CONTROL OF FOODBORNE PATHOGENS BY BACTERIOCIN-LIKE SUBSTANCES FROM LACTOBACILLUS SPP. IN COMBINATION WITH HIGH PRESSURE PROCESSING

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ABSTRACT

Lactic acid bacteria (LAB) and their bacteriocins have been used as natural food preservatives to improve food safety and stability but only few reports are available concerning inactivation of gram negative bacteria by LAB bacteriocins. The objectives of this study were (1) to screen food for bacteria with activity against gram-negative pathogens, (2) to measure the sensitivity of selected foodborne pathogenic targets to the new antimicrobial agents, (3) to evaluate a synergy between the antimicrobial agent and high pressure processing (HPP) against the targeted pathogens, and (4) to study the combined effect of antimicrobial agent and HPP on components of bacterial cell.

In search of food grade bacteria that produce bacteriocins active against gram-negative pathogens, foods were screened and *Lactobacillus curvatus* OSY-HJC6 and *L. casei* OSY-LB6A were isolated and identified. The cell-free extracts (CFE) of these isolates showed inhibition against *Escherichia coli* p220, *E. coli* O157, *Salmonella enterica* serovar Enteritidis, *Salmonella Typhimurium*, and *Listeria monocytogenes*. The activity of the CFE from *Lb. curvatus* and *Lb. casei* was completely eliminated by pronase, and pronase and lipase, respectively. The antimicrobial nature of CFE from both isolates was confirmed by eliminating the inhibitory effect of acid, hydrogen peroxide, and lytic bacteriophages.
Lactobacillus curvatus was evaluated for production of antimicrobial activity extracellularly and intracellularly. The extracellular fraction of Lb. curvatus was bactericidal against E. coli O157:H7 and Salmonella Enteritidis in TSBYE but was not active against gram positive bacteria. Intracellular fraction (CFE) had a bacteriostatic action against E. coli in a growth-permitting medium but showed bactericidal activity against the bacterium in buffer.

Lactobacillus casei produces broad-spectrum complex bacteriocin-like substances. The CFE of Lb. casei showed inhibitory effect against E. coli O157:H12, O157:H7, and two O157 meat isolates (O157-M1 and O157-M2). The effect of CFE was bactericidal against E. coli p220 in buffer, but exhibited bacteriostatic mode of action in growth-permitting media. The combination treatment of CFE (32 CEAU/ml) and HPP (350 MPa for 1-20 min) caused viability loss of 4.4-4.6 and 4.8-5.1 log_{10} CFU/ml O157:H12 and O157:H7, respectively, and decreased O157-M1 population under detectable levels (p < 0.05). A significant synergistic effect of CFE (100 CEAU/g) and HPP (400 MPa, 1 min) was observed against E. coli O157 inoculated in a meat product.

The CFE of the strain showed bactericidal activity against L. monocytogenes inoculated in a meat product. The effectiveness of CFE in combination with high pressure processing against the pathogens in cell suspension and meat product was similar to that against E. coli O157 strains. Combination of Lb. casei CFE (32 CEAU/ml) and high pressure processing (350 MPa for 1-20 min) had synergistic bactericidal effect against three strains of L. monocytogenes and decreased the pathogens’ population > 5 log_{10} CFU/ml (p < 0.05). The synergistic effect was most pronounced in pressure resistant L. monocytogenes OSY-8578 strain in culture suspension. Differential scanning calorimetry
thermogram suggested that ribosome and DNA in *Listeria* were damaged by the presence of CFE during HPP, compared to ribosome denaturation only by HPP. Combinations of CFE (100 CEAU/g) and HPP (500 MPa for 1 min) caused a reduction of > 5 log_{10} CFU/ml *L. monocytogenes* in meat products. No survivors were detected on both selective and nonselective media. The synergy between the CFE of *Lb. casei* and HPP suggests potential use of the combination treatment in food applications.
To my parents
Whom I honor and love
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INTRODUCTION

For the past decades, food safety and food control have been an important issue in many countries. According to a report of the World Health Organization, hundreds of millions of people worldwide suffer from foodborne diseases (http://www.who.int/archives/inf-pr-1997/en/pr97-58.html). Foodborne diseases cause approximately 76 million illnesses, resulting in 325,000 hospitalizations, and 5,000 deaths each year in the US alone (Mead et al., 1999).

*Listeria monocytogenes* and enterohemorrhagic *Escherichia coli* are some of the important foodborne pathogens of gram positive and negative bacteria, respectively. They are ubiquitous in nature and in the slaughterhouse environment (Grau and Vanderlinde, 1992; Johnson et al., 1990). High incidence of *L. monocytogenes* was reported in minimally processed and refrigerated ready to eat meat products such as fermented sausages, and vacuum packaged meat (Farber et al., 1989). Since these products are usually consumed without reheating, presence of *Listeria* poses serious risk to consumers.

*Escherichia coli* O157 causes life-threatening conditions such as haemorrhagic colitis and haemolytic–uremic syndrome with a low infective dose (Girffin and Tauxe, 1991). Most of the foodborne infections associated with this pathogen include consumption of foods of animal origin such as raw milk and ground beef (Armstrong et
al., 1996; Philips, 1999). Some strains of *E. coli* O157 are acid resistant and can survive in acid foods, like apple juice, for long periods especially at low temperatures (Miller and Kaspar, 1994; McCarthy, 1996).

To inactivate foodborne pathogens, novel technologies such as biopreservation systems, nonthermal technologies, or combined treatments have been studied. Use of lactic acid bacteria or their antagonistic metabolites such as lactic acid, hydrogen peroxide and bacteriocins is an example of biopreservation. Lactic acid bacteria (LAB) are generally regarded as safe as they have been associated with production of fermented foods in many centuries. For this reason, these bacteria are attractive as a means of naturally controlling growth of pathogenic and spoilage organisms in a variety of foods (Harris et al., 1992; Roberts and Zottola, 1993). Bacteriocins of LAB are known to be bactericidal to closely related gram positive bacteria (Nielsen et al., 1990) and few reports are available on their activity against gram negative microorganisms *per se* due to outer membrane acting as a permeability barrier.

New nonthermal technologies such as high pressure processing (Hite, 1899; Cheftel, 1995; Knorr, 1994), pulsed electric field (Castro et al., 1993; Zimmermann, 1986), and irradiations (Thayer, 1990; 1994) are very reliable in destroying harmful and deleterious microorganisms. They are alternatives to conventional thermal processing with minimized adverse effects on product quality such as taste, flavor or vitamin content (Smelt, 1998).

Foods contain many nutrients that can interact with biological antimicrobial substances like bacteriocins. Under certain conditions, the bactericidal effect of bacteriocins is degraded by food components during storage. Efficacy of bacteriocins
may be enhanced when food is treated simultaneously with additional preservation factors. Hurdle concept involves using two or more antimicrobial factors at suboptimal conditions to destroy spoilage and pathogenic bacteria (Leistener, 1985). By the combination treatment, microorganisms injured sublethally by one agent are destroyed by the other mild treatment (Kalchayanand et al., 1998).

The objectives of this study include (1) screening fermented foods for microorganisms that produce antimicrobial agents with activity against gram negative bacteria, (2) characterizing and testing the antimicrobial activities against broad range of gram positive and gram negative bacteria, (3) enhancing the efficacy of antimicrobial agents from the isolate by high pressure processing, and (4) investigating the effect of combination treatments on cellular structure of *Listeria* and *E. coli* by differential scanning calorimetry.
CHAPTER 1

LITERATURE REVIEW

Bacteriocins of lactic acid bacteria

Lactic acid bacteria. Lactic acid bacteria (LAB) are important groups of microorganisms in food fermentation. A wide variety of strains are routinely used as starter cultures, to manufacture dairy, meat, vegetables, and bakery products. In addition to their health and nutritional benefits (Gilliland, 1990; Sandine, 1990), LAB also contributes to safety of fermented foods. These bacteria produce organic acids, diacetyl, hydrogen peroxide, and antimicrobial proteins during fermentation (Lindgren and Dobrogosz, 1990). These compounds not only contribute to the desirable effect on food flavor and texture (Nettles and Barefoot, 1993), but also inhibit undesirable microflora, extending products shelf life. Growth of spoilage and pathogenic bacteria in the fermented foods is inhibited due to the production of antimicrobial agents by LAB as well as their competition for nutrients (Ray and Daeschel, 1992).

Bacteriocins of gram positive bacteria. Bacteriocins are proteinaceous antimicrobial substances produced by many bacterial species. Bacteriocins produced by LAB have been widely studied due to their potential use in food preservation as natural preservatives. Compared to classical peptide antibiotics, which are made through
enzymatic condensation of free amino acids, the bacteriocins of gram positive bacteria are ribosomally synthesized peptides or proteins with antimicrobial activity against other bacteria (Montville and Kaiser, 1993; Klaenhammer, 1993).

Bacteriocins of LAB are generally active against species that are closely related to the producing strain (Tagg et al., 1976). The producing stains are protected from the action of the bacteriocins by an immunity protein that is specific to its own bacteriocin (Quadri et al., 1995). Bacteriocins have variable inhibitory spectra. A few LAB bacteriocins such as bulgaricin, or acidophilin are active against a broad spectrum of gram-positive, or gram negative bacteria, as well as yeasts and molds (Klaenhammer 1993; Jack et al., 1995; Nemcova, 1997). Bacteriocins with broad inhibitory spectrum are potentially useful food additives for inhibiting spoilage and pathogenic bacteria (Nettles and Barefoot, 1993; De Vuyst and Vandamme, 1994).

Two plasmids in *Lactobacillus acidophilus* were found to be essential for the production of lactacin F and the immunity of the producing cells (Muriana and Klaenhammer, 1991a). Schillinger and Lucke (1989) reported presence of a plasmid in *Lb. sake* that may be involved in the formation of sakacin and immunity protein. Currently, it is believed that most bacteriocins are coded on plasmids (Vidaver, 1983; Jack et al., 1995).

**Classes of LAB bacteriocins.** Four classes of bacteriocins were identified by Klaenhammer (1993). Group I bacteriocins are known as lantibiotics. They are small peptides with dehydrated residues such as dehydroalanine, dehydrobutyryne, lanthionine, and β-methyl lanthionine, introduced by posttranslational modifications (Jung and Sahl,
Nisin is the most studied lantibiotic bacteriocins (Jung and Sahl, 1991), along with lactocin S (Nes et al., 1994). Group II bacteriocins are small heat stable peptides with molecular weight in the range of 2-6 kDa. Majority of bacteriocins produced by Lactobacillus species belongs to this group. Group II bacteriocins include pediocin PA-1 (Henderson et al., 1992), lactococcin A (Holo et al., 1991), curvacin A (Tichaczek et al., 1993), lactacin B (Barefoot and Klaenhammer, 1983), lactocin S (mortvedt et al., 1991), and sakacin P (Tichaczek et al., 1992; 1994). Lactacin F is a well-known two-component bacteriocin produced by Lb. johnsonnii (Muriana and Klaenhammer 1991a, b). Group III bacteriocins are large heat-labile proteins. Helveticin J (37kda, Joerger and Klaenhammer, 1990), acidophilin (Toba et al., 1991), or casecin (40kda, Rammelsberg et al., 1990) are large thermolabile bacteriocins with similarity to colicins produced by Escherichia coli (Morlon et al., 1983). Group IV bacteriocins are proteins complexed with lipid or carbohydrate moiety. Plantaricin S and lactocin 27 belong to group IV bacteriocins (Klaenhammer, 1993).

**Class I bacteriocins.** Nisin was first discovered in 1928 (Rogers and Whittier, 1928). It was considered as a toxic substance present in milk that affects performance of starter cultures. Hirsch and Mattick (1949) evaluated nisin as a clinical antibiotic in the 1940s, but later on, nisin was proved to be suitable for food preservation (Hurst, 1981). Nisin is a peptide produced by several strains of Lactococcus lactis. It has 34 amino acid residues with antimicrobial activity against a wide range of gram positive bacteria (Hurst, 1981; Jung and Sahl, 1991). Nisin is ribosomally synthesized and undergoes posttranslational modifications of the amino acids, serine, threonine, and cysteine (Liu...
and Hansen, 1990). As a result, it displays several unusual features such as the dehydrated residues (dehydroalanine and dehydrobutyrine from serine and threonine residues, respectively) and lanthionine and β-methyllanthionine residues which form 5 intramolecular thioester bridges (Jung and Sahl, 1991).

Nisin disrupts the proton motive force in energized membrane vesicles, inhibits uptake of amino acids by bacterial cell, and causes release of accumulated amino acids (Jung and Sahl, 1991). It was reported that nisin induces cell hydrolysis and causes partial leakage of cellular ATP (Abee et al., 1995; Jung and Sahl, 1991). Abee et al. (1995) reported that nisin caused an immediate loss of cellular potassium ions and depolarization of the cytoplasmic membrane in *Listeria monocytogenes*. Most gram positive bacteria showed sensitivity to nisin, including *Lactococci, Micrococci, Bacilli, Staphylococcus aureus, L. monocytogenes* and *Clostridium botulinum* (Hurst, 1981, 1983).

Factors associated with resistance to nisin include low cellular phospholipid contents, an altered membrane fatty acids composition (Ming and Daeschel, 1995; Mazzotta and Montville, 1997; Crandall and Montville, 1998), and changes in cell wall composition (Davies et al., 1996). Breuer and Radler (1990) reported that anionic polysaccharides bound to the cell wall of *Lb. casei* was shown to be associated with increased nisin resistance. Currently, nisin is approved as food preservatives in more than 40 countries worldwide (Delves-Broughton, 1993).

Other group I bacteriocins include lacticin 481, produced by *L. lactis*, lactocin S, by *L. sake* and carnocin UI49, by *Carnobacterium piscicola*. These class I bacteriocins showed similar molecular structures to nisin (Piard et al., 1992; Mortvedt et al., 1991; Stoffels et al., 1992; Klaenhammer, 1993).
**Class II bacteriocins.** Class II LAB bacteriocins are small heat stable bacteriocins with 3 subgroups; Ila, IIB, and IIc. Best studied bacteriocin in this group is Pediocin PA-1, which is a ribosomally synthesized bacteriocin by *Pediococcus acidilactici* (Gonzalez and Kunka, 1987; Marugg et al., 1992). Pediocin like bacteriocins belong to subclass Ila, which contains a consensus motif (YGNGV).

Pediocin PA-1 is a highly hydrophobic, positively charged peptide with 44 amino acids (Motlagh et al., 1992; Chikindas et al., 1993). Pediocin PA-1 was shown to be identical to Pediocin AcH (Klaenhammer, 1993; Ray and Daeschel, 1992). These bacteriocins form amphiphatic helices, with varying amounts of hydrophobicity, and β-sheet structure (Abee et al., 1995). The target of pediocin PA-1 is the cytoplasmic membrane of sensitive cells. Pediocin PA-1 forms hydrophilic pores in cytoplasmic membrane, dissipates ion gradients, and inhibits transport of amino acids. Studies suggested that sakacin A and P (Chikindas et al., 1993), leuconcin, and carnobacteriocin B2 and BM1 (van Belkum and Abee, unpublished data) might function like pediocin PA-1 based on membrane permeabilizing effects.

Unlike group I bacteriocins, pore formation by pediocin PA-1 was mediated by protein receptor and occurred in a voltage-independent manner (Chikindas et al., 1993). The role of protein receptor in the action of Lcn A, a bacteriocin produced by *Lc. lactis*, was reported. When LcnA was tested with liposomes composed only of phospholipids from sensitive lactococcal cells, LcnA could not permeabilize the liposomes in the absence of proteins, suggesting that a protein receptor is involved in pore formation of LcnA (van Belkum et al., 1991).
Pediococci are widely applied in meat and vegetable fermentation. The use of pediocin PA-1 is covered by several European and US Patents (Boudreaux and Matrozza, 1992; Gonzalez, 1988; Vandenberghe et al., 1989). Pediocins have inhibitory activity against *Listeria* spp. (Klaenhammer, 1993). Many studies on model food systems reported that pediocin-like bacteriocins are better at destroying pathogens in meat products than is nisin (Baccus-Taylor et al., 1993; Leisner et al., 1996; Montville and Winkowski, 1997; Nielsen et al., 1990).

