA, USA) in triplicates.

3.2.7 Mobile Phase Condition

A: 75% water, 20% acetonitrile, 5% THF and 50 mM ammonium acetate

B: 50% water, 40% acetonitrile, 10% THF and 50 mM ammonium acetate (The pH values of both mobile phases were adjusted to 3.0 using TFA).

3.2.8 Elution Condition

The solvent gradient consisted of 10% mobile phase B at 0 min, 50% mobile phase B at 5 min, 70% mobile phase B at 15 min, 100% mobile phase B at 25 min, and 100% mobile phase B at 35 min. The EC detector cell was set at the detecting potentials of 100, 200, 300, 400, 500, 600 and 700 mV separately. Flow rate and injection volume were set to 1 ml/min and 10 µl, respectively.

3.2.9 Data analysis

All experiments were repeated 3 times. The data were collected and the mean OD and standard deviation were calculated. In the comparison of the effect of curcumin on *L. monocytogenes* LM21 and s22-11G, the normalized growth and biofilm OD treated at the same curcumin concentration were compared by 2-tailed, paired T-test. In the comparison of the effect of curcumin from Bepharm and Acros company, the normalized growth and biofilm OD from both curcumin at the same concentration were compared by 2-tailed, paired T-test. Also, in the assay for the effect of DMSO, the normalized growth and biofilm OD at each DMSO concentration was compared with growth and biofilm OD without DMSO. The results were considered significantly different when P-values were lower than 0.05 ($P < 0.05$).

3.3 Results and Discussion

In the microtiter plate assay for assessment of the curcumin effect, 1% (v/v) of *L. monocytogenes* LM21 and s22-11G were treated with Bepharm's curcumin at the concentration ranging from 1 to 32 $\mu\text{g/ml}$ and were incubated at 32°C for 48 hours. According to the results, a significant difference between the growth of strain LM21 and s22-11G was not observed at each curcumin concentration ($P > 0.05$) (Figure3.1). The MIC of curcumin against both strains of *L. monocytogenes* was 64 $\mu\text{g/ml}$ while the half maximal inhibitory concentration (IC_{50}) was 32 $\mu\text{g/ml}$ in this research.

According to the microtiter plate assay for assessment of curcumin effect against biofilm formation of *L. monocytogenes*, a significantly greater biofilm reduction of *L. monocytogenes* s22-11G compared to *L. monocytogenes* LM21 was observed ($P < 0.05$)

(Figure 3.2). In *L. monocytogenes* s22-11G, sortase A (SrtA), a transpeptidase that cleaves the LPXTG motif and catalyzes the covalent linkage of LPXTG surface proteins to the cell wall (5), was interrupted by a mariner-based transposon (10). According to Bierne *et al.*, the $\Delta srtA$ mutant of *L. monocytogenes* EGDe, in contrast to a $\Delta inlA$ mutant, lost the ability to colonize the liver and spleen after oral inoculation in mice suggesting that *srtA* is also required for the cell wall anchoring of other LPXTG proteins involving in infections (7). Thus, LPXTG-containing proteins in the strain s22-11G may not be covalently linked to the cell wall and led to reduced biofilm production compared to the strain LM21 (wild type). Guiton *et al.* (2005) reported that deletion of *srtA* encoding SrtA in *Enterococcus faecalis* led to a deficiency in biofilm production (20). This suggests that SrtA is involved in biofilm formation and also explains why the biofilm reduction of *L. monocytogenes* s22-11G was higher than that of *L. monocytogenes* LM21 when treated with each curcumin concentration. Since curcumin was reported to mainly inhibit SrtA which is defective in *L. monocytogenes* s22-11G (11, 32), the substantial reduction in biofilm formation of *L. monocytogenes* s22-11G treated with curcumin may be due to other mechanisms as well. One of them might be the inhibition of SrtB, a transamidase in gram-positive bacteria which involves in the attachment of a subset of proteins to the cell wall (6).

Since DMSO was used to dissolve curcumin in this research, the microtiter plate assay was also performed to observe the effect of DMSO against growth and biofilm formation of *L. monocytogenes* LM21. The result showed that although there was a slight reduction of growth and biofilm in the presence of DMSO, it was not a statistically significant effect ($P > 0.05$) against growth and biofilm formation of *L. monocytogenes*

LM21 (Figure 3.5 and 3.6). According to Jacob and Herschler (1986), DMSO at concentration of 30-50% (v/v) exerted a marked inhibitory effect on a wide range of bacteria and fungi (21). This also suggests that DMSO concentrations used in this experiment should not interfere with the curcumin effect.

In the microtiter plate assay for assessment of the curcumin effect from Bepfarm and Acros company, 1% (v/v) of *L. monocytogenes* LM21 was treated with curcumin from each manufacturer at the concentration ranging from 1 to 128 µg/ml and were incubated at 32°C for 48 hours. The results showed that the growth of *L. monocytogenes* LM21 treated with both curcumin were not statistically different ($P > 0.05$) (Figure3.3) and the MIC of both curcumin against strain LM21 was 64 µg/ml. Therefore, curcumin from Bepfarm and Acros company did not have significantly different effects on the growth of *L. monocytogenes* LM21.

According to the microtiter plate assay for assessment of curcumin from different manufacturers on biofilm formation of *L. monocytogenes*, biofilm reduction of strain LM21 treated with Bepfarm's curcumin was statistically higher than those treated with Acros's curcumin ($P < 0.05$) (Figure3.4). Therefore Bepfarm's curcumin possessed more significantly effective biofilm inhibitory activity on *L. monocytogenes* LM21 than Acros's curcumin.

Reversed-phase HPLC was also performed to observe the differences between curcumin from Bepfarm and Acros company. It was previously reported that commercially available curcumin consists of three curcuminoid compounds which are curcumin, bisdemethoxycurcumin and demethoxycurcumin (Figure3.7) (23-24).

From Figure 3.8, both Bepfarm's and Acros's curcumin generated three peaks at

the retention time of 23.2 min (peak 1), 26.1 min (peak 2) and 28.9 min (peak 3). In HPLC, the same retention time indicates the same type of compound and the peak height indicates the peak intensity of each compound. Since the stationary phase of reversed phase HPLC is non-polar, the non-polar compounds have a better affinity to the stationary phase and stay in the column longer than polar compounds. Thus the retention time of polar compounds is shorter than non-polar compounds. From the curcuminoid structures (Figure 3.7), curcumin contains two methoxy groups. Therefore, it is more non-polar than demethoxycurcumin and bisdemethoxycurcumin respectively. According to this, peak 1, 2 and 3 should represent bisdemethoxycurcumin, demethoxycurcumin and curcumin respectively.

According to the peak area calculation (from 100-700 mV) of Bepfarm's curcumin, area of peak 1 (bisdemethoxycurcumin), peak 2 (demethoxycurcumin) and peak 3 (curcumin) were 116.6 μC (50.6%), 34.1 μC (14.9%) and 78.4 μC (34.5%) respectively. For the peak area calculation of Acros's curcumin, area of peak 1 (bisdemethoxycurcumin) peak 2 (demethoxycurcumin) and peak 3 (curcumin) were 196.6 μC , (81.4%), 42.6 μC (17.7%) and 2.3 μC (0.9%) respectively. This indicated that bisdemethoxycurcumin was the major constituent of both curcumin. Bepfarm's curcumin contained higher amount of curcumin (34.5%) than Acros's curcumin (0.9%) and also had more efficient biofilm inhibitory effect on *L. monocytogenes* LM21. Therefore, curcumin might be the most effective curcuminoid compound in curcumin for biofilm inhibition.

Curcumin was reported to have antibiofilm activities. However, to our knowledge, there were no direct studies about the inhibitory effect of pure curcuminoid

compounds on bacterial biofilm production. Park *et al.* (2005) used curcuminoid compounds (curcumin, demethoxycurcumin and bisdemethoxycurcumin) from dried rhizomes of *C. longa* to inhibit SrtA and adhesion of *Staphylococcus aureus* ATCC6538P to fibronectin. The result showed that curcumin ($IC_{50} = 13.8 \pm 0.7 \mu\text{g/ml}$) could inhibit SrtA more efficiently than demethoxycurcumin ($IC_{50} = 23.8 \pm 0.6 \mu\text{g/ml}$) and bisdemethoxycurcumin ($IC_{50} = 31.9 \pm 1.2 \mu\text{g/ml}$). Also, a potent inhibitory effect of curcumin against fibronectin adhesion was observed (9). Guiton *et al.* (2009) reported that deletion of SrtA in *Enterococcus faecalis* led to deficiency in biofilm production (20). These studies might support the hypothesis that curcumin was the most potent curcuminoid compound that could inhibit *L. monocytogenes* biofilm by the mechanism of SrtA inhibition.

3.4 Conclusion

At the same sub-inhibitory concentration of curcumin, the growth of *L. monocytogenes* LM21 and s22-11G (SrtA defective mutant) were not statistically different. However, the greater biofilm reduction in the strain s22-11G was observed indicating that SrtA plays an important role in biofilm formation. DMSO (at the final concentration of 0.50% to 0.0039% v/v) which was used to dissolve curcumin did not exhibit statistically significant inhibitory effects against growth and biofilm of *L. monocytogenes*. The statistically different effect of curcumin from Acros and Bepfarm company against the growth of the strain LM21 was not observed. Nevertheless, Bepfarm had more efficient biofilm inhibitory activity. The reversed-phase HPLC analysis indicated that Bepfarm's curcumin contained higher amount of curcumin (1 of

the 3 curcuminoid compounds in curcumin) than Acros's curcumin. These data suggests that curcumin is the most effective compound for biofilm inhibition.