**Mode of action.** The bactericidal action of bacteriocin occurs at the cytoplasmic membrane of target cells (Montville et al., 1995; Abee et al., 1995; Moll et al., 1996). In general, bacteriocins act by binding to the charged headgroups of phospholipids of the membrane or to a proteinaceous receptor, inserting into the membrane, and forming pores in the cytoplasmic membrane. This results in a depletion of the proton motive force, which interferes with cellular biosynthesis and causes cell death (Ruhr and Sahl, 1985).

Nisin (Ruhr and Sahl, 1985) and pediocin JD (Christensen and Hutkins, 1992) dissipated membrane potential and caused a collapse of proton motive force (Bruno and Montville, 1993). Pediocin AcH’s activity was related to inhibition of ATP synthesis, impairment of transport systems, or damage to permeability barriers of the cytoplasmic membrane (Bhunia et al., 1991). Upreti and Hidsdill (1975) reported that lactocin 27, produced by some strains of Lactobacilli, inhibited synthesis of protein and nucleic acids and the incorporation of leucine into proteins was inhibited more rapidly than that of thymidine into DNA and uracil into RNA. Bacteriocin, C3603, from *S. mutans* C3603 (Takata et al., 1984) and mutacin from *S. mutans* MT3791 (Hamada et al., 1986) caused
immediate inhibition of the synthesis of proteins, DNA and RNA in sensitive cells. Similar mode of action was reported in other bacteriocins (Jack et al., 1995; Chikindas et al., 1993; Tagg et al., 1976).

**Regulation of bacteriocin production.** Bacteriocin production by many gram positive bacteria is regulated by virtue of quorum sensing, a way of cell to cell communication; the expression of genes and synthesis of various proteins depend on cell density in the medium (Kuipers et al., 1998). Microorganisms get information on cell density in the medium by the presence of a signal peptide. A gene for the signal peptide is situated within an operon, and biosynthesis is autoregulated.

Quorum sensing is generally regulated by two-component signal transduction system, a sensor protein and a response regulator (Stock et al., 1990). A small signal molecule is synthesized, transformed in the cells, and secreted through an ATP dependent transporter. The signal molecule is sensed by membrane-located histidine kinase, which is phosphorylated upon interaction with the peptide and the signal is transmitted to an intracellular response regulator by phosphorylation. The response regulator of the system binds to certain direct repeats situated upstream of the promoter in a bacteriocin operon (Jack et al., 1995; Guder et al., 2000), activates the transcription of target genes, and regulates expression of various genes including the gene encoding the peptide precursor of the signal molecule (Kotelnikova and Gelfand, 2002).

**Regulation of nisin biosynthesis.** In general, genes for bacteriocin production are organized in clusters with genes coding for structure, immunity, processing, and transport
Genes encoding nisin-production are organized in a cluster (nis ABTCIPRKFEG) (Kuipers et al., 1995) in which genes for modification (nis B and C), processing (nis P), immunity (nis I), and regulation (nis R and K) are situated near the structural gene (nis A) (Siezen et al., 1996) (Figure 1.1a). Sometimes genes of the two-component signal transduction system or other regulatory system are added, and form a cluster with several transcriptional units either on chromosomes or on mobile elements, such as plasmids or transposons (Siezen et al., 1996).

In the autoregulation process of nisin in *Lc. lactis*, the two component signal transduction machinery, nis K and R, is important for transcription activation and production of nisin. Nis K, a histidine kinase gene senses presence of nisin in the medium and autophosphorylates itself (Engelke et al., 1994). The phosphoryl group is then transferred to nis R, a response regulator, which activates the transcription of the genes under control of the nis A and nis F promoters. This is followed by mRNA synthesis, and ribosomal synthesis of precursor nisin (De Ruyter et al., 1996). The precursor is modified by the putative enzymes nis B and C (Hess et al., 1988; Engelke et al., 1992) and translocated across the membrane. The fully modified nisin is then released by ABC transporter, nis T (Hess et al., 1988), and extracellularly processed by nis P (van der Meer et al., 1993), resulting in the release of active nisin. Nis I, together with nis F, nis E, and nis G protect producing cells from the bactericidal action of nisin (Engelke et al., 1994; Siegers and Entian, 1995).

Within the nisin gene cluster, the integrity of structural gene nis A is important. When the nisin structural gene was rendered defective by deleting 4 bp, the transcription of this gene was abolished (Kuipers et al., 1993). However, when a small amount of nisin
A was added to the medium, production of the transcript was restored (Kuipers et al., 1995). Nisin Z and several nisin mutants also induce the transcription of nisin. Kuipers et al. (1995) reported that the amount of transcripts produced correlated with the amount of nisin A added. However, when nis K gene of the strain *Lc. lactis* NZ9800 was inactivated, the transcription of nis A was no longer induced. Introduction of a plasmid containing nis A or nis Z failed to restore transcription of nis A, suggesting the importance of nisK (Kuipers et al., 1995). Transcription of nisin A (Figure 1.1b) is autoregulated by the signal transduction of fully modified peptide present in extracellular environment. This cell communication results in production of high quantities of antimicrobial peptides and increased immunity to nisin in neighboring cells (Kuipers et al., 1995).

**Bacteriocins of gram negatives bacteria**

**Colicins.** Colicins are large molecular weight proteins with antimicrobial properties. They are produced by strains of *Enterobacteriaceae* carrying specific plasmids called colicinogenic factors (Morlon et al., 1983). Colicin was first discovered in 1925 (Gratia, 1925) as a highly specific antibiotic produced by stains of *E. coli* and active against other strains in the same species. Seventeen colicins (Colicins A, B, D, E1 to E9, Ia, Ib, K, M, and N) have been studied. In addition to colicins, pyocin, produced by *Pseudomonas aeruginosa* (formerly pyocynia), and cloacin by *Entrobacter cloacae* belong to this group of bacteriocins (De Graaf and Oudega, 1986).

Colicins were classified based on the specificity of their adsorption to a protein receptor, and were further subgrouped according to the specificity of their immunity (Fredericq, 1957). For example, E group colicins all bind to the same protein receptor, a
product of the chromosomal \textit{btuB} gene, which is responsible for \text{vitB}_{12} transport in \textit{E. coli} (Di Masi et al., 1973). The E group colicins were subdivided into nine types (Col E1 to Col E9) based on the immunity test. Colicins E2, E7, E8, and E9 has their immunity proteins, Im2, Im7, Im8, and Im9, respectively. Similarly colicin Ia and Ib belong to group I, sharing a same receptor, but they are distinguished from each other by their immunity specificity (Watson et al., 1981; Cooper and James, 1984).

**Colicin’s mode of action.** Bacteriocins of gram negative bacteria generally exert their action by adsorption to their specific receptors on the sensitive cells and are translocated to their targets within the cells (Koniski, 1982; Riley and Gordon, 1996). Synthesis of colicins may be induced by stress factors such as ultraviolet light and mitomycin C. This results in rapid release of colicin into the environment. Colicin recognizes specific receptor on the surface of the sensitive cells, and destroys them by one of the following four mechanisms (Braun, 1995; Parker et al., 1989; Larkey et al., 1992; Lazdunski, 1995; Gouaux, 1997).

(I) Formation of ion-permeable channels (Colicins E1, A, B, Ia, Ib, 5, 10, K, and N) (Schwartz and Helinski, 1971; Cramer et al., 1983; Lazdunski, 1995).

(II) Inhibition of DNA synthesis and non-specific degradation of cellular DNA (Colicins E2, E7, E8, and E9) (Schaller and Nomura, 1976; Toba et al., 1988; Eaton and James, 1989; Chak et al., 1991).
(III) Inhibition of protein synthesis through the specific cleavage of 16s rRNA (Colicins E3, E5, E6, and colicin D) (Boo, 1971; Bowman et al., 1971; Senior and Holland, 1971; Akutsu et al., 1989).

(IV) Cell lysis resulting from inhibition of peptidoglycan synthesis (colicin M) (Harkness and Braun, 1989).

The primary target of colicin A, E1, K, Ia, and Ib is the cytoplasmic membrane (Cramer et al., 1983; Lazdunski et al., 1988). These colicin variants form voltage dependent channels in the phospholipids bilayer membranes, cause disruption of active transport, and result in leakage of ions. This results in the destruction of cell’s membrane potential which causes cell death (Schein et al., 1978; Konisky, 1982). Bourdineaud et al. (1990) reported that channel activity of colicin A is voltage dependent but translocation may be voltage independent.

R pyocin, produced by P. aeruginosa, inhibits sensitive strains by inactivating ribosomes, which result in a complete inhibition of protein synthesis (Kaziro and Tanaka, 1965a, b). Despite their different mode of action, most colicins have the same domain structures with N terminal, central, and C-terminal regions. N terminal region of colicins is important for uptake of colicins into sensitive cells. A central region is involved in binding to the receptors in outer membrane and C-terminal region is essential for exerting its activity. When colicin derivatives, mutated in the uptake domain (N terminal) and the receptor binding domain (central region), were translocated into cells by induced permeabilization using osmotic shock, the derivatives showed killing activity in vitro or in vivo (Tilby et al., 1978; Braun et al., 1980).
Immunity proteins. All colicin producing cells synthesize immunity proteins; these protect producing cells from the cytotoxic action of the related colicins (Wallis et al., 1992; Jakes et al., 1974). Therefore, each colicin has its own immunity proteins (Weaver et al., 1981a, b; Vander Goot et al., 1991) and the immunity proteins interactivate the corresponding colicin by direct interaction.

Pore forming colicins E1, A, B, Ia, Ib, K, L, M, and N are active in the cytoplasmic membrane. The immunity proteins of these colicins are found in the cytoplasmic membrane. These colicins are secreted without bound immunity proteins (Baty et al., 1987; Song and Cramer, 1991; Pugsley, 1984). In case of colicin A, the immunity proteins are composed of 4 transmembrane helices, of which three helices (H2, H3, and H4) and the periplasmic loop between the helices H3 and H4 are important for interaction with the colicin A. It recognizes the hydrophobic hairpin of the pore forming domain at C terminal (helices H8 and H9) (Geli and Lazdunski, 1992b) (Figure 1.2). This interaction between the immunity proteins and the pore forming domains takes place at the inner membrane to prevent pore formation in that membrane (Tokunda and Knoniski, 1978; Weaver et al., 1981b; Geli and Lazdunski, 1992a).

Colicins with nuclease activities (e.g. colicins D, E2 to E9, etc) bind to the immunity proteins in the cytoplasm, and are released as an equimolar colicin-immunity protein complex into the medium (Akutsu et al., 1989). The E colicins are plasmid encoded bacteriocins. The plasmid also codes for the production of a specific immunity protein. Upon synthesis, the immunity protein binds to the C terminal domain of its cognate (Di Masi et al., 1973). James et al. (1992) studied the genetic aspect of interaction between colicin E9 and its immunity proteins. ColE9-J plasmid encodes the
structural and immunity genes of the colicin in the operon. The promoter of E9imm genes is located within the colicin E9 structural gene. The transcription of the promoter allows constitutive expression of the immunity protein and provides immunity to colicin E9. Then transcription from the inducible promoter results in the expression of the genes of the operon, leading to the synthesis and secretion of the colicin immunity protein complex (Chak and James, 1986).

**Genetic aspects.** Bacteriocins differ in their inhibitory spectrum as well as in the location of their genes, either chromosomal or plasmid borne. The genetic determinants of colicins are plasmid borne whereas pyocins and pneumocin are chromosomally located. The genes coding for a bacteriocin determine the chemical composition of the bacteriocin, and regulate its biosynthesis, its release from the producing cells, and the host cell immunity to its own bacteriocin (Kageyama, 1975; Quirantes et al., 1994).

Genes for plasmid encoded bacteriocins can be easily transferred either by conjugation or transduction from bacteriocin producing cells to compatible recipient strains with all the features encoded. Colicins, A, D, E1 to E9, K, and N are encoded on small multicopy plasmids, which are ampiclicable in the absence of protein synthesis and are not self-transmissable. These small plasmids also encode a lysis protein, known as a bacteriocin release protein. The gene is located near the colicin activity gene and helps release the colicin from the producing cells (Braun et al., 1994). Colicins B, Ia, Ib, and M are encoded on large low copy plasmids, which are transferred by conjugation and are not amplifiable. These large plasmids do not encode lysis proteins. Therefore, these colicins are released without the help of lysis protein (Braun et al., 1994).
The genetic determinants of pyocins are located on the chromosome. Loci of pyocin vary for each pyocins; R and F are between alternation of trpCD and trpE (Kageyama, 1975; Shinomiya et al., 1983). Sano et al. (1990) cloned pyocin genes on appropriate plasmids and found homology in the amino acid sequences among these pyocins and colicins. These similarities in amino acid sequence are indicative of their similar function.

Inhibition of gram negatives by lactic acid bacteria. Inhibition of gram-negative bacteria by lactic acid bacteria has been studied. The inhibition may be due to the production of organic acids such as lactic and acetic acids (Sorrels and Speck, 1970; Gilliland and Speck, 1977), hydrogen peroxide (Gilliland and Speck, 1977), or bacteriocins (Klaenhammer, 1988).

Lactic acid is produced by lactic acid starter culture and is generally recognized as safe. Doores (1993) reviewed inhibitory effect of lactic acid on growth of many types of food spoilage bacteria including gram-negative species. Lactic acid has been widely used as a biopreservative in naturally fermented products (Ray and Sandine, 1992), and it is also applied for decontamination of meat (Dickson and Anderson, 1992; Greer and Dilts, 1995; Van Netten et al., 1994, 1995).

Synergistic effect of lactic acid with other antimicrobial factors such as diacetyl, hydrogen peroxide, lactoperoxidase systems, and reuterin was studied, in which lactic acid helps potentiate antimicrobial activity of other components against gram-negative bacteria. (Ray, 1992; Daeschel and Penner, 1992).
Jin et al. (1996) reported inhibition of strains of *E. coli* by organic acid produced by *Lactobacillus* spp. isolated from chicken intestine. Inhibitory effect of organic acids produced by *Lactobacillus* spp. against pathogenic bacteria was reported (Hechard et al., 1990).

**LAB bacteriocins against gram negatives.** The inhibitory activity of LAB bacteriocins is generally confined to gram positive bacteria. Most LAB bacteriocins (e.g. nisin) are not active against gram negative bacteria (Tagg et al., 1976; Skytta et al., 1993). It was reported that nisin formed pores in liposomes made of gram negative cell components (Garcia-Garcera, et al., 1993) and sublethally heat shocked gram negative bacteria (Boziris et al., 1998; Kalchayanand et al., 1992). Nisin was active against gram negative bacteria in the presence of EDTA (Stevens et al., 1991). The authors demonstrated the sensitivity of LPS mutants of *Salmonella* Typhymurium to nisin (Stevens et al., 1992). These studies show that outer membrane of gram negative bacteria acts as a permeability barrier to the action of nisin on the cytoplasmic membrane.

A large number of studies was published in recent decades on bacteriocins but these include only few reports concerning LAB bacteriocins against gram negative bacteria. Bacteriocins active against gram negative bacteria include those produced by *Streptococcus thermophilus* 81, plantaricin LP84, by *Lb. plantarum* NCIM 2084, and a bacteriocin AS-48 by *Enterococcus faecalis* (Ivanova et al., 1998; Suma et al., 1998; Galvez et al., 1989a, b).