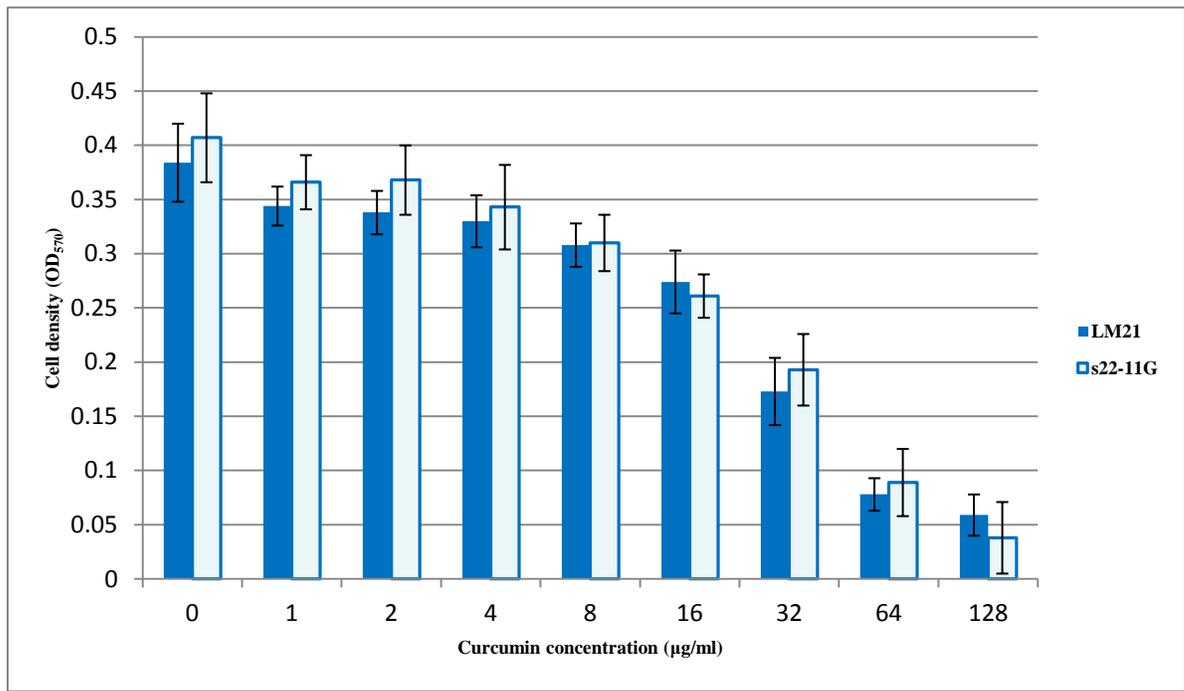


Figure 3.1: Cell density (measured at OD₅₇₀) of *L. monocytogenes* strain LM21 and s22-11G treated with each curcumin concentration and incubated at 32°C for 48 hr. *L. monocytogenes* LM21 is represented in dark blue and *L. monocytogenes* s22-11G is represented in light blue bar.

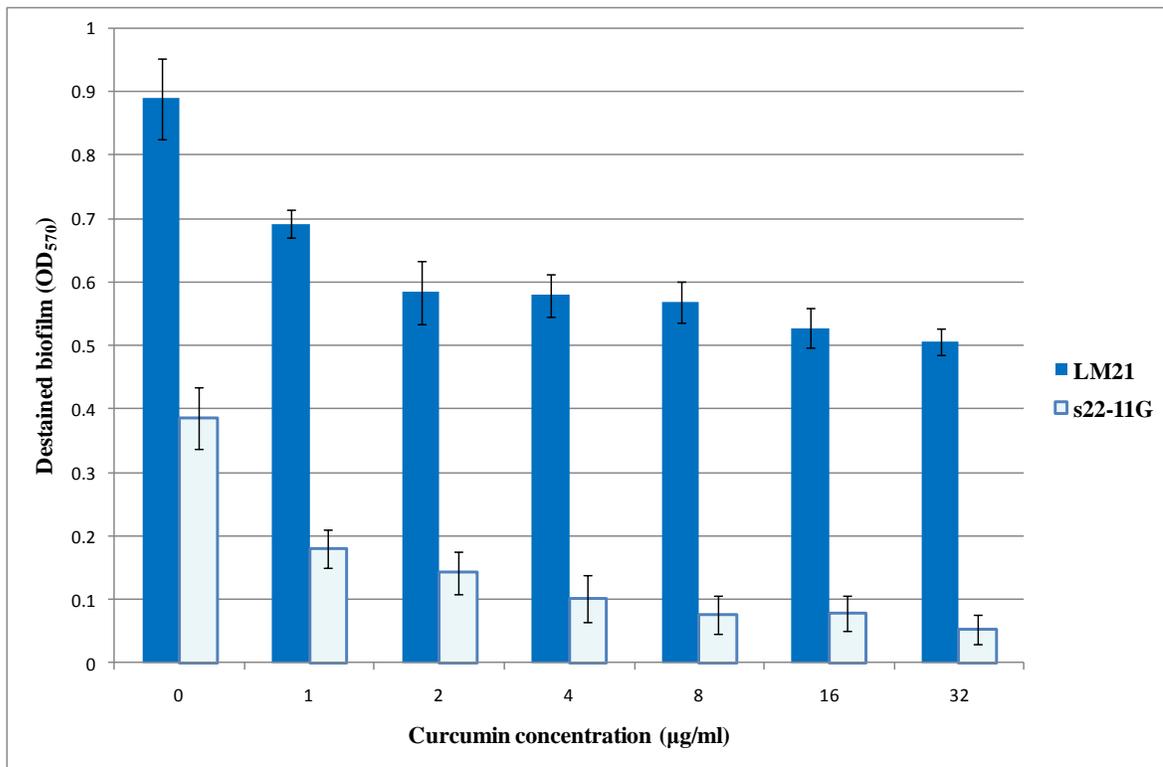


Figure 3.2: Destained biofilm (measured at OD₅₇₀) of *L. monocytogenes* strain LM21 and s22-11G treated with each curcumin concentration and incubated at 32°C for 48 hr. *L. monocytogenes* LM21 is represented in dark blue and *L. monocytogenes* s22-11G is represented in light blue bar.

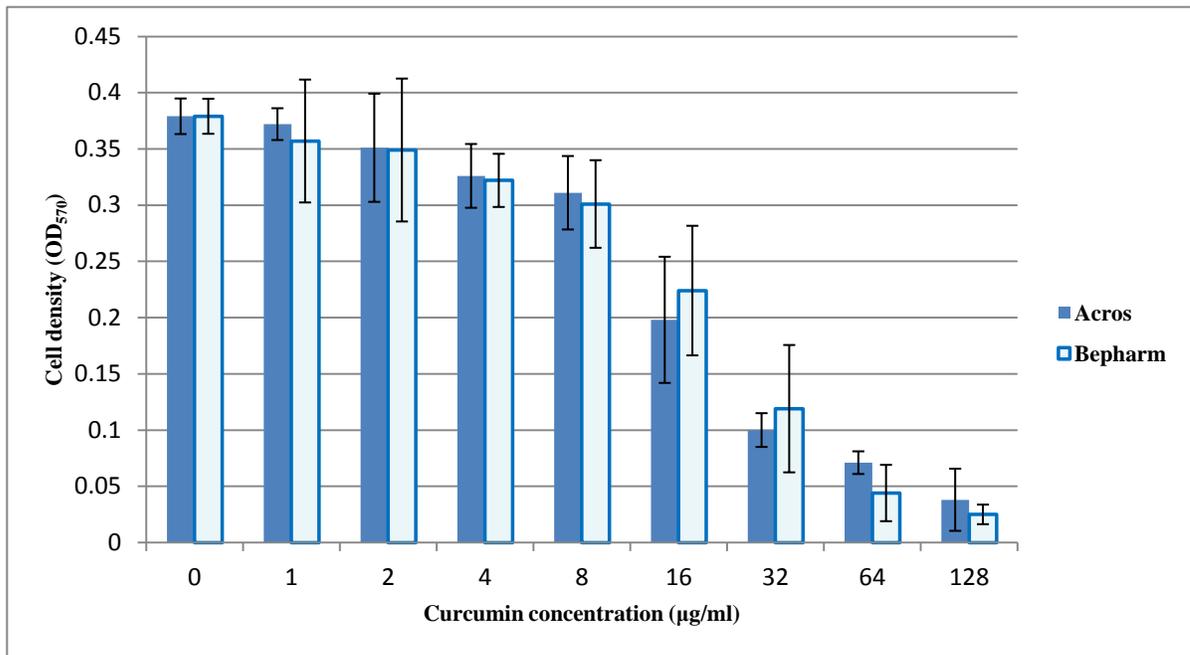


Figure 3.3: Cell density (measured at OD₅₇₀) of *L. monocytogenes* LM21 treated with Acros's and Bepharm's curcumin and incubated at 32°C for 48 hr. *L. monocytogenes* LM21 treated with Acros's curcumin is represented in dark blue and *L. monocytogenes* LM21 treated with Bepharm's curcumin is represented in light blue bar.

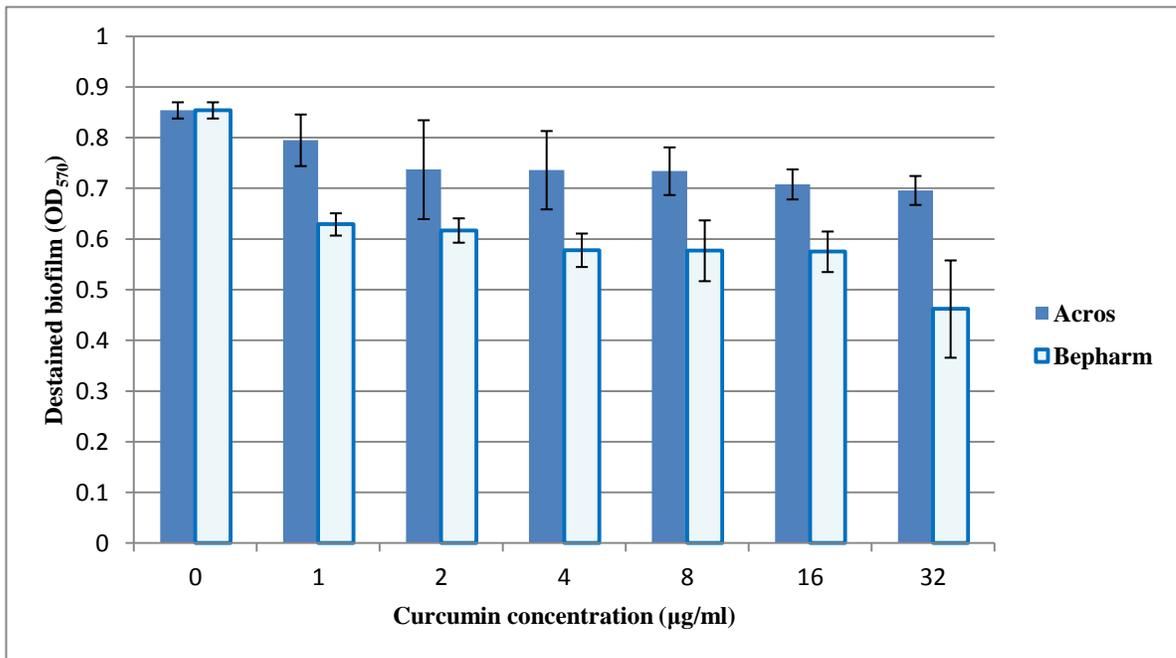


Figure 3.4: Destained biofilm (measured at OD₅₇₀) of *L. monocytogenes* LM21 treated with Acros's and Bepharm's curcumin and incubated at 32°C for 48 hr. *L. monocytogenes* LM21 treated with Acros's curcumin is represented in dark blue and *L. monocytogenes* LM21 treated with Bepharm's curcumin is represented in light blue bar.