*Streptococcus thermophilus* 81 produces 32-amino acid peptide that has a broad inhibitory spectrum against gram positive and gram negative bacteria. The peptide
showed bacteriostatic mode of action against several Bacillus species, L. monocytogenes, Salmonella Typhimurium, E. coli, Yersinia pseudotuberculosis and Y. enterocolitica (Ivanova et al., 1998).

*Lactobacillus plantarum* NCIM 2084 produces plantaricin LP84, a small molecular-weight bacteriocin. It inhibited gram positives including B. cereus, S. aureus, B. licheniformis, B. subtilus, P. aeruginosa, and Lb. amylovorus but showed relatively moderate inhibition against E. coli C21 (Suma et al., 1998). Plantaricin LP84 and lactic acid present in the culture supernatant showed inhibitory activity against the gram negative Pantoea agglomerans (Niku-Paavola et al., 1999).

Gálvez et al. (1989a, b) reported a bacteriocin, AS-48, produced by *Enterococcus faecalis* subsp. liquefaciens with a broad spectrum of activity against gram-positive and gram-negative bacteria. The primary target of AS-48 is the cytoplasmic membrane. Gálvez et al (1988) reported that at least 10 times higher concentration of AS-48 was required to inhibit gram negative bacteria compared to gram positive bacteria. The resistance of gram negative bacteria is attributed to their outer membrane (Gálvez et al., 1991).

**Gram positive and gram negative bacteria**

**Cell envelop.** The cell envelopes of gram-positive bacteria consist of cytoplasmic membrane, peptidoglycan layer, and teichoic acids attached to the peptidoglycan layer. The thick peptidoglycan layer comprises about 90% of the cell wall of gram-positive bacteria (Beveridge, 1999). The structure of gram-negative bacterial cell envelopes is much more complex than that of gram positive bacteria. This explains low sensitivity of
gram negatives to LAB bacteriocins. Cell envelop of gram-negative bacteria consists of peptidoglycan layer, and outer membrane. The peptidoglycan layer of gram-negative cell wall is very thin, comprising only 10% or less of the cell wall. In addition, instead of teichoic acids, gram-negative bacteria cell wall has lipoprotein molecules on the inner surface of the outer membrane. The fatty acids in the lipoprotein associate with the hydrophobic portion of the outer membrane. They are also bonded to the backbone of the peptidoglycan layer. Therefore, the peptidoglycan layer is attached to an outer membrane via lipoproteins. The space between the outer membrane and the cytoplasmic membrane is called the periplasmic space (Beveridge, 1995). Together, the cell wall (outer membrane, peptidoglycan layer, and periplasm), and the plasma membrane constitute the gram negative envelope (Beveridge, 1981; Beveridge and Graham, 1991) (Figure 1.3).

**Outer Membrane and Lipopolysaccharide as Permeation Barrier.** The outer membrane covers the peptidoglycan layer and the cytoplasmic membrane of gram-negative cells. The outer membrane is composed of phospholipids, proteins, and lipopolysaccharides (LPS) (Nikaido and Vaara, 1985). It is an asymmetrical membrane with the LPS molecules in its outer leaflet, instead of phospholipids, and glycerophospholipids in its inner leaflet. The LPS is a complex molecule composed of 3 distinct regions with lipid A, core polysaccharide, and O-antigen. The lipid A is the innermost portion of the LPS, and anchors the LPS to the hydrophobic portion of the outer membrane. External to lipid A towards the cell exterior, the polysaccharide portion of the LPS consists of a core polysaccharide and a repeat polysaccharide called O-antigen or O-polysaccharide. The polysaccharide is composed of sugars such as glucose,
galactose, and some unusual sugars. The composition varies from one gram-negative bacterium to another. This complex heteropolysaccharide provides the surface of the LPS with a hydrophilic character (Nikaido, 1996). In addition, the heteropolysaccharide group contains phosphate and carboxyl groups, which are electrostatically linked by divalent cations especially Mg$^{2+}$ or Ca$^{2+}$. Therefore the polyanionic nature of the LPS forms the molecular basis of the integrity and stability of the outer membrane (Nikaido, 1990), and the hydrophilic nature of the LPS makes the outer membrane a barrier to hydrophobic molecules (Helander and Mattila-Sandholm, 2000a). Small molecules below 600 Da can diffuse freely through the porins in the outer membrane. However, most bacteriocins produced by lactic acid bacteria are too large to reach the cytoplasmic membrane, with the smallest bacteriocins being approximately 3 kDa (Klaenhammer, 1993; Stiles and Hastings, 1991).

**Biopreservation with bacteriocins**

**Hurdle technology: Induced Permeabilization of Gram-negative bacteria.**

The susceptibility of microbial cells to antimicrobial agents can be improved through application of the hurdle concept. According to this concept, two or more antimicrobial agents at suboptimal levels can act synergistically than each of them alone at the optimal level (Leistner and Gorris, 1995). Cells sublethally treated by different stressing conditions may become sensitive to physical and chemical agents to which healthy cells are resistant under normal conditions (Ray, 1992). Many researchers have tried to overcome the penetration barrier in gram-negative bacteria to sensitize them to bacteriocin using chemical, physical, and genetic methods. *Salmonella* species and other
gram negative bacteria were sensitized to nisin and other bacteriocins after exposure to treatments that change the permeability barrier properties of the outer membrane (Stevens et al., 1991). It includes the combination of physical treatments such as hydrostatic pressure, heat, freezing, and thawing as well as chemical treatment with an addition of EDTA or ethyl maltol (Stevens et al., 1991; Kalchayanand et al., 1992, 1994; Hauben et al., 1996; Schved et al., 1996).

**Chemical treatments.** Permeabilizers can sensitize bacteria to other agents that are unable to penetrate into bacterial targets at sublethal concentration (Helander and Mattila-Sandholm, 2000b). Ethylene diamine tetraacetic acid (EDTA) is a known permeabilizer, functioning by chelating divalent cations (Vaara, 1992). It removes stabilizing cations such as magnesium ions in the LPS layer from the outer membrane of the gram-negative bacteria (Stevens et al., 1991; Delves-Broughton, 1993; Schved et al., 1994; Boziaris and Adams, 1999). This results in destabilization of the outer membrane through release of the part of LPS molecules, and phospholipids from the inner leaflet appear on the surface of the outer membrane. Therefore, the membrane cannot function as a penetration barrier to entry of hydrophobic compounds into the cell (Hancock, 1984; Vaara, 1992).

Stevens et al. (1991) reported inhibition of EDTA-treated cells of *Salmonella* and *E. coli* by nisin. Helander and Mattila-Sandholm (2000b) supported this with the result that EDTA promotes bacteriocin’s action against gram-negative bacteria in addition to its permeabilization effect (Boziaris and Adams, 1999; Abriouel et al., 1998; Gao et al., 1999).
Although EDTA is approved as a food additive to prevent discoloration in foods, it is not a natural additive. The drawback of the EDTA is that it sequesters a wide range of metals including irons (Shelef and Seiter, 1993).

Polycations are permeabilizers. They can displace cations in the outer membrane of gram-negative bacteria by different mechanism from EDTA. Interaction of polycations (e.g., polylsine and protamine) with outer membrane results in release of LPS. Other polycations (polymyxin B nonapeptide and polyethyleneimine) intercalate into the outer membrane without LPS release (Vaara, 1992; Helander et al., 1998). However, these compounds are not widely used as they could be hazardous as food ingredients (Helander et al., 1997, 1998; Vaara, 1992).

Organic acids such as citric and lactic acid are food grade permeabilizers. They sensitize gram-negative bacteria to nisin (Alakomi et al., 2000). The organic acids showed increased permeabilization effect on gram-negative bacteria by disrupting the outer membrane (Delves-Broughton, 1993; Alakomi et al., 2000). The effect of Tris on permeabilization of the outer membrane has been reported. Tris also helps release proteins, lipopolysaccharide (LPS), and alkaline phosphatase from the cell envelope (Irvin et al., 1981; Vaara, 1992). Galvez et al. (1989a, b) reported an enhanced antimicrobial activity of some bacteriocins in the presence of Tris. Synergistic effect of nisin with other metabolites such as pediocin and lactoperoxidase system has been reported, when the combinations were tested against gram negative bacteria (Hanlin et al., 1993; Bycroft et al., 1991).
**Physical treatments.** Sublethally heat shocked *Salmonella* Enteritidis was inhibited by nisin A (Boziaris, et al., 1998; Kalchayanand et al., 1992) and Pediocin PA-1/AcH (Kalchayanand et al., 1992). Kordel and Sahl (1986) studied that *E. coli* became susceptible to nisin when the outer membrane was altered by osmotic shock. Kihm et al. (1994) reported that the combination of chelating agents with heat treatment caused a greater permeabilization effect on gram negative bacteria, otherwise the cell wall disruption caused by heat alone could be repaired in the presence of sufficient divalent cations. This was supported by the results of Abriouel et al. (1998), in which sublethal heat treatment combined with EDTA increased sensitivity of *Salmonella* to bacteriocin AS-48.

Recently, high pressure processing and pulsed electric field (PEF) have been studied as nonthermal methods of food preservation (Morris, 1993). These methods inactivate microbial cells by destabilizing the structural and functional integrity of the cytoplasmic membrane (Knorr, 1994; Castro et al., 1993; Mertens and Knorr, 1992). Combination treatment of bacteriocins with HPP and PEF showed greater antimicrobial effects than each treatment alone (Knorr, 1994; Castro et al., 1993). Gram negative bacteria, sublethally injured by these treatments showed sensitivity to pediocin AcH/PA-1 and nisin (Kalchayanand et al., 1994). Nisin and lysozyme were combined with high pressure processing to destroy pressure-resistant *E. coli* strains (Kalchayanand et al., 1994, 1998; Hauben et al., 1996; Garcia-Graells et al., 1999). The pressure-resistant *E. coli* mutants were sensitized to nisin and lysozyme under high pressure. However, permeabilization by high pressure seemed transient, as the sensitivity of *E. coli* to lysozyme and nisin disappeared immediately upon relief of pressure (Hauben et al., 1996).
Combination of high pressure processing with slightly elevated temperature was studied (Sonoike et al., 1992; Patterson and Kilpatrick, 1998; Garcia-Graells et al., 1999).

**Genetic methods.** Gram-negative bacteria are resistant to lysozyme. When lipopolysaccharide mutants *Salmonella* and *E. coli* were developed by deleting part of the o-side chain in their lipopolysaccharide, these mutants became sensitive to lysozyme. Their sensitivity to lysozyme was correlated to the hydrophobicity of the outer membrane (Sanderson et al., 1974). A modified lysozyme with increased hydrophobicity either by chemically cross-linking a fatty acid to lysine residue (Ibrahim et al., 1993; Nakamura et al., 1996) or through genetic incorporation of a hydrophobic C-terminal peptide (Ibrahim et al., 1994; Ito et al., 1997) showed increased interaction with outer membrane, passed through the membrane, and exerted bactericidal effect against gram negative bacteria.

**Purification of bacteriocins**

Despite many studies on bacteriocins of lactic acid bacteria, a limited number of these studies focused on biochemical structures. Many challenges are associated with the purification of the antimicrobial peptides. Successful purification sometimes results in a limited amount of pure peptides and characterization of the bacteriocin is hampered (Carolissen-Mackay et al., 1997). Because of the heterogeneous nature of bacteriocins, a variety of combination of procedures has been suggested for their purification with varying success (Tagg et al., 1976; Klaenhammer, 1993). A number of important considerations for purification of bacteriocins will be discussed.
**Production of bacteriocins.** Bacteriocins produced by gram positive bacteria are not inducible or only slightly inducible (Hardy, 1982). In most cases, relatively low titres of activity are detected in broth cultures. For production of bacteriocin, it is recommended to start with large batches of cultures. Production studies are necessary as conditions of incubation, such as pH and temperature influence the yield of active bacteriocins (Laukova, 1992; Carolissen-Mackay et al., 1997; Franz et al., 1997).

A composition of the growth medium has a great effect on the production of bacteriocins (Tagg et al., 1976). In general, complex growth media, such as MRS, M17, and ATP (All purpose tween) are commonly used to grow lactic acid bacteria (Parente and Hill, 1992). These media are rich with peptides in the molecular weight range of most bacteriocins (3000-6000 Da); this interferes with the purification process (Barefoot and Klaenhammer, 1984). Rammelsberg et al. (1990) considers complex media unsuitable for the purification of some bacteriocins such as caseicin 80 due to unknown macromolecular components. In contrary, Yang and Ray (1994) reported that complex media were necessary to produce high titres of bacteriocins.

Media constituents such as Tween 80, has been shown to either enhance or interfere with the production and subsequent purification of bacteriocin (Mortvedt et al., 1991; Muriana and Klaenhammer, 1991b; van Laack et al., 1992). Tween 80 helps stabilize the bacteriocin activity by forming micelles with the proteins in the medium (Garver and Muriana, 1994). The production of lactococcin G (Nissen-Meyer et al., 1992), and lactacin F (Murianan and Klaenhammer, 1991b) was increased by the addition of Tween 80 in the media. The effect of Tween 80 on production of pediocin A (Piva and Headon, 1994), and lactocin S (Mortvedt et al., 1991) was negative.
Addition of Tween 80 in the growth medium might interfere with subsequent purification procedures. Ammonium sulfate precipitation of culture supernatants containing Tween 80 resulted in 3 distinct phases after centrifugation, a surface pellicle, a bottom pellet, and the supernatant (Muriana and Klaenhammer, 1991b; Mortvedt et al., 1991; van Laack et al., 1992). Majority of activity of lactacin F produced by *Lb. acidophilus* 11088 was recovered in the surface pellicle (Muriana and Klaenhammer, 1991b), whereas, lactacin F and lactocin S produced by *Lb. sake* L45 (Mortvedt et al., 1991) were lost in the floating pellet. Therefore these authors suggested to collect pellicle instead of the pellet for further purification. Effect of other media components on bacteriocin production was studied. Production of piscicoline 61 produced by *C. piscicola* LV 61 increased in the presence of peptone in MRS broth (Schillinger et al., 1993). Addition of yeast extract to MRS, whey, and whey permeate increased the activity of mesenterocin 5, produced by *Leuconostoc mesenteroides* subsp. *mesenteroides* UL5 (Daba et al., 1991). Supplementation of MRS with 4 % NaCl increased activity of plantaricin S by *Lb. plantarum* LPCO10 (Jiménez-Díaz et al., 1993).

**Purification techniques.** The size of bacteriocins ranges from small peptides to large proteins complexed with lipid and/or carbohydrate moieties. The majority are small cationic and hydrophobic molecules. The hydrophobic nature of the bacteriocins has complicated the purification process because bacteriocins tend to aggregate and sometimes nonspecifically adhere to materials that are employed during purification procedures. In some cases, bacteriocins are copurified with other cellular proteins (Jack et al., 1995).
Purification of bacteriocins includes many steps such as ammonium sulphate precipitation, ion-exchange chromatography, hydrophobic interaction, size exclusion chromatography, and reverse-phase HPLC. The type and sequence depends on their biological and physico-chemical properties such as molecular size, net charge, biospecific characteristics, and hydrophobicity (Kennedy, 1990; Garcia and Pires, 1993).

Size exclusion chromatography might negatively affect productivity due to the limited sample volume that can be loaded. Loading volume is limited to a maximum of a few hundred microliters. For this reason, it is necessary to concentrate the sample prior to column loading. Loading volume greater than 5% of the overall column volume often results in diffusion-related band spreading and dilution. Since size exclusion chromatography removes protein aggregates and elutes the purified proteins into the final formulation buffer, it is recommended that this technique be used as a final purification step (Klimchalk and Wang, 1997).