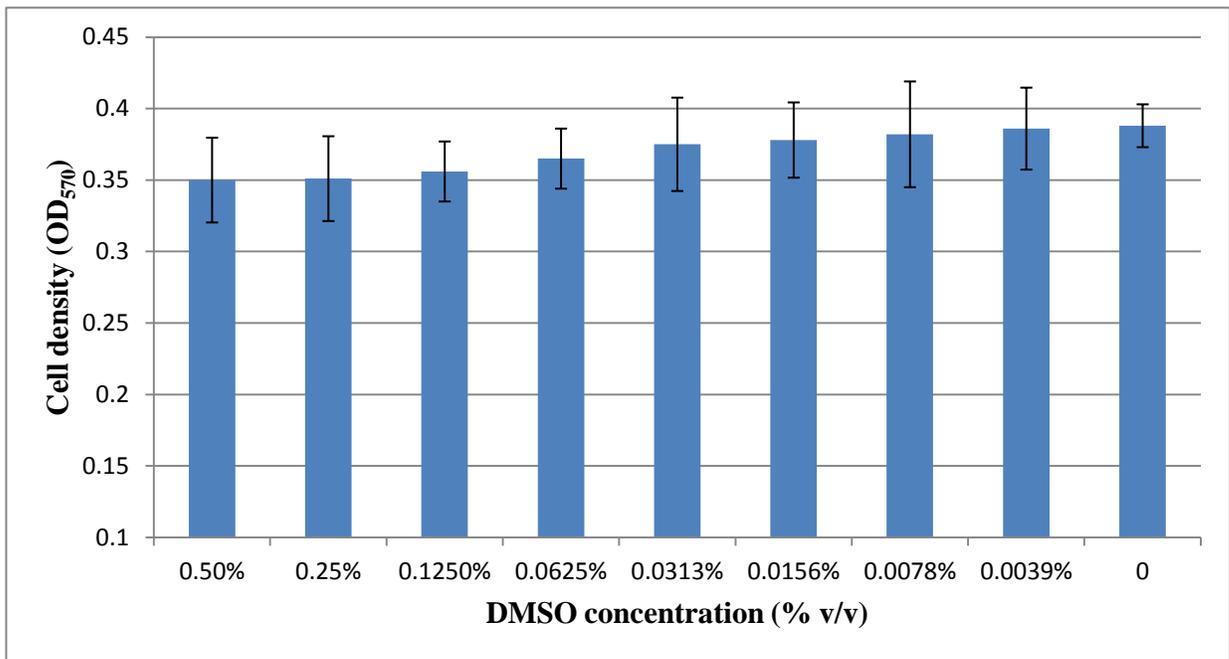


Figure 3.5: Cell density (measured at OD₅₇₀) of *L. monocytogenes* LM21 treated with each DMSO concentration and incubated at 32°C for 48 hr.

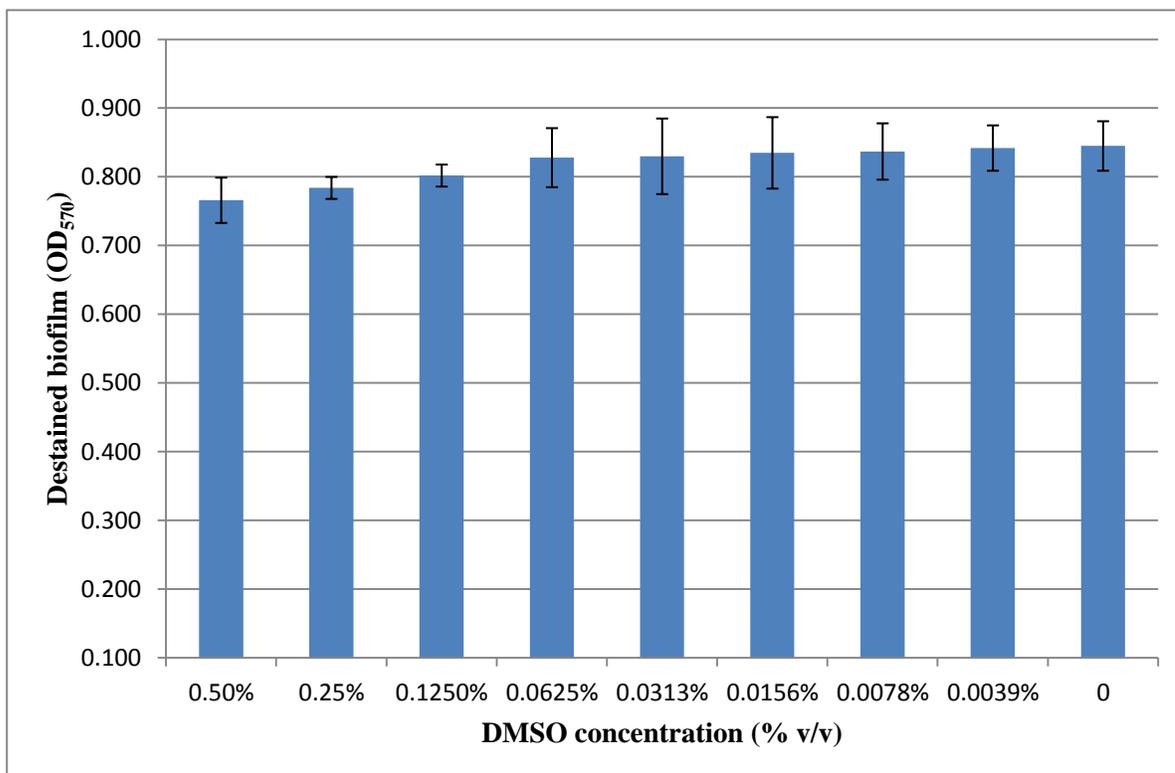
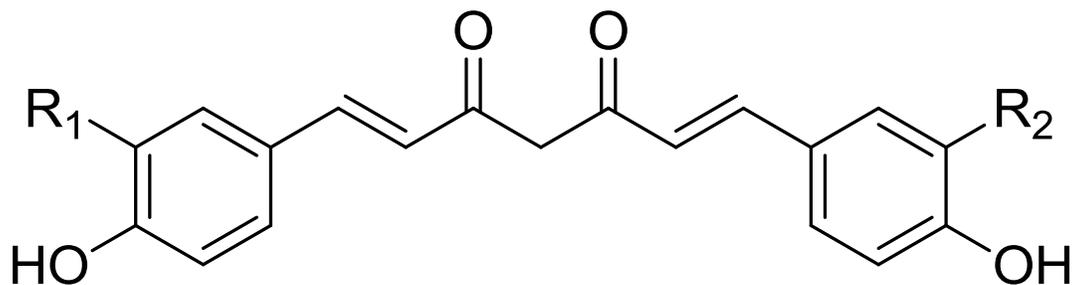


Figure 3.6: Destained biofilm (measured at OD₅₇₀) of *L. monocytogenes* LM21 treated with each DMSO concentration and incubated at 32°C for 48 hr.



Curcumin (**1**): R₁=R₂=OMe

Demethoxy-curcumin (**2**): R₁=H, R₂=OMe

Bisdemethoxy-curcumin (**3**): R₁=R₂=H

Figure 3.7: Structures of curcuminoid compounds

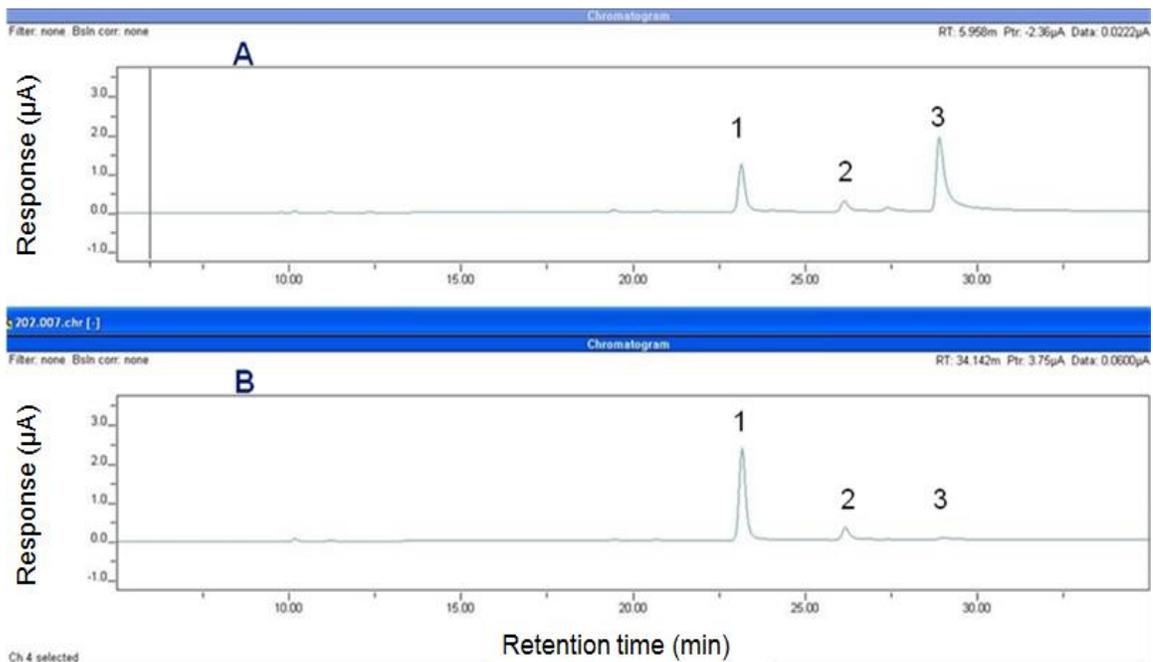


Figure 3.8: Graphs from reversed phase HPLC: Graph A = Bepharm's curcumin, Graph B = Acros's curcumin. Peaks from 400 mV response were chosen as a representative.

CHAPTER 4

ASSESSMENT OF THE EFFECT OF CURCUMIN ON SURFACE PROTEINS OF *LISTERIA MONOCYTOGENES* BY TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS (2D-GE)

4.1 Introduction

Two-dimensional polyacrylamide gel electrophoresis of bacterial proteins was introduced more than 25 years ago. This technique separates proteins based on pI (in the first dimension) and molecular weight (in the second dimension). In previous research, many studies have been done on responses of *L. monocytogenes* proteins to stresses including pH stress, high salinity, antimicrobials and temperature shocks. However, these analyses focused on total proteins or cellular proteins and rather than on surface proteins (18, 35, 37). Also, the response of *Listeria monocytogenes* to curcumin has never been studied using 2D-GE technique.

This experiment focused on the responses of surface proteins of *L. monocytogenes* LM21 to curcumin (*Curcuma longa*). The proteins of *L. monocytogenes* s22-11G, a sortase *mariner* transpon mutant were also studied and the protein spots were analyzed by the computer software.

4.2 Material and Methods

4.2.1 Culture preparation

Two strains of *Listeria monocytogenes* (LM21 and s22-11G) were stored in trypticase soy broth-0.6% yeast extract (TSBYE) with 12.5% glycerol at -75°C. Monthly,

the working cultures were transferred on TSAYE slants (Difco, Detroit, MI) and incubated at 32°C for 24 hours. The working cultures were stored at 4°C for 30 days. Prior to every experiment, a loopful of cultures from the slants were transferred to 10ml of TSBYE and were incubated at 32°C for 18 hours. Erythromycin was added to growth of *Listeria monocytogenes* s22-11G to a final concentration of 10 µg/ml.

4.2.2 Surface protein extraction

Three different protein extraction methods were performed as follows.

4.2.2.1 Method 1

The protein extraction was adapted from Mujahid *et al.* (2007) (28). Overnight cultures of two strains were grown in 400 ml of TSBYE at 37°C at 165 rpm until mid-exponential phase ($OD_{600} \sim 0.9$, the cell density $\sim 10^9$ CFU/ml). Cells were harvested by centrifugation at 2600 x g for 15 minutes and were washed twice with Tris-buffered sucrose (pH 7.0, 10 mM Tris, 250 mM sucrose). After washing, cells were resuspended in 60 ml of digestion buffer containing 20% sucrose in 20 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetic acid, 1 mM, pepstatin A, and 10 mM 1,10-phenanthroline), and 5000 U of mutanolysin. Enzymatic digestion was allowed to proceed for 2 hours at 37°C. The soluble surface proteins were separated from cell debris and intact protoplasts by centrifugation at 2900 x g for 1 hour. The supernatant containing solubilized proteins was centrifuged at 16,000 x g for 30 minutes to remove remaining cell debris and protoplasts. The protein solution was concentrated by Savant SpeedVac Concentrator (Thermo Fisher, Pittsburgh, PA) and the protein concentration was measured with the RC DC protein

assay kit (Bio-Rad, Hercules, CA)

4.2.2.2 Method 2

This protein extraction method was performed as described in method 1 (section 4.2.2.1) but the digestion buffer also included 10 mg/ml lysozyme (Sigma Chemical Co., St. Louis, MO). Also, prior to 2-hour incubation at 37 °C, the digestion buffer was subject to sonication (4 x 45 seconds on ice, at power level 5) by Microson (Misonic, Farmindale, NY)

4.2.2.3 Method 3

This extraction method was adapted from McLandsborough *et al.* (1995) (27). Overnight cultures of two strains were grown in 100 ml of TSBYE at 37°C and 170 rpm with a shaker until mid-exponential phase ($OD_{600} \sim 0.9$, the cell density $\sim 10^9$ CFU/ml). Cells were harvested by centrifugation at 10,000 rpm for 10 minutes and were washed twice with cold pH 5.2, 0.2 M sodium acetate buffer. After washing, the cells were resuspended in 0.5 ml of digestion buffer containing 20% sucrose, 1mM EDTA, pH 7.0, 200 U mutanolysin, 10 mM Tris and protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetic acid, 1 mM pepstatin A, and 10 mM 1,10-phenanthroline) (28). The digestion solution was incubated at 37°C for 2 hours. The soluble surface proteins were separated from cell debris and intact protoplast by centrifugation at 1300 x g for 5 minutes. Amicon Ultra-0.5 (MW cut off = 10 kDa) (Millipore, Bedford, MA) was used to concentrate and desalt the supernatant containing soluble cell surface proteins. The protein retentates were dissolved in IEF rehydration buffer containing 50 mM DTT, 0.2% 100 x Bio-Lyte 3/10 ampholyte and ASB-14: 7 M urea, 2 M thiourea, 1% ASB-14. Protein concentration was measured with the RC DC

protein assay kit (Bio-Rad, Hercules, CA)

4.2.2.4 Extraction of surface proteins of *L. monocytogenes* LM21 treated with curcumin

Curcumin powder (Bepharma Ltd., Shanghai, China) was dissolved in DMSO to prepare a stock solution of 102.4 mg/ml. Overnight cultures of *L. monocytogenes* LM21 was grown in 100 ml of TSBYE supplemented with 64 µg/ml curcumin (1/4 MIC in TSBYE) at 37°C at 165 rpm with a shaker until mid-exponential phase ($OD_{600} \sim 0.7$). Cells were suspended with pH5.2, 2M sodium acetate buffer to obtain $OD_{600} \sim 0.9$. Then, protein extraction was performed as described in section 4.2.2.3.

4.2.3 One dimensional sodium dodecyl sulfate gel electrophoresis (1D SDS-PAGE)

Prior to 2D-GE, 1D SDS-PAGE was performed to observe the presence of protein bands of *L. monocytogenes* LM21 and s22-11G from section 4.2.2.1, 4.2.2.2, 4.2.2.3. Proteins were diluted with Laemmli buffer (Bio-Rad, Hercules, CA) in the ratio of 1:1 and were then heated for 12 minutes. Proteins were loaded into the well of Any kD™ Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad, Hercules, CA), which the maximum volume of a well was 30 µl, and were run with Mini-PROTEAN electrophoresis cells (Bio-Rad, Hercules, CA) at 160 V. EZ-Run Pre-stained Rec Protein Ladder (Fisher Bioreagents, Pittsburgh, PA) containing proteins from 11 kDa to 170 kDa was used as a protein marker. Gels from all three methods were stained with Coomassie Brilliant Blue R-250 (Sigma Chemical Co., St. Louis, MO). Gels from method 3 were also silver stained with Silver Stain Plus Kit (Bio-Rad, Hercules, CA).

4.2.4 Two-dimensional gel electrophoresis

For IEF, approximately 60 µg of surface proteins of *L. monocytogenes* LM21 and s22-11G were loaded onto ReadyStrip™ IPG Strip (7 cm, pH 4-7) (Bio-Rad, Hercules, CA). The strips were rehydrated for 16 hours at 23 °C at 50 V. IEF was performed using a PROTEAN IEF Cell (Bio-Rad, Hercules, CA) as follows: 250 V for 15 min, followed by voltage ramping, linear mode, to 4000 V for 2 h, and final focusing at 4000 V for 20000 V-h. The current was limited to 50 mA per IPG strip, and the temperature was maintained at 23°C for all focusing steps.

To obtain a better resolution of the gel images, 11 cm IPG strips were also utilized. Approximately 90 µg of surface proteins from each treatment (*L. monocytogenes* LM21, *L. monocytogenes* s22-11G, *L. monocytogenes* with 64 µg/ml curcumin) were loaded onto ReadyStrip™ IPG Strip (11 cm, pH 4-7) (Bio-Rad, Hercules, CA). The strips were rehydrated for 16 hours at 23 °C at 50 V. With PROTEAN IEF Cell (Bio-Rad, Hercules, CA), IEF was conducted as follows: 250 V for 15 min, followed by voltage ramping, linear mode, to 8000 V for 2.5 h, and final focusing at 8000 V for 35000 V-h. The current limited to 50 mA per strip was applied, and the temperature at 23°C was maintained for all focusing steps.

The strips were stored at -80°C after focusing steps. Before performing the second dimension, strips were thawed and equilibrated with Equilibration Buffer I and II from ReadyPrep 2-D Starter Kit (Bio-Rad, Hercules, CA) for 20 minutes. The proteins from 7 cm strips and 11 cm strips were run with Any kD™ Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad, Hercules, CA) and Criterion™ TGX Any kD Stain-Free™ Precast Gel (Bio-Rad, Hercules, CA) respectively. EZ-Run Pre-stained Rec Protein Ladder (Fisher

Bioreagents, Pittsburgh, PA) was used as a marker for 11-cm IPG strip gel. The 7-cm IPG strip gels and 11-cm IPG strip gels were respectively run in Mini-PROTEAN® Tetra Cell and Criterion™ Cell at 160 V. Gels were stained with Silver Stain Plus Kit (Bio-Rad, Hercules, CA) and the gel images were taken with Kodak Image station 4000MM (Eastman Kodak, Rochester, NY, USA). Gels of each treatment were run in three replicates.

4.2.5 Gel Image Analysis

Two-dimensional gels prepared using 11-cm IPG strips were analyzed by PDQuest™ 2-D Analysis Software version 8.0.1 (Bio-Rad, Hercules, CA). Three gels of each experimental condition (LM21 surface proteins, LM21 + curcumin, and mutant s22-11G). Only protein spots that appeared consistently in three replicates were selected for comparison. Protein spots of *L. monocytogenes* LM21 supplemented with 64 µg/ml curcumin and *L. monocytogenes* s22-11G were compared with those of *L. monocytogenes* LM21 by student's T-test (significance level of 95%). The standard spot numbers (SSP number) were automatically assigned to the selected spots by the software.