Ultrafiltration method is one way to concentrate the sample. However, non-specific binding of the protein to the membrane during the process can cause productivity loss (Klimchalk and Wang, 1997). Hydrophobic Interaction chromatography (HIC) is useful to separate solutes molecules based on differences in their surface hydrophobicity under nondenaturating condition. HIC often generates long tailing peaks. Protein aggregation, conformational variations during elution, large elution volume and relatively more adsorption/desorption steps cause these broad peaks during HIC, compared to other chromatographic method (Chicz and Regnier, 1990).

For purification of curvacin A, sakacin P, lactosin S, and bavaricin A, ammonium sulphate precipitation, ion-exchange chromatography, hydrophobic interaction, and
reverse-phase HPLC were used (Tichaczek et al., 1992; Mortvedt et al., 1991; Larsen et al., 1993a). For helveticin J and lactacin F, ammonium sulphate precipitation and gel permeation chromatography were used (Joerger and Klaenhammer, 1986; Muriana and Klaenhammer, 1991b). Ion-exchange chromatography, ultrafiltration, and gel permeation chromatography were used to purify lactacin B (Barefoot and Klaenhammer, 1984). Ultrafiltration resulted in great success in the case of lactacin F (Muriana and Klaenhammer, 1991b) and leucocin LA44A (van Laack et al., 1992), but activity of caseicin 80 was lost during the ultrafiltration (Rammelsberg et al., 1990). Hastings et al. (1991) reported a loss of activity due to dialysis even when a membrane with a 2000 Da cut-off was used. The authors also reported the importance of pH during purification. Techniques with large change in pH generally cause great losses in activity of bacteriocins and low pH (pH 2-4) during the procedure resulted in good yields (Hastings et al., 1991).

Visual examination and stability of bacteriocins. Visualization of bacteriocin after SDS-PAGE is not always successful because small hydrophobic peptides like bacteriocins often diffuse out of the polyacrylamide gel during the staining (Carolissen-Mackay et al., 1997). Hastings et al. (1991) suggested to use a more active fixative (e.g. formaldehyde), instead of the normal fixative (aceto-methanol) to visualize the bands of leucocin A-UAL 187. Mesentericin Y105, lacticin 481 and lactacin B were not visualized after silver stain or coomassie dye. A band of lacticin 481 was seen after electroblotting onto PVDF membrane (Piard et al., 1992; Barefoot and Klaenhammer, 1984).
Low concentrations of lactacin B were suggested as a reason for the non-detection by silver staining (Barefoot and Klaenhammer, 1984). Lactacin F was only detected by silver staining (Muriana and Klaenhammer, 1991b).

Presence of contaminating proteins causes a problem with SDS-PAGE. Daba et al. (1991) observed a smear rather than a specific band of mesenteroicin 5 due to contaminating proteins. Co-migration pattern of curvaticin FS47 with Tween 80 micelle complex was reported at the molecular mass close to that of the Tween 80 monomer (M. W. 1300) (Garver and Muriana, 1994). Tween 80 was also thought to inhibit the silver staining of bound proteins on SDS-PAGE (Garver and Muriana, 1994).

In the case of pediocin A, direct SDS-PAGE analysis detected the activity with difficulty. This was probably due to either the indicator strain is sensitive to SDS or the SDS inhibits the bacteriocins (Piva and Headon, 1994). However, direct detection and identification of mesentericin Y105, bavaricin A, and acidocin B was successful on SDS-PAGE (Héchard et al., 1992; Larsen et al., 1993b; Van der Vossen et al., 1994).

During the purification, bacteriocins become unstable due to loss of cofactors, or modifications on the catalytic site (Héchard et al., 1992). Purified mesentericin Y105 (Héchard et al., 1992), leucocin A-UAL 187 (Hastings et al., 1991), and carnobacteriocins A1, 2, and 3 (Worobo et al., 1994) were very unstable. Purified diplococcin lost activity after 1 week at 4ºC or at room temperature (Davy and Richardson, 1981). Improved stability was observed at low pH and by addition of BSA to pure bacteriocin (Tagg et al., 1976; Hastings et al., 1991).
Figure 1.1: (A) Organization of nisin biosynthesis gene cluster, (B) Model for nisin biosynthesis and regulation (Kuipers et al., 1995; Kuipers et al., 1998).
Figure 1.2: (A) Three-dimensional structure of the pore-forming domain of colicin A in buffer solution as determined by X-ray analysis, (B) Umbrella model, (D) penknife model, two alternative models of how colicin A (closed form) might be inserted into the cytoplasmic membrane through helices 8 and 9, (C) open pore formed by colicin A starting from the ‘umbrella model’ (Braun, 1994).
Figure 1.3: Schematic presentation of the cell envelope of gram positive and negative bacteria (Abee et al., 1995).
LIST OF REFERENCES


Beveridge, T. J. 1995. The periplasmic space and the concept of the periplasm in gram positive and gram negative bacteria. ASM News. 61, 125-130.


Smelt, J. P. P. M. 1998. Recent advances in the microbiology of high pressure processing. Trends Food Sci. Technol. 9, 152-158.


CHAPTER 2

INHIBITORY ACTIVITY OF BACTERIOCIN-LIKE SUBSTANCE FROM
LACTOBACILLUS CURVATUS AGAINST FOODBORNE SPOILAGE
AND PATHOGENIC BACTERIA

ABSTRACT

Isolates from fermented foods were screened for antimicrobial activity against gram negative bacteria. The most active isolate was identified as a *Lactobacillus curvatus* by biochemical analysis and ribotyping, and the isolate was designated as OSY-HJC6. *Lactobacillus curvatus* OSY-HJC6 was further tested for production of antimicrobial activity extracellularly and intracellularly. A bactericidal antimicrobial activity of *Lb. curvatus* OSY-HJC6 was detected on MRS agar medium. A reduction of >8 log_{10} cfu/ml occurred in *Salmonella enterica* serovar Enteritidis and *Escherichia coli* O157:H7 in the presence of *Lb. curvatus* culture supernatant (1:1, v/v). Gram positive bacteria were not sensitive to the culture supernatant. Intracellular antimicrobial activity was tested in the strain’s cell free extract (CFE) and a broad inhibitory spectrum against gram positive and gram negative bacteria was detected. Culture supernatant and CFE retained the antimicrobial activity after heating at 60-100°C for 10-20 min but not after treating with a
protease. The CFE of *Lb. curvatus* retarded the growth of *E. coli* p220 in broth medium and food extracts (i.e., bacteriostatic action), but showed bactericidal activity against the bacterium in phosphate buffer.

**INTRODUCTION**

Presence of gram-negative bacteria in minimally processed foods raises concern about products safety and quality. Pathogens such as *Salmonella* spp. and pathogenic *E. coli* as well as spoilage microorganisms may multiply in these products during extended refrigerated storage, thus threatening consumer’s safety (Motlagh et al., 1991). These microorganisms are abundant in the environment and are naturally present in human and animal intestine. Cross contamination during slaughtering and carcass processing has a significant effect on the microbiological quality of meat (Borch and Arinder, 2002).

Lactic acid bacteria are beneficial microorganisms that are used in many types of food fermentations. These bacteria produce antimicrobial metabolites such as lactic acid, hydrogen peroxide, and bacteriocins that are inhibitory to undesirable microorganisms (Daeschel, 1989). Bacteriocins are proteinaceous agents with antimicrobial activity against closely related species to the producer strain (Tagg et al., 1976). Bacteriocins or bacteriocin-producing strains are of interest in this respect, as they may prevent growth of spoilage and pathogenic bacteria and may serve as natural antimicrobials with generally recognized as safe (GRAS) status.
Bacteriocins of LAB are commonly active against gram-positive spoilage and pathogenic bacteria but these bacteriocins exhibit no antimicrobial action against gram-negative bacteria per se (Abee et al., 1995). Recently, a nisin-like bacteriocin, produced by Lactococcus lactis subsp. lactis A 164 (isolated from Kimchi), was reported with activity against closely related lactic acid bacteria and Salmonella Typhimurium (Choi et al., 2000).

Gram-negative bacteria are resistant to several antimicrobial agents due to the presence of outer membrane (Nikaido, et al., 1985; Vaara, 1992). The lipopolysaccharide layer of the outer membrane acts as an effective permeability barrier rendering them resistant to many antimicrobial agents (Hancock, 1984). Therefore, in order to sensitize gram-negative bacteria to bacteriocins, researchers have attempted to permeabilize the outer membrane by osmotic shock, heat shock, freezing, and the use of acid and chelating agents (Kordel and Sahl, 1986; Stevens et al., 1991, 1992; Kalchayanand et al., 1992; Delves-Broughton, 1993). Sublethal injury sensitized gram-positive and gram-negative bacteria to LAB bacteriocins (Kalchayanand et al., 1992; Ray, 1993).

The objectives of this study include (a) screening food-grade bacteria for bacteriocins active against gram-negative bacteria, (b) evaluating extracellular and intracellular antimicrobial activity of the isolate against a broad spectrum of foodborne pathogens, and (c) testing the activity against gram-negative foodborne pathogens in defined media and food extracts.
MATERIALS AND METHODS

Screening for antimicrobial activity against Gram-negative bacteria

Samples of fermented Asian foods were homogenized in a stomacher, 10-fold serially diluted with 0.1% peptone water, and plated on Trypticase soy agar (BBL, Sparks, MD) supplemented with 0.6% Yeast Extract (Difco Laboratories, Sparks, MD) (TSAYE) or MRS agar (Difco). Selected isolates were evaluated for inhibitory activity against *E. coli* p220, a non-pathogenic strain, and *E. coli* O157:H7, a pathogenic strain. The isolates were spotted or stabbed into MRS agar, and plates were incubated at 37°C for 24 hours. Growth on MRS agar was killed by 15-min exposure to chloroform vapors. The agar plates, containing killed colonies, were overlaid with 5-ml TSAYE soft agar (0.75% agar) seeded with 5 µl targeted microorganism. Plates were incubated at 37°C for 24 hours and zones of inhibition were sought (Galiano and Hinsdill, 1970) (Figure 2.1).

Characterization and identification of an antimicrobial-producing isolate

An isolate with strong antimicrobial properties was identified as *Lactobacillus curvatus* using colony and cell morphology, biochemical analysis (API 50 CH strip, bioMérieux, Inc., Hazelwood, MO) and ribotyping (Kraft Foods Basic Science, Glenview, IL). The isolate was designated as *Lb. curvatus* OSY-HJC6. The isolate was compared with *Lb. curvatus* ATCC 25601 and other lactobacilli using pulsed field gel electrophoretic typing technique as described by Tynkkynen et al., with modifications (Tynkkynen et al., 1999). Briefly, bacterial DNA was prepared from *Lactobacillus* spp. and embedded in agarose gel. Agarose-imbedded DNA was digested with 2 restriction
enzymes, ApaI and SmaI (Invitrogen Life Technologies, Carlsbad, CA). The resulting restriction fragments were separated on agarose gel by pulsed filed gel electrophoresis (PFGE) apparatus (Electrophoresis Cell, Bio-Rad, Hercules, CA). The electrophoresis conditions were 1.6% agarose gel, 0.5 x TBE buffer (45 mM Tris-borate, 1mM EDTA), 5 V/cm, 1-15 s switching time for 42 hours (Jenkins et al. 2002).

**Microorganisms**

*Lactobacillus curvatus* OSY-HJC6 was isolated from food and identified during the course of this study. Lactic acid bacteria (*Lb. curvatus* ATCC 25601, *Lb. leichmannii*, *Lb. acidophilus* OSU133, *Pediococcus pentosaceus* and *Lc. lactis* ATCC 11454) were obtained from the culture collection of the Food Safety Laboratory, Ohio State University. Cultures were grown in MRS broth. Working cultures were maintained on slant of MRS agar, and stored at 4°C.

Other bacteria tested include *E. coli* (p220, O157:H7, 395T4958, K12), *L. monocytogenes* (Scott A and Ohio), *L. innocua* ATCC 33090, *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Bacillus cereus* 14759, and *Pseudomonas fluorescens* OSU 608. These also were obtained from the culture collection of the Food Safety Laboratory, Ohio State University. Cultures were grown in Trypticase Soy Broth (BBL), supplemented with 0.6% Yeast Extract (TSBYE). Working cultures were maintained on slant of TSAYE and stored at 4°C. Each stock culture was maintained in the respective growth media, containing 20% glycerol, and stored at −80°C. Before use in experiments, the strains were transferred into fresh growth media and incubated at 37°C for 18 hours;
this was followed by two consecutive transfers in the medium and incubation under the conditions just indicated. The final counts of cultures were approximately $10^9$ CFU/ml.

**Extracellular antimicrobial activity**

*Detection of antimicrobial activity in culture supernatant.* *Lactobacillus curvatus* OSY-HJC6 was inoculated in MRS, M17, APT, and glucose broth (GB) (Suma *et al.*, 1998) (All these media were obtained from Difco). In addition, MRS supplemented with Glycine (Fisher Scientific, Fair Lawn, NJ) and Triton X-100 (Fisher Scientific) was used to grow *Lb. curvatus* (Yang *et al.*, 1998). Triton X-100 (0.5-1%, v/v) was added to buffered MRS medium (pH 6.5) before inoculation or when the culture was grown to an Absorbance (at 600nm) of 0.6 (Spectronic 1201, Milton Roy Co., Rochester, NY) to minimize the inhibitory effect of the supplement. Cultures were incubated at 37°C for 18 hours. Aliquots were taken at time intervals and the supernatant was tested for antimicrobial activity against *E. coli* p220 using well diffusion assay with modification (Lyon and Glatz, 1993).

Fifteen ml TSAYE soft agar (0.75% agar) plates seeded with 15 µl *E. coli* p220 (OD$_{600}$=0.4) were prepared. Wells, 6-mm diameter, were cut with sterile cork-borer and 50 µl of the supernatant, either pH-adjusted to 6.5 with 1N NaOH or non-adjusted, were added. The plates were stored at 4°C for 4 hours, to allow antimicrobial agents to diffuse, and incubated at 37°C for 16 hours. Colicin produced by *E. coli* 395T4958 in TSBYE was used as a positive control.
**Antimicrobial activity against Gram-negative pathogens.** The antimicrobial activity of culture supernatant from glucose broth (GB) was evaluated against actively growing *Salmonella Enteritidis* and *E. coli* O157:H7 cells ($10^8$ CFU/ml). Cell suspension of each culture was prepared in 0.1% peptone water, and mixed with active culture supernatant at 3:1 and 1:1, v/v, ratios. Glucose broth, pH adjusted to the same pH value of the culture supernatant (i.e., pH 4.2) by adding sterile lactic acid, was mixed with pathogen’s culture (1:1, v/v) and used as a negative control. Another control treatment received 0.1% peptone water (3:1, v/v), instead of the culture supernatant. The mixtures were incubated at 37°C for 8 hours, samples were taken at time intervals and plated on selective and nonselective agar media to detect pathogen’s inactivation and injury (Ray, 1993). Survivors were enumerated on TSAYE and Xylose Lysine Desoxycholate (XLD, Difco) agar for *Salmonella Enteritidis* and TSAYE and Eosin Methylene Blue (EMB, Difco) agar for *E. coli* O157:H7 (Ellison and Tatini, 1999).

**Intracellular antimicrobial activity**

**Preparation of cell free extract.** The cell free extract (CFE) of *Lb. curvatus* was prepared by the method described by Sanz et al. (1999) with modifications (Figure 2.2). Briefly, *Lb. curvatus* was inoculated in 2 liters of MRS media with or without 1% NaCl supplementation (Casla et al., 1996). The mixture was incubated for 18 hours at 30°C. Cells were harvested by centrifugation at 13,200 x g for 20 min at 4°C and washed twice in 20 mM Sodium Phosphate Buffer (SPB) at pH 7.0. Cell pellet was treated with 5mg/ml lysozyme (Sigma, St. Louis, MO), 0.6 M sucrose and 5 mM MgCl₂ and incubated at 30°C for 3 hours. Incubated pellet was washed twice in 20 mM sodium phosphate buffer
(pH 7.0), suspended in 50 ml of the same buffer, cooled in a water/ice mixture bath, and sonicated using an ultrasonic processor (Torbeo; Cole Parmer, Vermon Hill, IL) at setting 5, for 5 minutes while maintaining the cooling. The supernatant was collected by centrifugation at 34,200 x g for 20 min, freeze-dried, and stored at –80ºC until use (Figure 2.2). The freeze-dried powders (0.2g) were resuspended in 1 ml of the same buffer; this solution was designated as cell free extract (CFE).

**Antimicrobial activity assay.** The antimicrobial activity of CFE was tested in a microtiter plate assay system with modifications (Jimenez-diaz et al., 1995). Each well of the microtiter plate (Becton Dickinson, Franklin lakes, NJ) contained 25 µl 2X TSBYE medium, 25 µl CFE or its two-fold dilutions, and 10 µl of the indictor strain, *E. coli* p220 (10⁶ CFU/ml). The control sample was made of 25 µl TSBYE medium, 25 µl SPB, with or without added inocula (10 µl). The microtiter plate cultures were incubated at 37°C for 15 hours, and growth inhibition of the indicator strain was measured spectrophotometrically at 600 nm in a microtiter plate reader (Vmax Kinetics Microplate Reader, Molecular Devices, Sunnyvale, CA). The absorbance value of each dilution without the indicator was subtracted from the corresponding value of the same dilution containing the indicator microorganism. The degree of growth inhibition of *E. coli* p220 caused by pure colicin (Sigma) was used as a reference value.

Commercial colicin is produced by some strains of *E. coli* and contains 20,000 units/mg pure proteins. One colicin unit per ml is the minimal concentration required to cause a zone of clearing on a lawn of *E. coli* ATCC9637 cells. The highest dilution of the CFE causing the inhibition of the indicator, *E. coli* p220, compared that of the colicin
control, was used to estimate activity in CFE in units equivalent to colicin. Therefore, antimicrobial activity in CFE was expressed as colicin equivalent activity units (CEAU)/ml or g.

**Characteristics of cell free extract.** The CFE was evaluated for inhibitory responses that may be attributed to H$_2$O$_2$ from the producing cells. The CFE and a solution containing 3% H$_2$O$_2$ were tested with or without added 2600 U/ml catalase (Sigma) (Liao et al., 1993; Parrot et al., 1990). Inhibition caused by H$_2$O$_2$ was tested using the microtiter plate method. Possible interference of lytic bacteriophage was tested by the method of Lewus et al. (1991), with a slight modification. Portions of agar (1 cm$^2$) were cut from the inhibition zones of *E. coli* p220 plates and transferred aseptically to 3 ml TSBYE broth, macerated, and incubated for 3 h at 37°C. Portion (100 µl) of the mixture and 10 µl overnight culture of the indicator strain, *E. coli* p220, were added to 5 ml TSBYE soft agar, overlaid on TSAYE plates, and incubated at 37 °C for up to 72 hours. Plates were inspected for possible plaques formation.

**Antimicrobial activity of cell-free extract against *Escherichia coli* p220.** The CFE of *Lb. curvatus* (0.2 g) was prepared in 1 ml, 20 mM, sodium phosphate buffer (pH 7.0) (320 CEAU/ml) and serial two fold dilutions were prepared in the same buffer. Overnight culture of *E. coli* p220 was diluted into fresh TSBYE medium or the phosphate buffer to a final concentration of ~10$^5$ cells/ml. Aliquots (0.1 ml) containing 2, 8, and 32 CEAU/ml of *Lb. curvatus* CFE, were added to 0.9 ml *E. coli* p220 cell suspension. The control received 0.1 ml buffer instead of the CFE. The mixtures were incubated at 37°C,
and aliquots were taken at 3-hours intervals during 18 hours of incubation. The viable count was determined by spread plating on TSAYE. The plates were incubated and survivors were counted.

**Preparation of food extracts**

The inhibitory activity of CFE from *Lb. curvatus* was tested using *E. coli* p220 in three food extracts. The food samples include milk permeate, cheese whey permeate, and filtered wiener (frankfurter) exudate. Milk permeate was prepared from pasteurized milk (2% fat) using a filtration unit (Filtron Technology Corporation, Northborough, MA) with a 100 kDa molecular weight cut off. Fifteen ml of milk were dispensed in the filter unit, centrifuged at 4,340 x g for 20 minutes and a clear milk permeate was obtained. Cheese whey permeate, from experimental Cheddar cheese made at the Ohio State University, was filter sterilized using 0.2-µm filters. The wiener exudates were obtained from a commercial frankfurter and filter sterilized using 0.2-µm filters. All three food extracts were stored at –20ºC until use. Growth inhibition of *E. coli* p220 by *Lb. curvatus* CFE in food extracts was tested using the microtiter plate method.

**Stability of antimicrobial agents**

The antimicrobial agents in supernatant of GB and CFE of cultures grown in MRS + 1% NaCl were tested for sensitivity to pepsin, trypsin, protease, chymotrypsin, bromelin, lipase, α-amylase, pronase, ficin, and papain (Lee et al., 1999). Enzymes were obtained from Sigma except pronase, ficin and papain, which were from Calbiochem (Sandiego, CA). Enzymes were applied at a final concentration of 1 mg/ml in sodium
phosphate buffer (pH 7), except lipase, which was added at 0.1 mg/ml. Mixtures of enzyme preparation and CFE or culture supernatant were incubated at 37°C for 2 hours. Fifty µl of the supernatant and enzyme mixture was assayed for the residual activity using the well diffusion assay. Portions of neutralized culture supernatant and CFE were subjected to heat treatment at 60-100°C for 10-20 minutes, and the residual activity was assayed.

**RESULTS AND DISCUSSION**

**Screening for antimicrobial activity against gram-negative bacteria.**

Fermented foods were screened in search of microorganisms with antimicrobial activity against gram negative bacteria. Food isolates were stabbed in TSAYE agar, plates were incubated to allow growth of isolates, resulting growth was exposed to chloroform vapor to kill the cells and help release antimicrobial agents into the medium. After plates were overlaid with gram-negative indicators (e.g., *E. coli p220*) and incubated, stabs exhibiting zones of inhibition were selected. Several isolates showed inhibitory activity against *E. coli p220*, *E. coli O157:H7* and *Salmonella Enteritidis* on TSAYE plates. Among these, an isolate from kimchi showed the most effective and consistent activity, thus it was further investigated. The isolate was a gram-positive, non-sporeforming, catalase-negative, rod-shaped bacterium. The isolate was characterized by biochemical testing using API system (API 50 CH strip), and ribotyping, and it was identified as *Lactobacillus curvatus*. 
Production of antimicrobial activity extracellularly

Colonies of *Lb. curvatus* OSY-HJC6 that exhibited zones of inhibition on TSAYE plates showed no inhibitory activity after subculturing in MRS broth. Similar result was reported by Daeschel and Klaenhammer (1985) who found *P. pentosaceous* FBB61 producing antimicrobial agents on agar but not in broth media. Garver and Muriana (1994) also reported that attempts to produce curvaticin FS47, produced by *Lb. curvatus* FS47, in MRS broth were unsuccessful.

Production of bacteriocin or bacteriocin-like substance is affected by environmental factors such as composition of the culture medium and incubation conditions (Laukova, 1992; Franz et al., 1997). Therefore, promising isolates were grown in APT, M17, glucose broth (GB) or buffered (pH 6.5) MRS broth, supplemented with a surfactant (0.5-1% Triton X-100) and glycine (2%). Yang et al. (1998) reported more than 170-fold increase in secretion of heterologous proteins of *E. coli* by supplementing the culture media with glycine and Triton X-100. Glycine and Triton X-100 influence permeability or integrity of cell wall (Fujiyama *et al.*, 1995; Yang et al., 1998). Glycine is incorporated into precursors of peptidoglycan, resulting in disruption of peptidoglycan cross linkage and cell membrane integrity (Hammes et al., 1973). Glycine and Triton X-100 synergistically enhance excretion of the protein (Yang et al., 1998). Supplementation of MRS with 0.5 and 1% Triton X-100 retarded the growth of *Lb. curvatus* appreciably; the bacterium required > 60 and 80 hours incubation in these media, respectively, before detectable growth (measured as absorbance at 600 nm) and antimicrobial activities were observed. Antimicrobial activity was satisfactory when MRS broth was supplemented with 2% Glycine and 1% Triton X-100 with the highest inhibitory activity appearing after
ca. 66 hours of incubation. Addition of 0.5% Triton X-100 after the culture reached $A_{600 \text{nm}}$ of 0.6, instead of adding the additives before inoculation, resulted in faster growth but a similar level of bacteriocin was detected (Table 2.1). Yang et al. (1998) found that addition of a membrane stabilizer, MgCl$_2$, to the modified culture medium, counteracted some of the membrane damaging effect of glycine and Triton X-100. Addition of the salt diminished the leakage of heterologous protein out of *E. coli* membrane (Yang et al., 1998; Pugsley et al., 1984).

Suma et al. (1998) reported production of plantaricin LP84 by *Lb. plantarum* NCIM 2084 using a simple glucose broth (GB) containing elevated glucose and low mineral concentrations. This medium contains Mg$^{++}$ concentration 10,000 times less than that in MRS broth. In GB, *Lb. curvatus* grew well and produced antimicrobial activity against *E. coli* p220, *E. coli* O157:H7, and *Salmonella* Enteritidis. No antimicrobial activity was detected from culture supernatants of APT or M17 media against *E. coli* p220. Supernatant of *Lb. curvatus* OSY-HJC6 culture, after incubation in GB, was used in the remainder of the study; this will be referred to as “antimicrobial culture supernatant” or ACS.

It is obvious that *Lb. curvatus* produces extracellular antimicrobial agent(s) more readily on solid than in liquid media. Low cell density may account for the limited production in liquid culture. Bacteriocin producing lactic acid bacteria are capable of controlling synthesis of various proteins depending on cell density in the medium, a phenomenon known as quorum sensing (Bassler, 1999; Miller and Bassler, 2001). Solid media, therefore, are most suitable for cell-cell communication which may affect production or release of antimicrobial agents in cell’s environment.
Intracellular antimicrobial activity

It is likely that *Lb. curvatus* OSY-HJC6 accumulates antimicrobial agents intracellularly and only limited quantities are excreted in the liquid media. Therefore, intracellular antimicrobial activity of the bacterium was investigated. Cell-free extracts, prepared from cells grown in MRS broth, supplemented with 1% NaCl, had pronounced antimicrobial activity (Table 2.1). Weak inhibitory effect was observed from intracellular activity using GB (data not shown). According to a previous study (Casla et al., 1996), greater intracellular antimicrobial activity was observed in cells grown in MRS broth supplemented with 1% NaCl than in non-supplemented medium. Beneficial effect of sodium chloride on bacterial growth and bacteriocin production was reported in lactic acid bacteria (Korkeala et al., 1992; Passos et al., 1993), including *Lb. sake* (sakacin P, Gänzle et al., 1996), *Lc. lactis* (lactacin 481, Uguen et al., 1999), and *Lb. amylovorus* DCE 471 (amylovorin L471, Neysens et al., 2003). However, disadvantages of adding NaCl on bacteriocin production were also reported (De Vuyst et al., 1996; Nilsen et al., 1998). Larsen et al. (1993) reported addition of 1% NaCl had no influence on bavaricin A production at 10ºC.

*Lactobacillus acidophilus* OSU133 was used in this study as a negative control to determine if the preparation procedure has any influence on inhibitory activity of CFE. *Lactobacillus acidophilus* OSU133 is a bacteriocin producer but does not have inhibitory activity against gram-negative bacteria. Therefore detection of antimicrobial activity from *Lb. acidophilus* against the indicator, *E. coli* p220, will question the validity of the extraction method. When both bacteria were treated similarly, the CFE of *Lb. curvatus* showed antimicrobial activities against *E. coli* p220, whereas the indicator was not
inhibited by the CFE of *Lb. acidophilus*. This suggests that the inhibitory activity of the CFE from *Lb. curvatus* cannot be attributed to the traces of lysozyme that may have remained during pellet washing or to non-specific intracellular components. Extraction of the antimicrobial agents from *Lb. curvatus* using acetic acid (Fang et al., 1997), or chloroform (Burianek and Yousef, 2000) was not successful (data not shown).

**Inhibitory spectrum**

The antimicrobial activity of ACS and CFE from *Lb. curvatus* OSY-HJC6 was tested using well diffusion assay and microtiter plate method, respectively, against gram negative and gram positive strains. The CFE of *Lb. curvatus* showed inhibitory activity against gram negative (*E. coli* p220, *E. coli* O157:H7, *Salmonella* Enteritidis, *Salmonella* Typhimurium, and *P. fluorescens*), and gram positive (*B. cereus*, *L. monocytogenes* Scott A, and *P. pentosaceus*) bacteria (Table 2.2). The CFE showed broader inhibitory spectrum than that of ACS; activity of the latter was limited to gram negative bacteria. The differences in their inhibitory spectrum could be due to the different methods used to detect inhibition. The conventional well diffusion assay is convenient to use but it has low sensitivity, whereas the microtiter plate method is sensitive and suitable for measuring antimicrobial activity quantitatively.

Bacteriocins produced by strains of *Lb. curvatus* have been reported earlier. These include curvaticin 13 (Sudirman et al., 1993), curvacin A (Tichaczek et al., 1992), curvaticin FS47 (Garver and Muriana, 1994), and bacteriocins from *Lb. curvatus* NCFB 2739 (Ha et al., 1994), *Lb. curvatus* IFPL105 (Casla et al., 1996), *Lb. curvatus* SE1 (Kim et al., 1998), and *Lb. curvatus* L442 (Mataragas et al., 2002). *Lactobacillus curvatus*
bacteriocins, reported in these studies, were active against closely related species and pathogens, such as *L. monocytogenes*, but no antimicrobial activity against gram negative bacteria was reported. This shows uniqueness of the antimicrobial agent reported in this study. *Lactobacillus curvatus* OSY-HJC6 was genotyped using pulsed field gel electrophoresis (PFGE). The strain produced a different DNA banding pattern, when compared with other antimicrobial-producing lactobacilli (data not shown).

**Characterization of antimicrobial agent**

The pH of culture supernatant of *Lb. curvatus*, grown in GB, was adjusted to pH 6.5 and its antimicrobial activity was compared with that of the non-neutralized supernatant using well-diffusion assay. The neutralized supernatant retained most of the inhibitory activity compared to the non-neutralized product. This confirms that the source of the antimicrobial activity cannot be attributed to the acidity of the culture.

The neutralized culture supernatant (pH 6.5) retained about 70% of the activity after heat treatment at 60-100°C for 10-20 minutes (data not shown). The supernatant, however, was sensitive to pronase. These data suggest that *Lb. curvatus* produces a bacteriocin-like substance extracellularly. The bacteriocin-like substance is active against pathogenic and non-pathogenic strains of gram negative bacteria including *E. coli*, and *Salmonella* Enteritidis. Colicin, which was used as a positive control, inhibited a non-pathogenic *E. coli* strain, but showed no activity against *Salmonella* Enteritidis and *E. coli* O157:H7 (data not shown). Similarly, the antimicrobial activity of CFE was eliminated completely by pronase and partially by trypsin. Most of the activity was retained after heat treatment at 60-100°C for 10 min (Table 2.3).
The antimicrobial agent produced by \textit{Lb. curvatus} OSY-HJC6 fits the definition of bacteriocins or bacteriocin-like substance, with respect to the proteinaceous nature and mode of action (Tagg et al., 1976; Jack et al., 1995). The inhibitory spectrum, however, seems broader than that observed with most bacteriocins. In this study, the antimicrobial activity was examined for its genetic determinant by a plasmid curing method (Muriana and Klaenhammer, 1987). The association of plasmid with the antimicrobial activity was not apparent (data not shown). Lack of bacteriocin’s genetic code in plasmids may not be essential for defining bacteriocins; production can be either chromosomally or plasmid mediated (Joerger and Klaenhammer, 1986; Ray et al., 1989).