4.3 Results and Discussion

Three methods of protein extraction from *L. monocytogenes* were compared. In the method 1, listerial cells grown in 400 ml TSBYE were spun down and suspended in 60 ml digestion buffer with 5000 U mutanolysin. Thus, final concentration of mutanolysin was 83.3 U/ml. Prior to concentration with SpeedVac Concentrator, protein concentration was too low to be measured ($OD_{750} < 0$). After concentration, the amount

of proteins was still relatively low and was not sufficient to be detected by Coomassie blue although the maximum amount of proteins that can be loaded in a 30 μ l well were used (10 μ g proteins of strain LM21 and 6.6 μ g proteins of strain s22-11G). Thus when run by 1D SDS-PAGE, no protein bands of either strain were observed (Figure4.1).

In the method 2, listerial cells grown in 400 ml TSBYE were spun down and suspended in 60 ml digestion buffer with 5000 U mutanolysin (final concentration = 83.3 U/ml) and 10 mg/ml lysozyme. Cells in digestion buffer were sonicated and were then incubated for 2 hours. Prior to concentration by SpeedVac Concentrator, protein concentrations of strain LM21 and s22-11G were about 11.2 mg/ml 10.9 mg/ml respectively (which included 10 mg/ml lysozyme). The Coomassie blue stained gel of unconcentrated proteins showed very light bands of *L. monocytogenes* LM21 and s22-11G and very dark bands of lysozyme (Figure4.2). In this experiment, the concentrated proteins could not be run by SDS-PAGE since proteins turned into insoluble aggregates during heat denaturation.

In the method 3, listerial cells grown in 100 ml TSBYE were spun down and suspended in 0.5 ml digestion buffer with 200 U mutanolysin (final concentration = 400 U/ml). Prior to concentration by Amicon Ultra-0.5, the protein concentrations of strain LM21 and s22-11G were about 800 μ g/ml 600 μ g/ml respectively. After concentration and desalting, proteins of strain LM21 were run by 1D SDS-PAGE and were stained with Coomassie blue. Bands were observed but were not really sharp (Figure4.3); thus, silver stain was also used to stain proteins of strain LM21 and s22-11G to obtain sharper bands. At the same protein concentrations, the band intensity of *L. monocytogenes* LM21 was darker than those of strain s22-11G (Figure4.4).

The protein extraction method 1 and 2 were performed based on Mujahid *et al.* (2007) (28) and did not seem to work in this research, and is likely due to the concentration of mutanolysin (83.3 U/ml) compared to the mutanolysin concentration in the method 3 (400 U/ml). Although the extraction method 2 also combined 10 mg/ml lysozyme and sonication, only 2-3 light bands of strain LM21 and s22-11G were observed (Figure 4.2). Thus 10 mg/ml lysozyme was not sufficient for the extraction either. In the method 3, the desalting column and the sufficiently high concentration of mutanolysin (400 U/ml) were used and gave the most desirable results. Despite the lower amount of mutanolysin (200 U) used in the method 3, the final concentration of mutanolysin (400 U/ml) was higher than those in the method 1 and 2 (83.3 U/ml). Mutanolysin, a 23 kDa muramidase from *Streptomyces globisporus* (42), is a very expensive enzyme. Thus, the method 3 not only gave the most desirable results but was also cost-effective. However, it might be unavoidable that other proteins besides surface proteins (e.g. cellular proteins) could be solubilized in the extraction solution although this method was optimized for surface protein extraction.

Initially, 7 cm gels with pH 4-7 IPG strip were used for 2-D gel analysis with 60 µg of proteins. The 7 cm gels of *L. monocytogenes* LM21 and s22-11G protein extracts are shown in Figure 4.5 and 4.6. Due to the small size of precast gels and IPG strips used, the protein spots were densely packed which made the image analysis more difficult. To obtain a better resolution of the gel images, 11 cm, pH 4-7 IPG strips were also utilized.

The larger, 11 cm, pH 4-7 IPG strip gels were loaded with 90 µg of proteins of *L. monocytogenes* LM21 (control), *L. monocytogenes* LM21 treated with 64 µg/ml of

curcumin and *L. monocytogenes* s22-11G (sortase mutant). Protein spots of *L. monocytogenes* LM21 with 64 µg/ml curcumin and *L. monocytogenes* s22-11G were compared with those of *L. monocytogenes* LM21 without curcumin. Compared to the protein spots of *L. monocytogenes* LM21 (Figure4.7), 5 proteins were up-regulated, 16 proteins were down-regulated and 4 proteins were absent in *L. monocytogenes* LM21 treated with 64 µg/ml curcumin (Table 4.1 and Figure4.8). One protein (SSP# 2005) was present in *L. monocytogenes* LM21 treated with 64 µg/ml curcumin but absent in *L. monocytogenes* LM21. Curcumin is a polyphenolic compound that can disrupt the cell membrane and cause leakage of cellular components, alteration of fatty acids and phospholipid profiles and damage of the energy metabolism and synthesis of genetic materials (15, 39). Thus 5 up-regulated proteins and 1 protein (SSP# 2005) that was only expressed in the presence of curcumin might function as stress proteins or virulence proteins that are necessary for survival of *L. monocytogenes* LM21 in the presence of a sub-lethal concentration of curcumin. One of them (SSP# 6702) may correspond to the protein encoded by lmo0355 (MW 54.43 kDa, pI 5.7) which is a surface protein of *L. monocytogenes* according to Mujahid *et al.* (28). The 16 down-regulated proteins may be due to the partial inhibitory effect of curcumin, so the use of lower sub-lethal concentration of curcumin may decrease the number of down-regulated proteins. Curcumin was also reported to have an ability to inhibit SrtA, a transpeptidase that is required for anchoring LPXTG-containing surface proteins to the cell wall of gram positive bacteria (7, 9). Thus some of the 16 down-regulated proteins may be SrtA and LPXTG-containing proteins. Besides SrtA and LPXTG proteins, other down-regulated proteins may not be necessary for survival of *L. monocytogenes*. From the 16 proteins,

protein SSP# 6501 had comparable molecular weight and pI (MW = 28.4 kDa, pI 5.42) to the underexpressed protein after salt stress (at 65 g/l NaCl) reported by Esvan *et al.* (16).

In *L. monocytogenes* s22-11G (Figure4.10) which is a SrtA defective mutant, 2 proteins were up-regulated, 8 proteins were down regulated and 3 proteins were absent respectively when compared to those of *L. monocytogenes* LM21 (Table 4.2 and Figure4.9). The three absent proteins may be the LPXTG-containing surface proteins that require SrtA for anchoring to the cell wall. The 8 down-regulated proteins suggested that *srtA* gene encoding SrtA may enhance in the expression of these proteins. Deletion of *srtA* led to 2 up-regulated proteins suggesting that *srtA* may be involved in repression of these proteins.

In this experiment, the protein spot identification was not performed. Thus the exact information (e.g. MW, pI, functions) could not be reported. The characteristics of the differentially expressed proteins discussed above still need to be confirmed by mass spectrometry (e.g. MALDI-TOF).

4.4 Conclusion

Three different methods were used to extract surface proteins of *L. monocytogenes*. The method 1 and 2 did not give desirable results due to the insufficient concentration of mutanolysin to lyse the cell wall. The method 3 gave the most desirable results since the final enzyme concentration was higher than the other 2 methods despite the lower amount of mutanolysin used. For 2D-GE, 11 cm IPG strips were also used to obtain a better image resolution compared to 7 cm IPG strips. Proteins of *L. monocytogenes* without curcumin, *L. monocytogenes* with curcumin and *L.*

monocytogenes s22-11G from the method 3 were run using 11 cm IPG strips and were analyzed by the computer software. 5 proteins, 16 proteins and 4 proteins were up-regulated, down-regulated and absent respectively in *L. monocytogenes* LM21 treated with curcumin while 2 proteins, 8 proteins and 3 proteins were up-regulated, down-regulated and absent respectively in *L. monocytogenes* s22-11G. To obtain the exact information about these differentially expressed proteins, protein identification by mass spectrometry is still required.

^a +, Proteins were up-regulated; -, proteins were down-regulated

Standard Spot Number (SSP Number)	Expression ^a
0104	+
0105	+
1402	+
2002	-
2005	absent in <i>L. monocytogenes</i> LM21
2103	-
3701	absent in <i>L. monocytogenes</i> LM21 treated with 64 µg/ml curcumin
4001	-
4302	absent in <i>L. monocytogenes</i> LM21 treated with 64 µg/ml curcumin
5402	-
5403	-
5501	-
5706	absent in <i>L. monocytogenes</i> LM21 treated with 64 µg/ml curcumin

Table 4.1: Selected proteins of *L. monocytogenes* LM21 treated with 64 µg/ml curcumin for comparison with proteins of untreated *L. monocytogenes* LM21 (run on 11 cm, pH 4-7 IPG strip).