\textbf{Efficacy of extracellularly-produced bacteriocin-like agent.}

Two pathogenic gram negative bacteria, \textit{Salmonella} Enteritidis and \textit{E. coli} O157:H7, were evaluated for sensitivity to the antimicrobial agent produced by \textit{Lb. curvatus} OSY-HJC6 in GB culture supernatant. Population of \textit{Salmonella} Enteritidis decreased 2 and > 8 log cfu/ml in the 3:1 and 1:1 pathogen-antimicrobial supernatant mixtures, respectively (Figure 2.3). The supernatant inhibited colony formation by \textit{Salmonella} Enteritidis in a concentration- and time-dependent manner. Lower counts of treated \textit{Salmonella} Enteritidis were observed on XLD than TSAYE, suggesting cell injury by the antimicrobial agent.

Inhibition of \textit{E. coli} O157:H7 by the culture supernatant was similar to that of \textit{Salmonella} Enteritidis. Population of \textit{E. coli} O157:H7 decreased 2 and > 8 log cfu/ml in the 3:1 and 1:1 pathogen-antimicrobial supernatant mixtures, respectively but required
longer incubation time than was the case with *Salmonella* Enteritidis. In general, *E. coli* O157:H7 showed more resistance to the culture supernatant and lactic acid than did *Salmonella* Enteritidis (Figure 2.4).

Lactic acid and other organic acids inhibit the growth of food spoilage bacteria including gram-negatives (Doores, 1993). The undissociated forms of weak organic acids can diffuse into the cytoplasmic membrane on account of its fat solubility, reduce the intracellular pH, slow down metabolic activities, and disrupt the transmembrane proton motive force (Doores, 1993). Roth and Keenan (1971) reported that lactic acid causes sublethal injury, possibly via disruption of the polysaccharide layer of *E. coli* outer membrane. In the current study, glucose broth adjusted to pH 4.2 using lactic acid inhibited both pathogens by only 2 log cfu/ml over 8 hours at 37°C (Figures 2.3 and 2.4). This rules out lactic acid as the main cause of the inhibitory activity of *Lb. curvatus* supernatant. However, presence of lactic acid in culture supernatants helps permeabilization of the outer membrane of gram negative bacteria, and enables the antimicrobial agent to penetrate the barrier and to exert its antimicrobial effect. This is supported by Alamoki et al. (2000) who found that lactic acid increases the permeability of the outer membranes of several gram-negatives, including *E. coli* O157:H7, *P. aeruginosa*, and *Salmonella* Typhimurium.

**Mode of antimicrobial action of cell-free extract**

Cell free extract’s mode of action was investigated using *E. coli* p220. Suspension of *E. coli* p220 (10⁵ CFU/ml) in TSBYE or sodium phosphate buffer (SPB) was treated with 32, 8, and 2 CEAU/ml at 37°C for 18 hours. When *E. coli* was present in a growth
permitting medium (TSBYE), CFE of *Lb. curvatus* (32 CEAU/ml) exhibited a bactericidal action initially, and a bacteriostatic action during longer incubation (Figure 2.5a). The lack of a considerable bactericidal action may resulted from an interaction between the CFE and ingredients of the nutritionally complex media, modifying its antibacterial activity (Liao et al., 1993, 1994; Bhunia et al., 1991). Treatment of *E. coli* cultures with 8 and 2 CEAU/ml showed no inhibitory effect, compared to the control (Figure 2.5a). The antimicrobial CFE (32 CEAU/ml) was bactericidal against the indicator bacterium in sodium phosphate buffer. A reduction of approximately 5 log cycles occurred in *E. coli* population during 18 hours of incubation. Treatment of cultures with low-activity CFE (8 and 2 CEAU/ml) seemed to stimulate the growth of *E. coli* culture in sodium phosphate buffer; the CFE may have served as a nutrient that supported cell growth (Figure 2.5b).

**Efficacy of the bacteriocin-like agent in food extracts.**

The inhibitory effect of CFE on the growth of *E. coli* p220 in filtered food extracts was tested using microtiter plate method. When milk permeate was used as a medium, growth of *E. coli* p220 was retarded about 50%, compared to that of the control by dilutions up to $2^{-9}$ in the presence of CFE of *Lb. curvatus* (Figure 2.6a). In cheese whey permeate, growth inhibition of *E. coli* p220 was less than that in milk permeate (Figure 2.6b). In the filtered wiener exudate, the limited growth of *E. coli* p220 was prevented by the CFE at dilutions up to $2^{-4}$ (Figure 2.6c). Considering data in Figure 2.5a, CFE of *Lb. curvatus* inhibited *E. coli* p220 in food extracts mainly by a bacteriostatic mode of action, similar to that in the TSBYE.
In conclusion, *Lb. curvatus* OSY-HJC6, a food isolate, is a new strain that produces a bacteriocin-like substance. The antimicrobial CFE showed a broader inhibitory activity than that of the extracellular culture supernatant. Both extracellular and intracellular antimicrobial activities of *Lb. curvatus* were heat stable but were sensitive to a protease. The CFE of *Lb. curvatus* exhibited bacteriostatic mode of action in growth permitting media including food extracts, but showed bactericidal activity in buffer.

REFERENCES


Table 2.1: Zones of inhibition obtained by *Lactobacillus curvatus* OSY-HJC6 in supplemented MRS media against *Escherichia coli* p220 at 37°C.

<table>
<thead>
<tr>
<th>Hour</th>
<th>MRS+Glycine+ 0.5% TX-100</th>
<th>MRS+Glycine+ 1% TX-100</th>
<th>MRS+Glycine+ 0.5% TX-100&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>10</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>47</td>
<td>12</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>66</td>
<td>14</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>82</td>
<td>11.5</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>124</td>
<td>12</td>
<td>14</td>
<td>12</td>
</tr>
</tbody>
</table>

<sup>a</sup>Triton X-100 was added when OD<sub>600</sub> of the culture reached 0.6
<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Media</th>
<th>Relative Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram positive bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em> Scott A</td>
<td>TSBYE</td>
<td>+</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> 14579</td>
<td>TSBYE</td>
<td>+++</td>
</tr>
<tr>
<td><em>Pediococcus pentosaceus</em></td>
<td>MRS</td>
<td>−</td>
</tr>
<tr>
<td><strong>Gram negative bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> p220</td>
<td>TSBYE</td>
<td>++</td>
</tr>
<tr>
<td><em>E. coli</em> K12</td>
<td>TSBYE</td>
<td>++</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>TSBYE</td>
<td>+</td>
</tr>
<tr>
<td><em>Salmonella Enteritidis</em></td>
<td>TSBYE</td>
<td>+</td>
</tr>
<tr>
<td><em>Salmonella Typhimurium</em></td>
<td>TSBYE</td>
<td>+</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>TSBYE</td>
<td>++</td>
</tr>
</tbody>
</table>

*a TSBYE = Trypticase soy broth + 0.6 % yeast extract, MRS = de man, Rogosa and Sharpe

Table 2.2: Inhibitory spectrum of cell free extract from *Lactobacillus curvatus* OSY-HJC6 as determined by microtitier plate method at 37°C.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative activity $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>++</td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
</tr>
<tr>
<td>60 °C for 10 min</td>
<td>++</td>
</tr>
<tr>
<td>70 °C for 10 min</td>
<td>++</td>
</tr>
<tr>
<td>80 °C for 10 min</td>
<td>++</td>
</tr>
<tr>
<td>90 °C for 10 min</td>
<td>++</td>
</tr>
<tr>
<td>100 °C for 10 min</td>
<td>++</td>
</tr>
<tr>
<td>Enzymes $^b$</td>
<td></td>
</tr>
<tr>
<td>Pepsin</td>
<td>++</td>
</tr>
<tr>
<td>Trypsin</td>
<td>–</td>
</tr>
<tr>
<td>Protease</td>
<td>++</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>++</td>
</tr>
<tr>
<td>Papain</td>
<td>++</td>
</tr>
<tr>
<td>Bromelin</td>
<td>++</td>
</tr>
<tr>
<td>Lipase</td>
<td>++</td>
</tr>
<tr>
<td>Pronase</td>
<td>–</td>
</tr>
<tr>
<td>Ficin</td>
<td>++</td>
</tr>
<tr>
<td>Amylase</td>
<td>++</td>
</tr>
</tbody>
</table>

$^a$ Activity retained (+), activity lost (–)
$^b$ Enzymes were applied at a final concentration of 1 mg/ml, except lipase was added at 0.1 mg/ml.

Table 2.3: Effect of heat and enzyme treatment on activity of cell free extract from *Lactobacillus curvatus* OSY-HJC6
Figure 2.1: Isolation of colonies from foods that have antimicrobial activity against *Escherichia coli* p220.
*Lactobacillus curvatus* OSY-HJC6 overnight culture grown in 2 liters of MRS + 1% NaCl, at 30°C for 18 hours

- Harvest cell pellets by centrifugation at 13,200 g for 20 min at 4°C
- Wash twice in 20 mM sodium phosphate Buffer (SPB), pH 7.0
- Incubate the pellets with 5 mg/ml lysozyme, 0.6 M sucrose and 5 mM MgCl₂, and incubated for 3 hours at 30°C
- Wash the pellets twice in 20 mM sodium phosphate buffer (pH 7.0)
- Resuspend in 50ml of the same buffer, cooled in a water/ice mixture bath
- Sonicate the pellets at setting 5 for 5-7 minutes on ice
- Collect the supernatant by centrifugation at 34,200 g for 20 min
- Freeze-dry the supernatant and store at –18°C until use

Figure 2.2: Protocol for preparation of antimicrobial cell free extract
Figure 2.3: Bactericidal effect of culture supernatant from *Lactobacillus curvatus* OSY-HJC6 against *Salmonella* Enteritidis in TSBYE (A) and in XLD agar (B). *Salmonella* Enteritidis was incubated with various concentrations of culture supernatant, and CFU/ml were counted. (⚫) Control cells (without culture supernatant), (■) 3:1 mixture (25% supernatant), (▲) 1:1 mixture, and (●) 1:1 mixture with pH adjusted glucose medium with lactic acid.
Figure 2.4: Bactericidal effect of culture supernatant from *Lactobacillus curvatus* OSY-HJC6 against *Escherichia coli* O157:H7 in TSBYE (A) and in EMB agar (B). *Escherichia coli* O157:H7 was incubated with various concentrations of culture supernatant, and CFU/ml were counted. (●) Control cells (without culture supernatant), (■) 3:1 mixture (25% supernatant), (▲) 1:1 mixture, and (♦) 1:1 mixture with pH adjusted glucose medium with lactic acid.
Figure 2.5: Bacteriostatic and bactericidal effect of cell free extract from *Lactobacillus curvatus* OSY-HJC6 against *Escherichia coli* p220 in TSBYE (A), and in sodium phosphate buffer (20 mM, pH 7) (B). Culture of *E. coli* p220 was incubated with various concentrations of cell free extract, and CFU/ml were counted. (●) Control cells (without cell free extract), (■) 32 CEAU/ml, (♦) 8 CEAU/ml, and (▲) 2 CEAU/ml.
Figure 2.6: Inhibitory activity of cell free extract from *Lactobacillus curvatus* OSY-HJC6 against *Escherichia coli* p220 in food filtrates. (a) milk permeate, (b) cheese whey permeate, and (c) wiener exudate.
CHAPTER 3

INACTIVATION OF ESCHERICHIA COLI O157 IN CULUTRE AND SUASAGE
BY LACTOBACILLUS CASEI ANTIMICROBIAL METABOLITES AND HIGH PRESSURE PROCESSING

ABSTRACT

An isolate from a mold-ripened cheese showed antimicrobial activity against gram-positive and gram-negative bacteria. The isolate was identified as Lactobacillus casei OSY-LB6A by biochemical method, ribotyping, and membrane lipid analysis. Culture’s cell free extract (CFE) has bacteriocin-like activity against Listeria monocytogenes, Salmonella spp., and Escherichia coli strains including O157:H7, O157:H12, and two meat isolates (O157-M1 and O157-M2). The CFE exhibited bactericidal mode of action against E. coli in a buffer solution but it was bacteriostatic in a growth permitting medium. Suspensions of E. coli O157 were treated with Lb. casei CFE containing 32 colicin equivalent activity units (CEAU)/ml, with or without pressure (350 MPa for 1-20 min) at 25°C. Combination of CFE and HPP for 20 min inactivated 4.4-4.6 log and 4.8-5.1 log E. coli O157:H12 and O157:H7, respectively, and almost eliminated populations of the O157-M1 and O157-M2. Combinations pressure (400-500 MPa for 1 min) and CFE (20 and 100 CEAU/g) were applied to meat products. Treating
the pressure-resistant *E. coli* O157:H12 with 100 CEAU/g and 400 MPa inactivated 4.0-4.9 log, compared to 1.3-1.6 log reduction by HPP alone. Synergy between CFE of *Lb. casei* OSY-LB6A and high pressure processing against *E. coli* O157 strains suggests the feasibility of using this combination to minimize the risk of transmission of *E. coli* O157 by food.

**INTRODUCTION**

Enterohemorrhagic *Escherichia coli* (EHEC) strains, including O157, are important human pathogens. They cause bloody diarrhea and serious complications such as hemolytic uremic syndrome (HUS), and hemorrhagic colitis (HC) with a low infectious dose (Girffin and Tauxe, 1991). Serotype O157:H7 is one of the most virulent verotoxin-producing *E. coli*. Foods with bovine origin such as ground beef and raw milk appear to be the major source of *E. coli* O157 infection (Philips, 1999; Bell et al., 1994).

Numerous intervention strategies have been proposed to eliminate EHEC in food. Bacteriocins of lactic acid bacteria (LAB) are useful for food biopreservation but these agents have no or limited activity against EHEC. Lactic acid bacteria produce antimicrobial substances such as lactic acid, acetic acid, diacetyl, and antimicrobial proteins, which inhibit or inactivate spoilage and pathogenic microorganisms. Meat microbiota includes bacteriocin-producing LAB such as *Lb. plantarum, Lb. sakei, Lb. curvatus, Pediococcus acidilactici, P. pentosaceus* (Hugus, 1998). Bacteriocins produced by LAB have been widely studied, due to their potential use as natural food preservatives.
(Lindgren and Dobrogosz, 1990; Stiles and Hastings, 1991; Ray, 1992). Inhibitory spectrum of LAB bacteriocins varies; some are active against diverse bacterial groups, whereas others only affect related species (Klaenhammer 1993; Jack et al., 1995; Nemcova, 1997). Bacteriocins with broad inhibitory spectrum are most useful in controlling growth of spoilage and pathogenic bacteria in foods (Nettles and Barefoot, 1993; De Vuyst and Vandamme, 1994).

Emerging technologies such as high pressure processing (HPP) and gamma radiation are effective against EHEC in food. High pressure processing targets foodborne pathogenic and spoilage microorganisms without altering food quality (Knorr, 1993; Cheftel, 1995). High pressure technology is commonly proposed for combination treatment as it inactivates microorganisms by acting against multiple targets including intracellular and membrane bound enzymes (Mackey et al., 1994; Wouters et al., 1998). Moderate HPP with other preservation factors have been investigated as synergistic antimicrobial combinations against pathogenic E. coli. In gram negative bacteria, membrane damages caused by HPP increased their sensitivity to nisin and lysozyme (Kalchayanand et al., 1994; Hauben et al., 1996). Others reported the use of HPP in combination with nisin and lysozyme (Masschalck et al., 2000), lysozyme (Masschalck et al., 2001), lactoperoxidase system (García-Graells et al., 2003), heats (Alpas and Bozoglu, 2000), and temperature and pH (Alpas et al., 2000). Most of the combination treatments showed significant synergistic effects against the target microorganisms. Combination treatments, however, may not eliminate the tailing behavior, i.e., a fraction of pathogen’s population remains viable after an extended treatment (Masschalck et al., 2001; Ludwig et al., 1992; Patterson et al., 1995a).
The objectives of this study include (a) screening for food-grade microorganisms that produce antimicrobials against foodborne pathogens, particularly gram-negative bacteria, (b) determining the efficacy of these strains against gram positive and negative bacteria, (c) investigating the feasibility of using the new antimicrobial agent, in combination with high pressure processing, to eliminate *E. coli* O157 strains in meat, and (d) to evaluate effect of the treatment on cellular components of the *E. coli* using differential scanning calorimetry.

**MATERIALS AND METHODS**

**Screening for antimicrobial-producing bacteria**

Food (Parmesan, Cheddar, mold ripened cheeses, and specialty cheeses) and environmental (meat and dairy plants, food processing facilities, and home kitchens) samples were collected and their microbiota were screened for ability to produce antimicrobials against a selected indicator strain. Screening and isolation of bacteria with antimicrobial activity was made using hydrophobic grid membrane filters, as described by Ryser and Richard (1992) with modifications. Briefly, samples were homogenized, diluted and dilutions were filtered through a hydrophobic-grid filter (QA Life Sciences Inc., San Diego, CA) mounted on an autoclaved filtering apparatus. Filters were overlaid on tryptose agar (TA; Difco Laboratories, Sparks, MO), incubated at 37 °C, and filters were removed. The incubated agar plates, which may contain antimicrobial metabolites,
were overlaid with soft tryptose agar (0.75% agar), seeded with *E. coli* p220 (~10^6 CFU/ml), the indicator bacterium. Inoculated plates were incubated and zones of inhibition were sought.

**Characterization and identification of an antimicrobial-producing isolate**

The isolate with strong antimicrobial properties was identified using colony and cell morphology, biochemical analysis (API 50 CH strip, bioMérieux, Inc., Hazelwood, MO), membrane lipid profile analysis (Microbial ID, Inc., Newark, DE), and ribotyping (Kraft Foods Basic Science, Glenview, IL). The antimicrobial-producing isolate was identified as *Lb. casei* OSY-LB6A. *Lactobacillus casei* OSY-LB6A was compared to *Lb. casei* ATCC 334 and other lactobacilli using pulsed field gel electrophoretic typing technique (Tynkkynen et al., 1999). Bacterial DNA was prepared for pulsed filed gel electrophoresis (PFGE) as described by Tynkkynen et al. (1999) with slight modification. Glycine and phenylmethylsulphonyl fluoride were omitted from the growth medium and the wash buffer, respectively. Agarose-imbedded DNA for PFGE analysis was digested with 2 restriction enzymes, ApaI and Smal (Invitrogen Life Technologies, Carlsbad, CA). The resulting restriction fragments were separated in agarose gel by PFGE. The electrophoresis conditions were 1.6 % agarose gel, 0.5 X TBE buffer (45 mM Tris-borate, 1mM EDTA), 5 V/cm, 1-15 s switching time for 42 hours (Jenkins et al. 2002).

**Microorganisms**

*Lactobacillus casei* OSY-LB6A was isolated from food and identified during the course of this study. *P. pentosaceus, Lb. casei* ATCC 334, *Lb. curvatus* OSY-HJC6, *Lb.
curvatus ATCC 25601, *Lb. Leichmannii*, and *Lactococcus lactis* ATCC 11454 were obtained from the culture collection of the Food Safety Laboratory, Ohio State University. Cultures were grown in MRS broth (Difco). Working cultures were maintained on slant of MRS agar, and stored at 4°C.

*Escherichia coli* (395T4958, K12, p220, O157:H7, O157:H12, O157-M1, and O157:M2), *L. monocytogenes* Scott A, *Bacillus cereus* 14579, *Pseudomonas fluorescens*, *Salmonella enterica* serovar Enteritidis, and *Salmonella Typhimurium*, were obtained from the culture collection of the Food Safety Laboratory, Ohio State University. Cultures were grown in Trypticase Soy Broth (BBL, Sparks, MD), supplemented with 0.6% Yeast Extract (Difco) (TSBYE). Working cultures were maintained on slant of Trypticase Soy Agar supplemented with 0.6% Yeast Extract (TSAYE) and stored at 4°C.

Each stock culture was maintained in the respective growth media, containing 20% glycerol, and stored at −80°C. Before use in experiments, the strains were transferred into fresh growth media and incubated at 37°C for 18 hours; this was followed by two consecutive transfers in the medium and incubation under the conditions just indicated. The final counts of cultures were approximately $10^9$ CFU/ml.

**Antimicrobial cell-free extract of *Lactobacillus casei* OSY-LB6A**

*Lactobacillus casei* was inoculated into 2 liters of MRS medium, supplemented with 1% NaCl (Casla et al., 1996), and the mixture was incubated at 30°C for 18 hours. Cells were harvested by centrifugation at 13,200 x g for 20 min at 4°C and washed twice in 20 mM sodium phosphate buffer (SPB), pH 7.0. The cell pellet was treated with 5 mg/ml lysozyme (Sigma, St. Louis, MO), 0.6 M sucrose and 5 mM MgCl₂, and incubated
at 30°C for 3 hours. After incubation, the pellet was washed twice in 20 mM sodium phosphate buffer (pH 7.0), suspended in 50 ml of the same buffer, cooled in a water/ice mixture bath, and sonicated (Torbeo ultrasonic processor, Cole Parmer, Vermon Hill, IL) at setting 5, for 5 minutes while maintaining the cooling. The supernatant was collected by centrifugation at 34,200 x g for 20 min, freeze-dried, and stored at –18°C until use. The freeze-dried powder (0.2 g) was resuspended in 1 ml of the same buffer; this solution was designated as cell-free extract (CFE).

**Stability of cell-free extract**

Portions of CFE were subjected to heat (100°C for 10 min) or treated at 37°C for 2 hours with pepsin, trypsin, protease, chymotrypsin, bromelin, lipase, α-amylase, pronase, ficin, and papain. Enzymes were obtained from Sigma except pronase, ficin, and papain, which were from Calbiochem (Sandiego, CA). Enzymes were applied at a final concentration of 1 mg/ml, except lipase, which was added at 0.1 mg/ml. Fifty µl of the supernatant and enzyme mixture was assayed for the residual activity using the well diffusion assay (Lee et al., 1999).

**Antimicrobial activity assay**

The antimicrobial activity of CFE was tested in a microtiter plate assay system, as described by Jimenez-diaz et al. (1995), with modifications. Each well of the microtiter plate (Becton Dickinson, Franklin lakes, NJ) contained 25 µl 2X TSBYE medium, 25 µl CFE or its two-fold dilutions, and 10 µl indicator strain, *E. coli* p220 (10⁶ CFU/ml). Positive and negative control wells contained 25 µl TSBYE medium, 25 µl SPB, with or
without added inoculums (10 µl), respectively. The microtiter plate cultures were incubated at 37°C for 15 hours, and growth inhibition of the indicator strain was assessed by measuring Absorbance at 600 nm in a microtiter plate reader (Vmax Kinetics Microplate Reader, Molecular Devices, Sunnyvale, CA). The absorbance value of each dilution without the indicator was subtracted from the corresponding value of the same dilution containing the indicator microorganism. The antimicrobial activity of colicin (Sigma) against *E. coli* p220 was used as a reference for measuring the activity in the CFE. Commercial colicin is produced by some *E. coli* strains and the powder contains 20,000 units/mg pure proteins. One unit per ml is the minimal concentration required to cause a zone of clearing on a lawn of *E. coli* ATCC 9637 cells. The highest dilution of the CFE causing inhibition of the indicator, compared to the positive control, was converted to colicin-equivalent activity units (CEAU).

**Preparation of food extracts**

The inhibitory activity of CFE from *Lb. casei* was tested using *E. coli* p220 in three food extracts. These include µ-filtered wiener (frankfurter) exudate, milk permeate, and cheese whey permeate. The food extracts were prepared as follows. The wiener exudates were collected from retail packages (Ball Park Brands) and sterilized using 0.2-µm filters. For milk permeate, a filtration unit (Filtron Technology Corporation, Northborough, MA) with a 100 kDa molecular weight cut off was used. Milk sample (15 ml, 2% fat) was dispensed in the filter unit, centrifuged at 4,340 x g for 20 minutes and a clear milk permeate was obtained. Cheese whey permeate was obtained during manufacture of experimental Cheddar cheese (Department of Food Science and
Technology, OSU) and filter sterilized. Food extracts were stored at -20°C until use. Growth inhibition of \( E. \ coli \) p220 by \( Lb. \ casei \) CFE in food extract was tested using microtiter plate method.

**Antimicrobial activity of Lactobacillus casei CFE against Escherichia coli p220**

Freeze dried CFE of \( Lb. \ casei \) (0.2 g) was prepared in 1 ml of 20 mM sodium phosphate buffer (pH 7.0) and two fold dilutions were prepared in the same buffer. Overnight culture of \( E. \ coli \) p220 was diluted into fresh TSBYE medium or 20 mM sodium phosphate buffer (pH 7) to a final concentration of \( \sim 10^6 \) cells/ml. Aliquots (0.1 ml) of 3 different concentrations of \( Lb. \ casei \) CFE (2, 8, and 32 CEAU/ml) were added to 0.9 ml \( E. \ coli \) p220 cell suspension. The control received 0.1 ml buffer instead of the CFE. The mixtures were incubated at 37°C, and aliquots were taken at 3-hours intervals, during 18 hours of incubation. The number of viable bacterial cells was determined by spread-plating on TSAYE agar. The plates were incubated at 37°C and the colony forming units were counted.

**Inactivation of Escherichia coli O157 strains with combinations of cell-free extract and high pressure processing**

Treatments are (a) control, (b) CFE alone, (c) HPP alone, and (d) combination of the treatments. Nine hundred µl aliquots of \( E. \ coli \) O157 cultures were placed in sterile stomacher bags (polyethylene, 4”x6”, Fisher Scientific International Inc., Pittsburgh, PA). Control was received 0.1 ml of sodium phosphate buffer (SPB, 20mM, pH 7). For CFE treatment, 0.1ml CFE (32 CEAU/ml) was added to the culture for 30 min at 25°C.
Control and CFE treatment samples were heat sealed, and placed on ice before analyzed. For HPP and combination treatments, 0.9 ml aliquots of *E. coli* culture were dispensed in sterile plastic bags and received 0.1 ml of SPB, or the same volume of CFE (32 CEAU/ml), respectively; bags were first heat sealed, double sealed in a bag containing a hypochlorite solution, and placed in water/ice bath until pressure treated. The sealed bags were high pressure processed at 350 MPa for 1-20 min, using a hydrostatic food processor (Quintus QFP6, Flow Pressure Systems, Kent, WA), containing a water/propylene glycol (Houghto-Safe 620-TY, Houghton International, Inc., Valley Forge, PA) mixture (1:1, v/v) as the pressure transmitting fluid. Adiabatic heating due to compression was taken into account by maintaining the samples at appropriate initial temperatures such that the final temperature during the pressurization was 30°C. The holding time at the desired pressure ranged from 1 to 20 min. For each pressure, an untreated sample acted as a control and was used to provide an estimate of the initial numbers of viable cells present in the sample before pressurization. Treated and non-treated samples were enumerated on TSAYE agar.

**Combination treatment of a meat product by CFE and HPP**

Commercially sterile canned pieces of sausage (Vienna sausages, Armour ®) were purchased from a local supermarket and used in a challenge study. Sausages were treated with (a) none (control), (b) CFE (20 and 100 CEAU/g), (c) HPP (400 and 500 MPa for 1 min at 25°C), and (d) combination of the treatments. One Vienna sausage was placed in a bag, weighed (~16g), and inoculated with *E. coli* O157 strains separately so that each product contained approximately 10^6 CFU/g. The inoculum was distributed
evenly in the bag contents by hand-mixing for 5 min. The bag contents were refrigerated for 30 min to allow attachment of the culture onto the sausage. Non-treated (control) and HPP treated *E. coli* O157 cultures received 0.1 ml SPB instead of CFE. For CFE treatment, the CFE (20 or 100 CEAU/g) was added to the inoculated products and the bag contents were refrigerated for 30 min. For combination study with HPP, 20 and 100 CEAU/g of CFE were used in combination 1 and 2, respectively. The sausage, mixed with *E. coli* O157 culture was high pressure processed at 500 MPa for 1 min or 400 MPa for 1 min in the presence or absence of two concentrations of CFE. The strains of *E. coli* in treated and non-treated samples were enumerated on a nonselective (TSAYE) and a selective (McConkey Sorbitol, MSA, Difco) agar and total and uninjured populations of the pathogen were determined, respectively.

**Preparation of *Escherichia coli* for thermal analysis by differential scanning calorimetry (DSC)**

*Escherichia coli* K12 was tested in these experiments. Cultures of *E. coli* K12 were grown in TSBYE for 13 hours at 37°C. Seventy ml of *E. coli* K12 culture was treated with *Lb. casei* CFE (32 CEAU/ml for 30 min), HPP (350 MPa, 1 min) or combination of the treatments. HPP treatment was carried out under the same conditions with *E. coli* O157 strains as described in previous method section. One ml aliquots of the culture were serially diluted and pour plated using a TSAYE and MSA to determine total and uninjured cell counts, respectively. Remaining cells were centrifuged at 10,000 x g for 10 min and their pellets were washed for differential scanning calorimetry (DSC) according to the procedure described by Lee and Kaletunc (2002). Cell pellets (~60 mg
wet weight) were transferred into DSC pans. Reference was prepared with water approximately 80% of sample weight (~48mg). Cell pellets in pans were heated in differential scanning calorimeter (DSC 111, Setaram, France) from 1 to 140°C with 4°C/min heating rate.

**Statistical analysis**

MINITAB statistical program (Minitab Inc., State College, PA) was used to analyze inactivation values. One-way analysis of variance was performed for the effect of high pressure and combination treatment with CFE against *E. coli*. When treatment factors were significant, Tukey’s range test was used for multiple comparisons of means. Variables compared include bacterial counts on TSAYE and MSA.

**RESULTS AND DISCUSSION**

**Isolation and identification of an antimicrobial-producing bacterium**

Analysis of more than 100 food and environmental samples produced a limited number of isolates exhibiting antimicrobial activity against the gram-negative indicator bacterium, *E. coli* p220. An isolate from a mold-ripened cheese showed antimicrobial activity against *E. coli* p220, *E. coli* O157 strains (O157:H7, O157:H12, O157-M1, and O157-M2), selected *Enterobactericeae*, and gram-positive bacteria. The CFE of *Lb. casei* inhibited *L. monocytogenes* Scott A and *B. cereus* strongly, but not *P. pentosaceus* (Table 3.1). The isolate was characterized by biochemical testing, using API system (API 50 CH strip), ribotyping, and membrane lipid profile analysis, and it was identified as *Lb. casei*. 

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The isolate was compared with antimicrobial-producing and non-producing lactobacilli, using PFGE, a genetic typing technique. Restriction enzymes generated different DNA banding patterns for the food isolate, compared with the other strains (Figure 3.1). The isolate was designated as *Lb. casei* OSY-LB6A.