^a +, Proteins were up-regulated; –, proteins were down-regulated

Standard Spot Number (SSP Number)	Expression ^a
5801	–
5805	absent in <i>L. monocytogenes</i> LM21 treated with 64 µg/ml curcumin
5905	–
6001	+
6501	–
6604	–
6702	+
7202	–
7204	–
7705	–
8102	–
8104	–
8208	–

Table 4.1: (continued)

^a +, Proteins were up-regulated; –, proteins were down-regulated

Standard Spot Number (SSP Number)	Expression ^a
0105	+
1005	+
2502	–
3201	–
3402	–
3701	–
3705	absent in <i>L. monocytogenes</i> s22-11G
5501	–
5603	–
7101	absent in <i>L. monocytogenes</i> s22-11G
7204	–
7205	absent in <i>L. monocytogenes</i> s22-11G
8102	–

Table 4.2: Selected proteins of *L. monocytogenes* s22-11G for comparison with proteins of *L. monocytogenes* LM21 (run on 11 cm, pH 4-7 IPG strip)

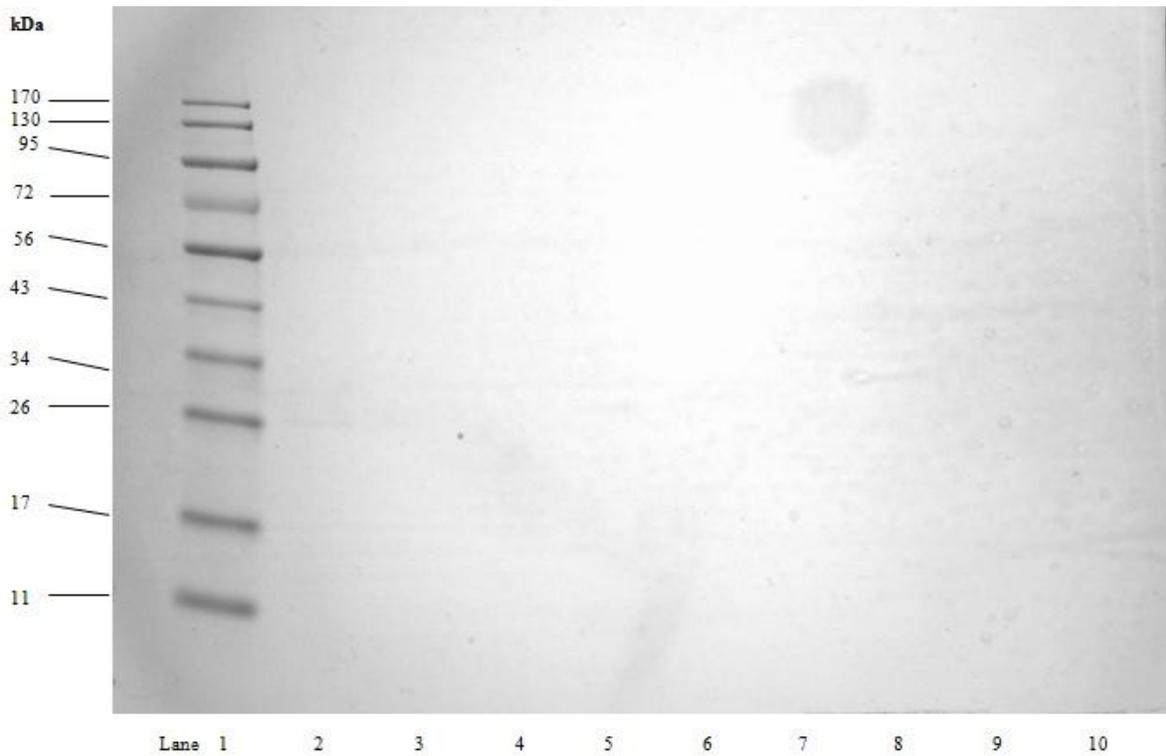


Figure 4.1: Coomassie blue-stained gel of proteins extracted by method 1. Lane 1: protein marker, Lane 2-6: 10 µg of proteins of *L. monocytogenes* LM21, Lane 7-10: 6.6 µg of proteins of *L. monocytogenes* s22-11G.

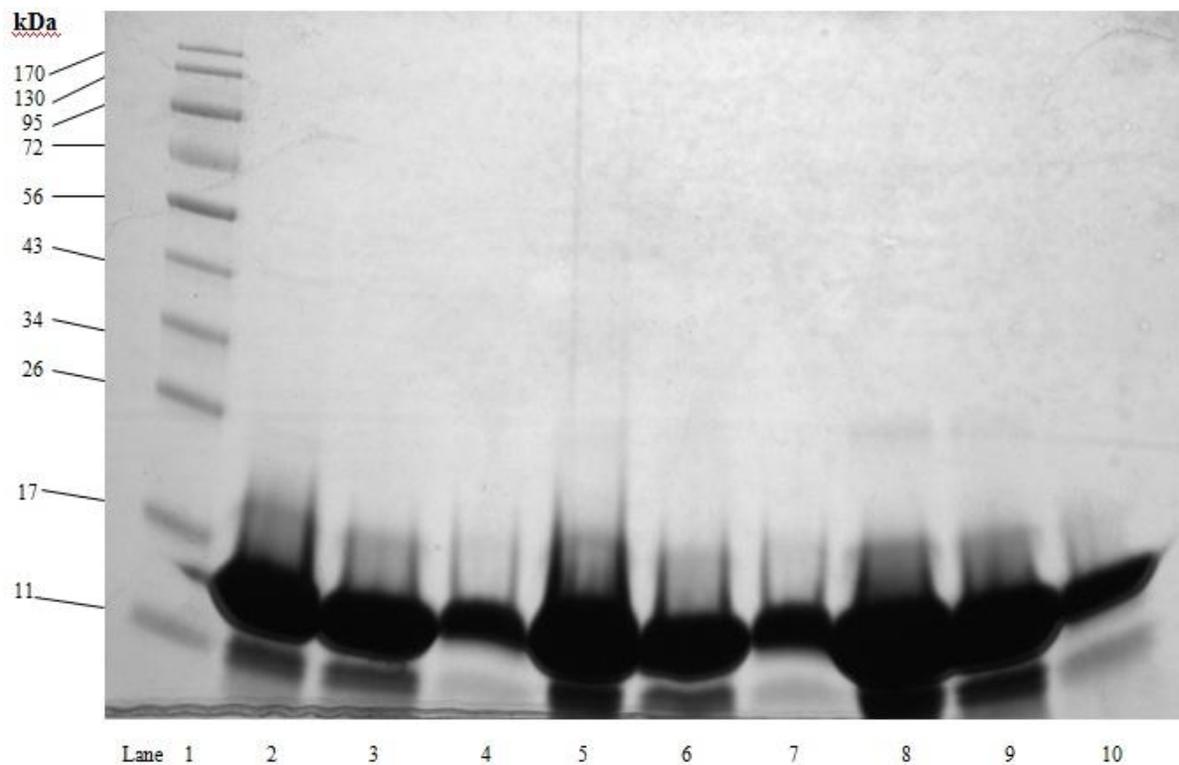


Figure 4.2: Coomassie blue-stained gel of proteins extracted by method 2. Lane 1: protein marker, Lane 2: 168 µg of proteins of *L. monocytogenes* LM21 and lysozyme, Lane 3: 84 µg of proteins of *L. monocytogenes* LM21 and lysozyme, Lane 4: 16.8 µg of proteins of *L. monocytogenes* LM21 and lysozyme, Lane 5: 163.5 µg of proteins of *L. monocytogenes* s22-11G and lysozyme, Lane 6: 81.75 µg of proteins of *L. monocytogenes* s22-11G and lysozyme, Lane 7: 16.35 µg of proteins of *L. monocytogenes* s22-11G and lysozyme. Lane 8: 150 µg of lysozyme, Lane 9: 75 µg of lysozyme, Lane 10: 15 µg of lysozyme

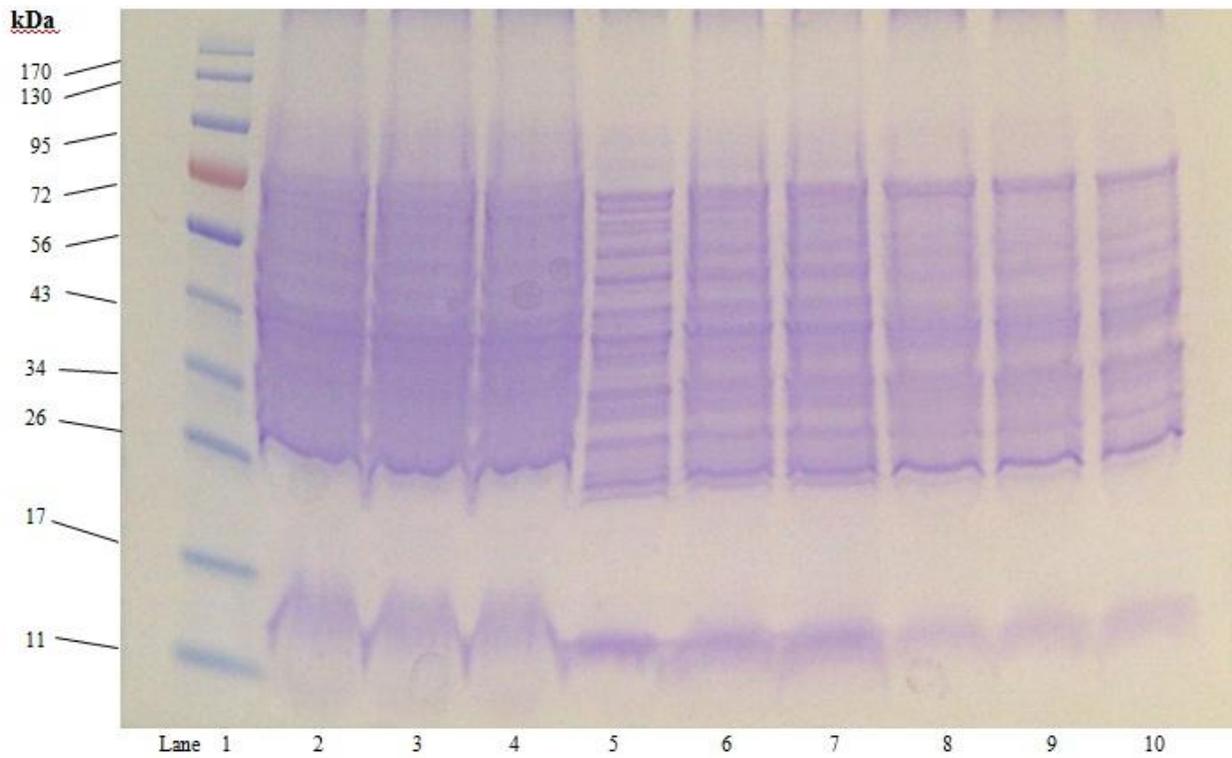


Figure 4.3: Coomassie blue-stained gel of proteins extracted by method 3. Lane 1: protein marker, Lane 2-4: 60 µg of proteins of *L. monocytogenes* LM21, Lane 5-7: 30 µg of proteins of *L. monocytogenes* LM21, Lane 8-10: 21.75 µg of proteins of *L. monocytogenes* LM21

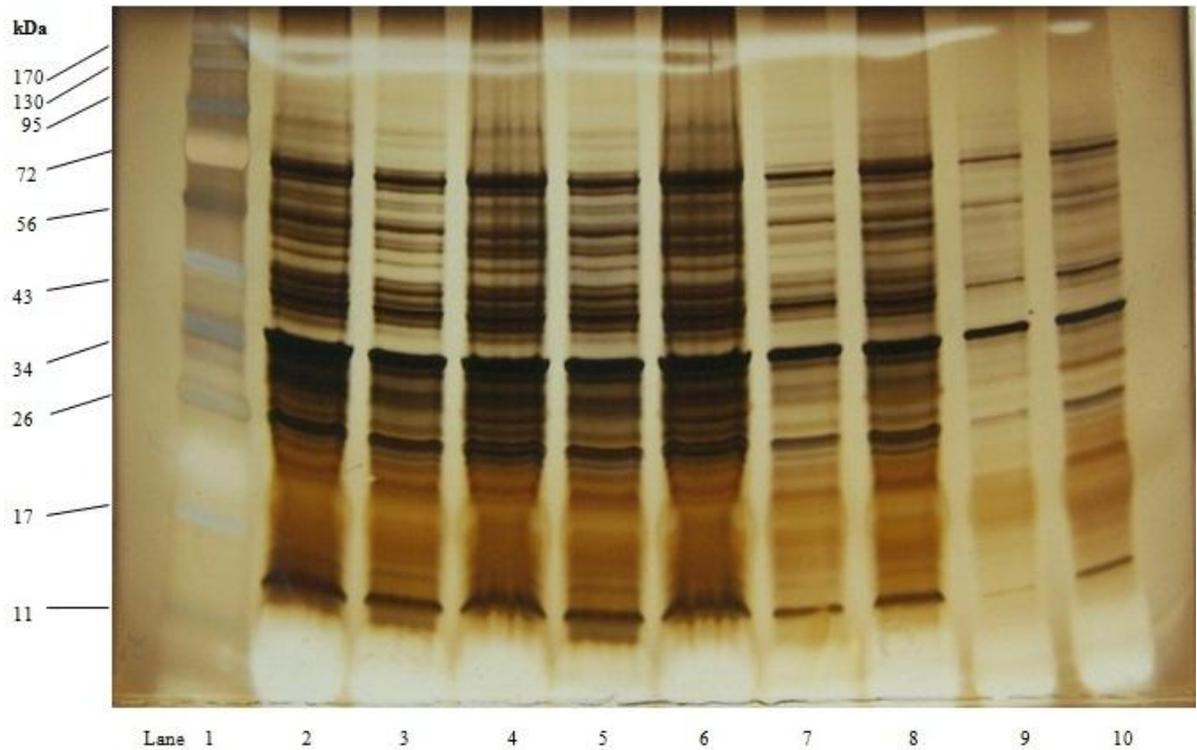


Figure 4.4: Silver-stained gel of proteins extracted by method 3. Lane 1: protein marker, Lane 2: 10 µg of proteins of *L. monocytogenes* LM21, Lane 3: 6 µg of proteins of *L. monocytogenes* s22-11G, Lane 4: 6 µg of proteins of *L. monocytogenes* LM21, Lane 5: 6µg of proteins of *L. monocytogenes* s22-11G, Lane 6: 6 µg of proteins of *L. monocytogenes* LM21, Lane 7: 3 µg of proteins of *L. monocytogenes* s22-11G, Lane 8: 3 µg of proteins of *L. monocytogenes* LM21, Lane 9: 1 µg of proteins of *L. monocytogenes* s22-11G, Lane 10: 1 µg of proteins of *L. monocytogenes* LM21

4

pH

7

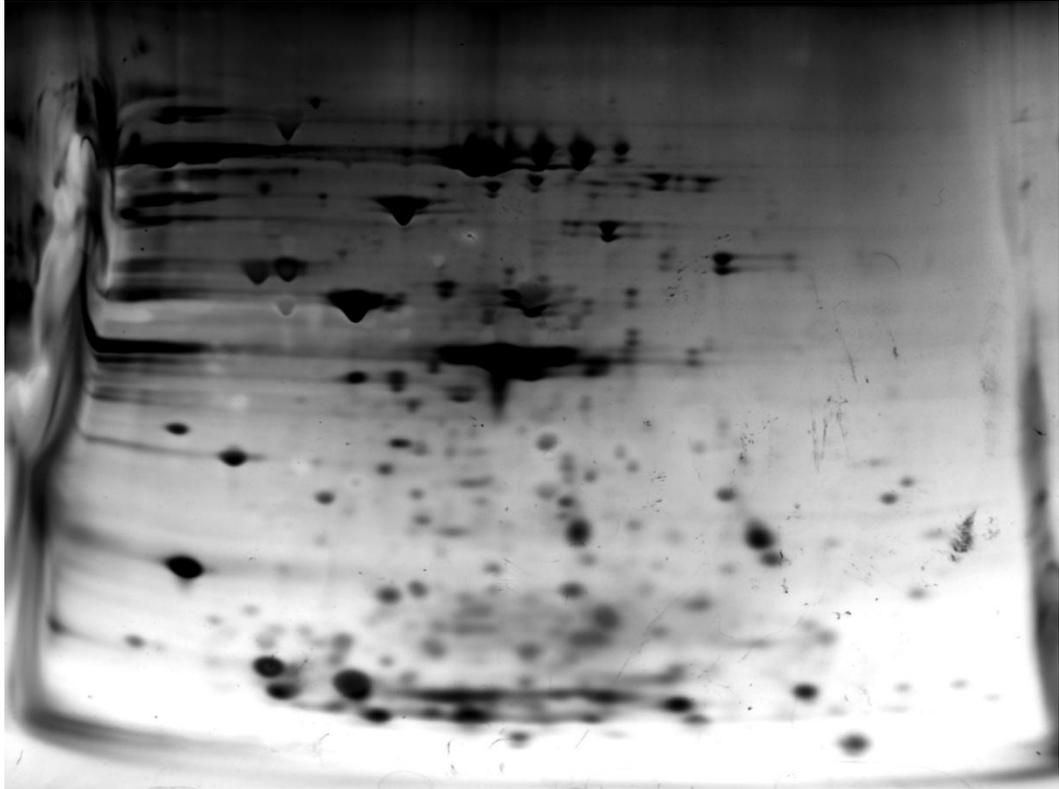


Figure 4.6: 2D-GE image of approximately 60 μg of proteins of *L. monocytogenes* s22-11G separated on pH 4-7, 7 cm IPG strip

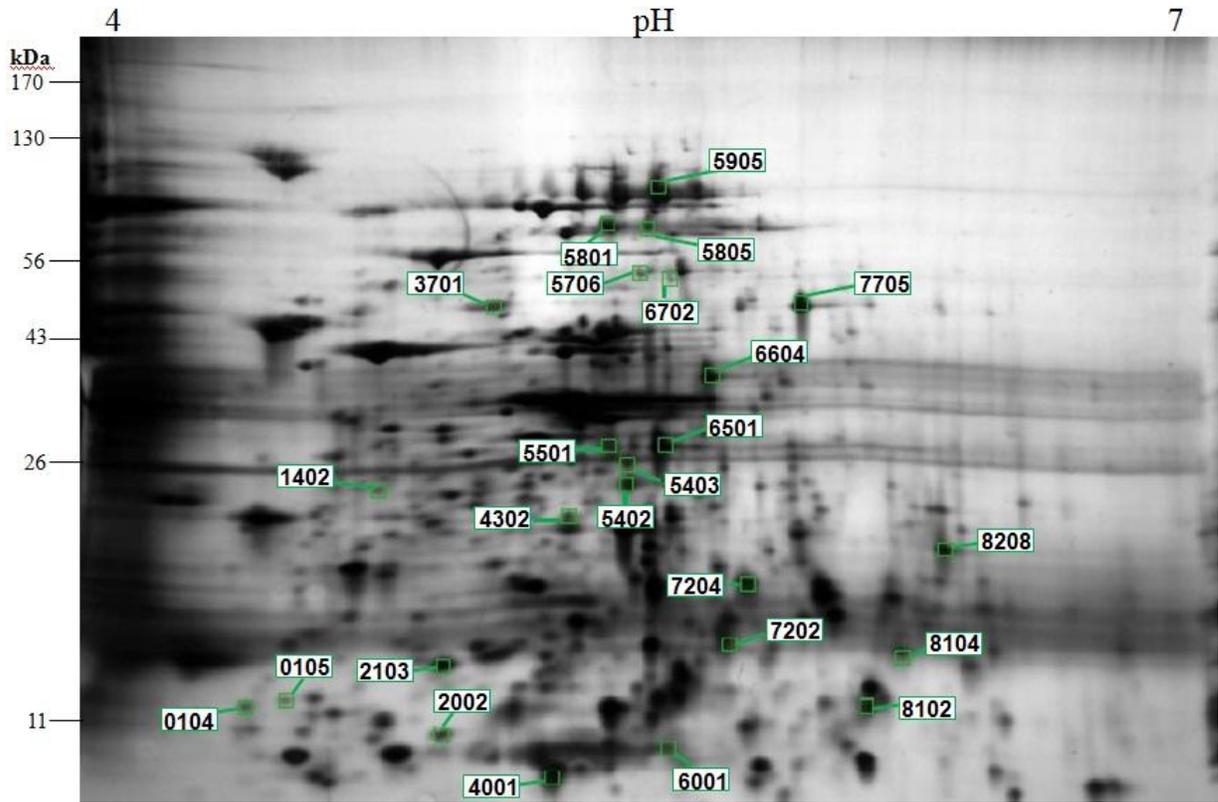


Figure 4.7: 2D-GE image of approximately 90 μ g of proteins of *L. monocytogenes* LM21 separated on pH 4-7, 11 cm IPG strip. The boxed spots represent spots that were chosen by the software when compared by student's T-test. The standard spot numbers (SSP number) were automatically assigned to the selected protein spots for comparison with proteins of *L. monocytogenes* LM21 treated with 64 μ g/ml curcumin (Figure 4.8)

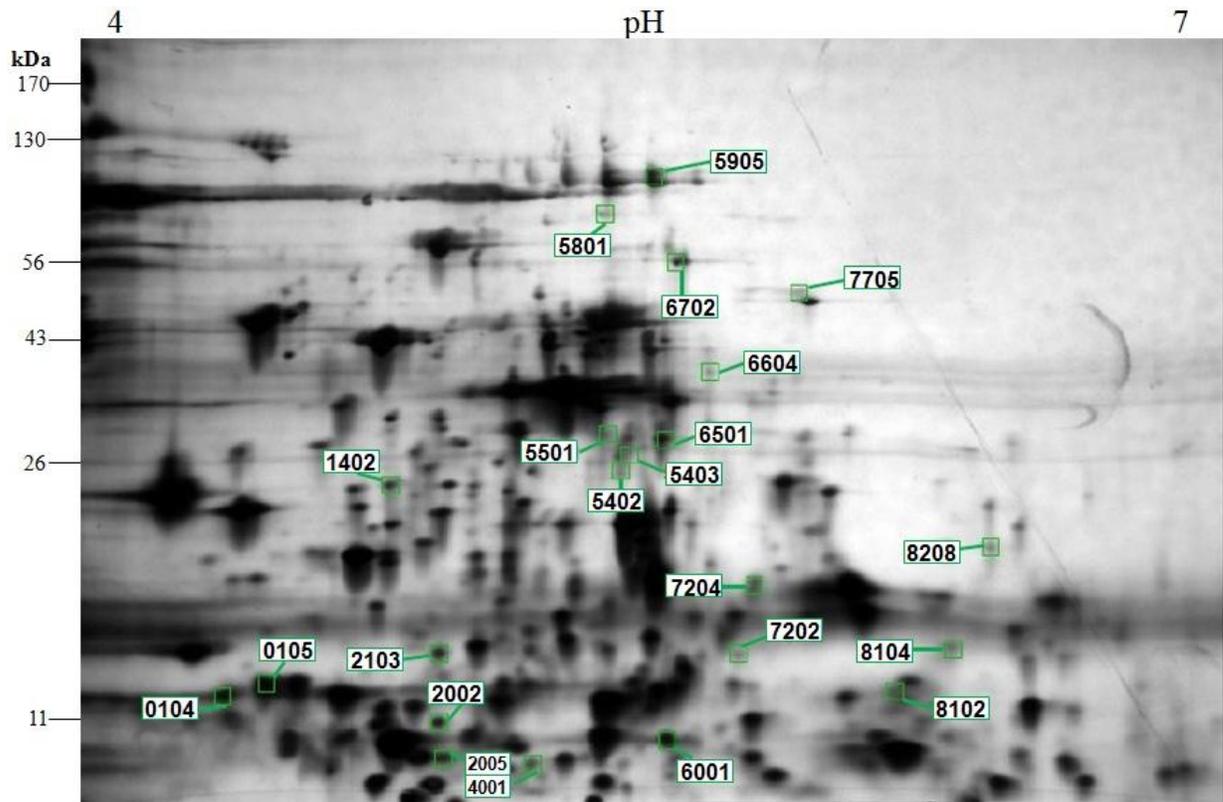


Figure 4.8: 2D-GE image of approximately 90 μg of proteins of *L. monocytogenes* LM21 treated with 64 $\mu\text{g}/\text{ml}$ curcumin separated on pH 4-7, 11 cm IPG strip. The boxed spots represent spots that were chosen by the software when compared by student's T-test. The SSP numbers were automatically assigned to the selected protein spots for comparison with proteins of *L. monocytogenes* LM21 (Figure 4.7)

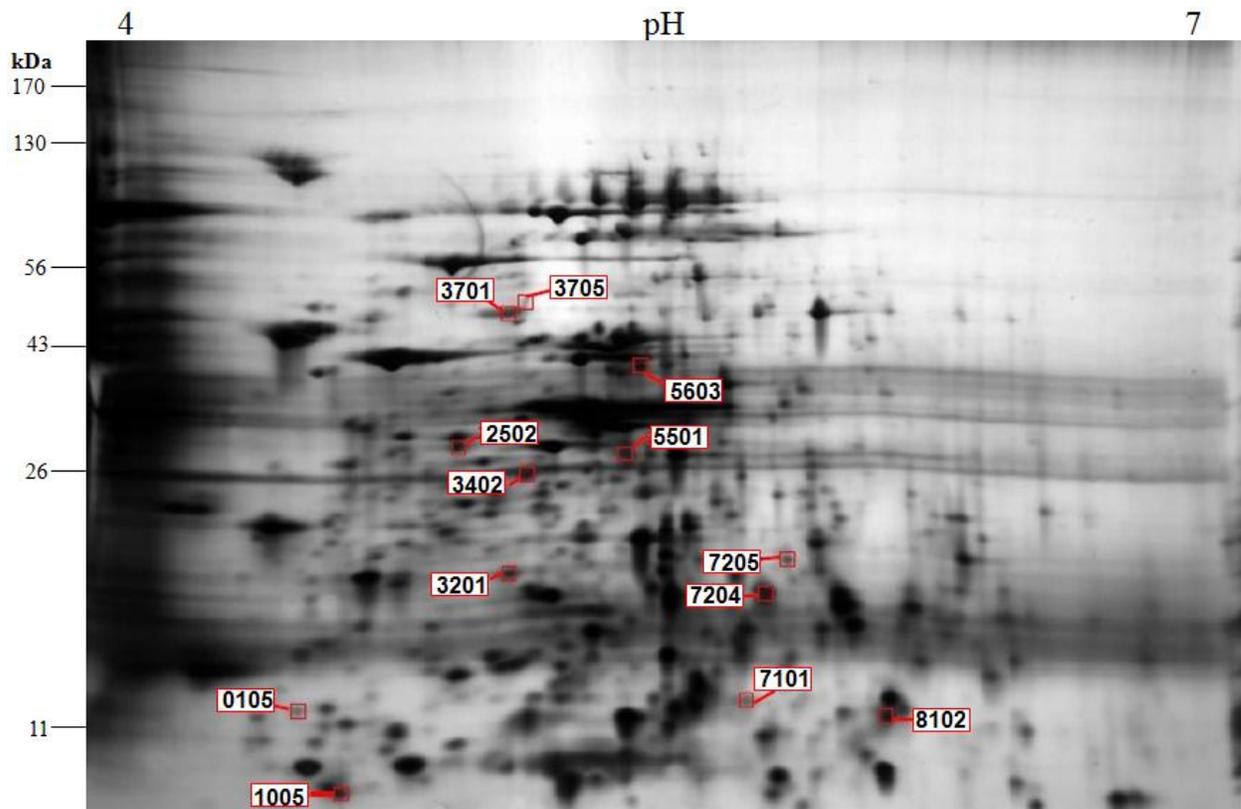


Figure 4.9: 2D-GE image of approximately 90 μ g of proteins of *L. monocytogenes* LM21 separated on pH 4-7, 11 cm IPG strip. The boxed spots represent spots that were chosen by the software when compared by student's T-test. The SSP numbers were automatically assigned to the selected protein spots for comparison with proteins of *L. monocytogenes* s22-11G (Figure 4.10)

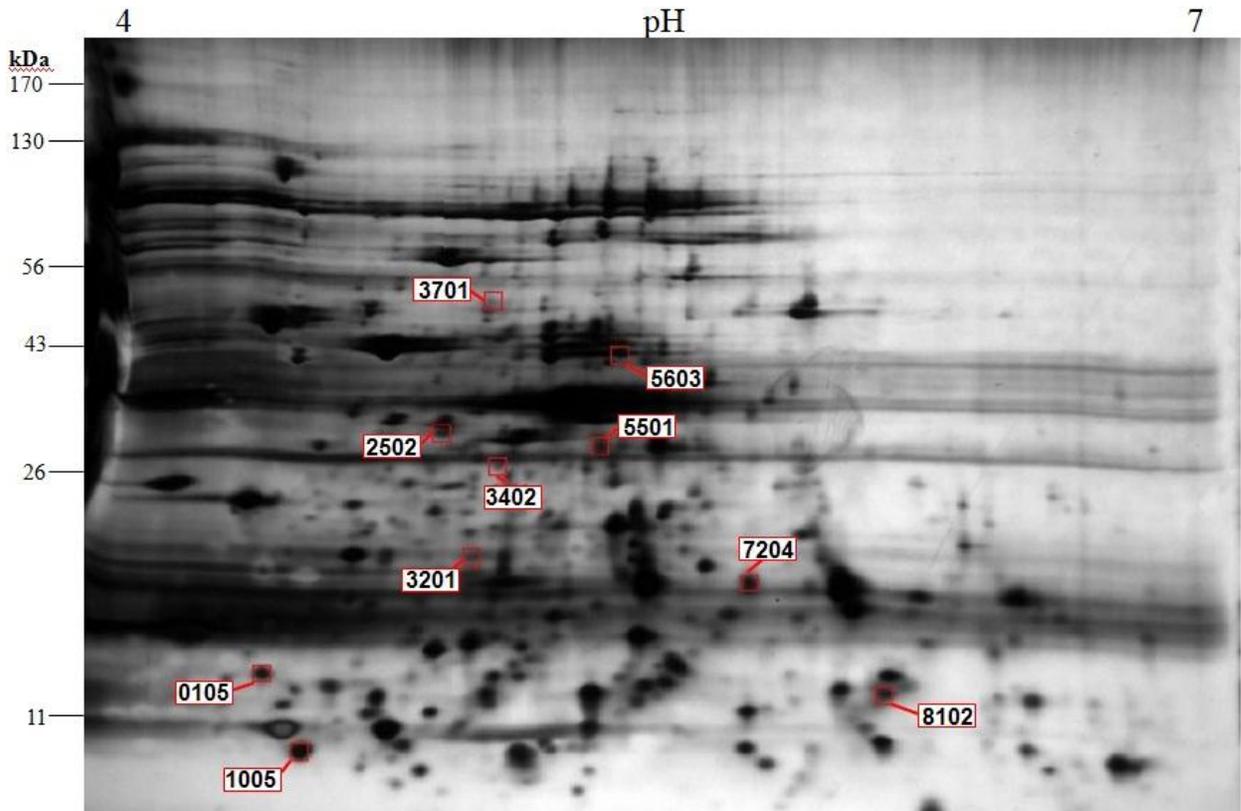


Figure 4.10: 2D-GE image of approximately 90 μ g of proteins of *L. monocytogenes* s22-11G separated on pH 4-7, 11 cm IPG strip. The boxed spots represent spots that were chosen by the software when compared by student's T-test. The SSP numbers were automatically assigned to the selected protein spots for comparison with proteins of *L. monocytogenes* LM21 (Figure 4.9)

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