**Inhibitory characteristics of cell free extract**

The inhibitory activity of CFE from *Lb. casei* OSY-LB6A was tested under conditions that eliminate possible inhibitory effect of organic acids (both neutralized CFE and acidified broth samples), and hydrogen peroxide (catalase treated CFE). The natural pH of *Lb. casei* OSY-LB6A CFE was 4.2. When neutralized to pH 7.2, the CFE retained most of its inhibitory activity, confirming that the source of the antimicrobial activity cannot be attributed to the low pH of the culture. Similar results were reported for several bacteriocins produced by lactic acid bacteria (Bhunia et al., 1988; Hurst, 1981; Toba et al., 1991). Catalase-treated *Lb. casei* CFE did not lose any activity compared to untreated CFE. Plaques were not detected on lawns of indicator strain prepared by mixing indicator cells with portion of agar that had been removed previously from clear zones of inhibition. This result rules out the possibility that a lytic phage is causing the inhibitory activity. The nature of antimicrobial agent was evaluated with various enzymes. The activity of *Lb. casei* CFE was eliminated by pronase, but showed resistance to most proteolytic enzymes. Lipase had also an influence on the activity of CFE, indicating that a lipid is critical to its biological activity (Table 3.2). When *Lb. casei* CFE (pH 4.2) was heated at 60, 80, 90 and 100°C for 10 min, the inhibitory activity of the extract against *E. coli* p220 remained unchanged compared to the unheated control (Table 3.2).
The data show that the inhibitory activity of CFE from *Lb. casei* OSY-LB6A is bacteriocin like substance. Bacteriocin is a protein or a peptide with a narrow spectrum of activity, a bactericidal mode of action, and plasmid encoded function (Jack et al., 1995; Tagg et al., 1976). Researchers have shown that plasmid borne genetic determinants may not be necessary, as bacteriocins from lactic acid bacteria include plasmids encoded bacteriocins such as pediocin AcH (Ray et al., 1989), and pediocin PA-1 (Bhunia et al., 1988), and chromosomal determinants in the case of lactacin B (Barefoot and Klaenhammer, 1983), and helveticin J (Joerger and Klaenhammer, 1986).

Bacteriocins produced by strains of *Lb. casei* have been reported. They are caseicin 80 (Rammelsberg et al., 1990; Rammelsberg and Radler, 1990), lactocin 705 (Cuozzo et al., 2000), and antimicrobial substances produced by *Lb. casei* ssp. *casei* LC-10 and *Lb. casei* ssp. *pseudoplantarum* LB1931 (Huttunen et al. 1995). Most of these antimicrobials are active against strains closely related to the producer but they were not active against gram-negative bacteria. Lactocin 705 is a two-component bacteriocin from *Lb. casei* CRL705, of which lac705α and 705β peptides are responsible for receptor recognition and antimicrobial activity on the cell membrane of target cells, respectively (Cuozzo et al., 2003). Although the antimicrobial agent addressed in the current study seems to be a bacteriocin, further investigation is needed to identify and fully characterize this agent.

**Antimicrobial assay of cell-free extract**

A conventional well diffusion assay is convenient to use but often generates ambiguous results due to its low sensitivity. The microtiter plate method made it easy to
compare the antimicrobial activity in liquid medium. When culture supernatant of nisin producing *Lc. lactis* was tested as a positive control against its known sensitive indicator, *Lb. leichmannii*, the growth of indicator was inhibited in a concentration dependent manner. Treatments of *E. coli* p220 cell suspension in TSBYE broth with CFE of *Lb. casei* using microtiter plate method showed delay of growth of the indicator bacterium, similar to that of culture supernatant by *Lc. lactis* (data not shown). Presence of CFE in milk permeate prevented the growth of *E. coli* p220 up to the $2^{-4}$ dilutions; higher dilutions allowed the growth of the indicator bacterium (Figure 3.2a). In cheese whey permeate, the inhibitory effect of the CFE from *Lb. casei* on the growth of *E. coli* p220 was similar to that in milk permeate (Figure 3.2b). Filtered wiener exudate did not support luxurious growth of *E. coli* p220. However, the limited growth of *E. coli* p220 was prevented or inhibited in the presence of CFE (Figure 3.2c). In general, the cell free extract from *Lb. casei* strongly inhibited growth of *E. coli* p220 in three filtered food extracts.

**Antimicrobial mode of Lactobacillus casei cell-free extract against Escherichia coli**

Cell free extract’s mode of action was investigated using *E. coli* p220, a nonpathogenic strain. Suspension of *E. coli* p220 ($10^6$ CFU/ml) in sodium phosphate buffer (SPB) or TSBYE was treated with 32, 8 and 2 CEAU/ml at $37^\circ C$ for 18 hours. The effect of CFE (32 CEAU/ml) was bactericidal against *E. coli* cells in buffer. A reduction of approximately 5 log cycles occurred in *E. coli* cells within 3 hours of incubation. Treatment of cultures with low concentrations of CFE (8 and 2 CEAU/ml) seemed to stimulate the growth of *E. coli* cells in sodium phosphate buffer; low concentration of
CFE may have served as a source of nutrients for the growing cells (Figure 3.3a). When *E. coli* was suspended in a growth-permitting medium (TSBYE), the CFE of *Lb. casei* (32 CEAU/ml) exhibited bacteriostatic mode of action against the indicator during 18 hours of incubation. This may be due to a possible interaction of CFE with ingredients of nutritionally complex media, modifying its antibacterial activity (Liao et al., 1993, 1994; Bhunia et al., 1991). Low concentration of CFE (8 CEAU/ml) showed inhibitory effect but not with 2 CEAU/ml. It is likely that CFE behaves bacteriostatically under growth permitting conditions like in foods. Therefore combined use of CFE with high pressure processing was considered in this study as a multiple hurdle approach (Leistner, 1985).

**Inactivation of *Escherichia coli* by cell-free extract and high pressure combination.**

*Escherichia coli* O157 strains in TSBYE were subjected to CFE (32 CEAU/ml for 30 min at 25°C), HPP (350 MPa for 1-20 min at 25°C), or to a combination treatment (Figure 3.4). Exposure of *E. coli* cultures to CFE for 30 min caused minimal effect on the pathogens’ viability, despite their sensitivity to CFE of *Lb. casei* in microtiter plate assay when incubated for 15 hours at 37°C (data not shown). This seems to be due to the bacteriostatic effect of CFE in TSBYE media, and to high inoculum (~10⁹ CFU/ml) used in this study than that in microtiter plate assay (~10⁶ CFU/ml). High count may decrease efficacy of the CFE against *E. coli* strains. Leriche et al. (1999) reported that the activity of nisin producing *Lc. lactis* against *L. monocytogenes* was greatly dependent on the inoculum size. Researchers observed similar results with other bacteriocins that inactivation was greater when low levels of *Listeria* were present in foods, such as meat and cheese (Nielsen et al., 1990; Maisnier-Patin et al., 1992; Goff et al., 1996).
Escherichia coli strains varied in sensitivity to HPP. Variable resistance to HPP among E. coli serotypes has been observed earlier (Patterson et al., 1995a,b; Alpas et al., 1999; Benito et al., 1999). Moreover, Hauben et al. (1997) reported that pressure sensitive E. coli strains could develop resistance to HPP by spontaneous mutation. In our study, the E. coli O157:H12 seems to be the most resistant, followed by strain O157:H7, and the meat isolates (O157-M1 and O157-M2) showed the greatest sensitivity to HPP. Pressure treatment less than 10 min at 350 MPa caused minimal inactivation and the viability loss of strains O157:H12 and O157:H7 remained below 1 log after 10 min of pressurization. However, increasing the pressurization time to 15 min or longer had a significant effect on the viability loss of the strains, which ranged from 1.1-1.2 in O157:H12 and O157:H7, to 4.1-5.5 in the meat isolates (p < 0.05).

Synergy between HPP and CFE was evident. The combination treatment caused a significant (p<0.05) inactivation in the pressure resistant O157:H12 strain, compare to pressure-treated control, and synergy increased with increase in treatment time, causing viability loss of 4.3-4.6 logs by combination treatment for 20 min (p < 0.05) (Figure 3.4a).

Pathogenic E coli O157:H7 showed inactivation pattern similar to that of strain O157:H12, with viability loss of 5.0-5.3 logs of the population by combination treatment for 20 min (p< 0.05) (Figure 3.4b). Treatment of the pressure sensitive strains (O157-M1 and O157-M2) with 350 MPa for 1 min in the presence of CFE (32 CEAU/ml) inactivated 2.4-3.2 logs. When the pressurization time increased to 15-20 min, the combination treatment decreased pathogen’s population >8 log (Figures 3.4c, and 3.4d).
These data indicate that the combination treatments had a significant synergistic effect against \textit{E. coli} O157 strains, including the enterohemorrhagic \textit{E. coli} (p < 0.05). Synergy was most evident in the pressure sensitive strains.

\textbf{Inactivation of \textit{Escherichia coli} O157 strains in meat products by cell-free extracts and high pressure.}

The combined effect of \textit{Lb. casei} CFE and HPP against \textit{E. coli} strains was tested in a commercial canned sausage (Vienna sausages). \textit{Escherichia coli} O157:H7, O157:H12, and O157-M1 are variable in pressure resistance and thus were tested in sausage. Sausages were inoculated to contain $10^6$ CFU/g \textit{E. coli} O157 strains. The inoculated product was treated with HPP (500 MPa for 1 min) in combinations with CFE (20 or 100 CEAU/g). Treatment with HPP inactivated 1.4 logs \textit{E. coli} O157:H12, 3.3 logs \textit{E. coli} O157:H7, and 4.5 logs \textit{E. coli} O157-M1 (Figure 3.5). No apparent synergistic effect was observed due to the combination treatments, compared to that caused by HPP alone. Inactivation by the combination treatment seemed to be caused mainly by HPP. However populations of the pathogen on nonselective and selective media were clearly different; this indicates that presence of CFE during the pressurization injured a fraction of the population. Although the extent of injury by low-and high-activity of CFE with pressure treatment was not very pronounced, the data suggested a possibility of synergistic effect by the combination treatments.

Pressure treatment of \textit{E. coli} O157 strains, at 400 MPa for 1 min, produced an inactivation pattern similar to that observed at 500 MPa (Figure 3.6). Combining HPP with low-activity CFE caused inactivation levels similar to these for HPP alone. However,
pressurization of cells with high-activity CFE significantly reduced the number of survivors by 2.0-2.9 logs in O157:H12 strain (p < 0.05). A significant increase in the injury (4.0-4.9 logs) by combination treatment with high-activity CFE was observed in the strain O157:H12, compared to that by HPP alone or combination with low-activity CFE (p < 0.05) (Figure 3.6a). In the case of strain O157:H7, inactivation by each treatment was similar to that of strain O157:H12, except that HPP alone had no significant effect on the cells’ viability and that less injury was observed than in strain O157:H12. This may imply the difficulties to inactivate the pathogenic E. coli O157:H7 in food system (Figure 3.6b). Similar observations were noticed when E. coli O157-M1 was tested by the pressure-CFE combination. This strain, however, is clearly sensitive to pressure and pressure-CFE combinations, when compared to the other E. coli O157 strains including the O157:H7 enterohemorrhagic strain (Figure 3.6c).

**Thermal analysis of cells by differential scanning calorimetry (DSC)**

The effect of CFE and pressure on cellular components of E. coli K12 was evaluated by DSC (Figure 3.7). Analysis of bacteria with DSC reveals the influence of thermal treatments on the structural components in relation to cell injury and death (Stephens and Jones, 1993; Mohacsi-Farkas et al., 1994; Niven et al., 1999; Lee and Kaletunc, 2002). When untreated E. coli was heated, endothermic transitions corresponding to denaturation of cell structures such as ribosome (peak a: 71°C), and DNA (peak b; 93°C), and outer membrane (peak c; 115°C) were observed (Figure 3.7a, curve 1). Denaturation of ribosome in E. coli was identified in the range between 55 to 90°C (Mackey et al., 1991). Exposure of cells to CFE (32 CEAU/ml) for 30 min caused
no apparent changes in the peak area in the DSC curve (Figure 3.7a, curve 2). Lack of viability loss by CFE concurs with unaltered DSC thermogram. Treatment with HPP at 350 MPa for 1 min inactivated cells’ population by 2 logs, and the peak associated with ribosome denaturation (peak a) decreased correspondingly (Figure 3.7a, curve 3). Damages in cellular ribosome and membrane by HPP are attributed to eventual cell death (Earnshaw et al., 1995; Patterson et al., 1995a; Niven et al., 1999). When *E. coli* was pressurized in the presence of CFE, the combination treatment caused a viability loss similar to that resulting from HPP treatment alone, (population decreased < 3 logs CFU/ml). This combination produced a thermogram showing the ribosome and DNA peaks, however, the ribosome denaturing peak (Figure 3.7a, curve 4) decreased, when compared with that resulting from the pressure treatment alone (Figure 3.7a, curve 3). The effect of HPP and CFE combination on the cellular components was more pronounced in *Listeria innocua* (Chung et al., 2004) than in *E. coli* under the same condition. The resistance of *E. coli* to the combination treatment could be attributed to the presence of the outer membrane in gram negative bacteria as an effective permeability barrier to the CFE. Changes associated with the damage of the cellular components in DSC thermogram correlate well with the loss of cell viability. The DSC data suggest that presence of CFE may cause considerable damage to cellular components during the high pressure treatment.

Gram negative bacteria are generally more pressure-sensitive than gram positive bacteria (Hoover et al., 1989). However, *E. coli* O157 strains used in this study appear to be more pressure resistant than is *L. monocytogenes* under the experimental condition. This was also supported by the result of DSC analysis of *E. coli* and *Listeria* (Chung et
al., 2004). It was reported that certain *E. coli* O157:H7 strains are among the most pressure resistant vegetative bacteria known (Benito et al., 1999; Patterson et al., 1995). Ludwig and Schreck (1997) reported that the sensitivity is correlated to cell morphology, with cocci being the most pressure resistant (Ludwig and Schreck, 1997). Garriga et al. (2002) supported this observation with their results in which *Staphylococcus* was the most resistant to HPP at 400 MPa for 10 min, followed by the *E. coli* strains, among 8 strains from various genus, including *Listeria, Salmonella, Staphylococcus, Escherichia, Lactobacillus, and Leuconostoc*. Alpas et al. (1999) studied variation in pressure resistance with different species of exponential phase cells. The authors reported that viability loss ranged from 0.9 to 3.5 in *L. monocytogenes* strains and 2.8-5.6 in six *E. coli* O157 strains at 345 MPa for 5 min in 1% pepton solution. The variation was diminished when the cells were pressurized at high temperature (50°C). As the sensitivity of microorganisms is influenced by factors such as pressurization time and temperature, pressure magnitude, microbial types and growth stages, suspending media, and presence of antimicrobial compounds (Mackey et al., 1995; Patterson et al., 1995b; Kalchayanand et al., 1998a, b), these factors should be taken into account for comparison.

The pressure treatment causes damage to the cell membrane, which may lead to death of bacteria (Smelt, 1998; Wouters et al., 1988). In addition, HPP may facilitate the access of the antimicrobial agents to the cytoplasmic membrane. Therefore sublethal pressure injury may sensitize bacterial cells to the action of antimicrobial agents (Kalchayanand et al., 1994; 1998a; 1998b). It was reported that a stiff membrane sensitizes microorganisms to HPP (Russell et al., 1995; Smelt et al., 1994). Presence of membrane-active antimicrobial agents, such as bacteriocins, may increase membrane stiffness by
binding to the phospholipid headgroups and locally immobilizing the cytoplasmic membrane (Ter Steeg et al., 1999). Moreover, modification of fatty acid by HPP has been reported in lipid bilayer and in the cells grown under pressure (Macdonald, 1992; Fujii et al., 1997). A relation has been reported between phospholipids composition of the membrane of the susceptible microorganisms and resistance to pressure and/or antimicrobial agents (Ludwig et al., 1992; Russell et al., 1995; Smelt et al., 1994). Factors including membrane fluidity and structure of the antimicrobial agents contribute to the sensitivity of cells to the combination treatments (Ter Steeg et al., 1999). Although the mechanism of \textit{Lb. casei} CFE action against \textit{E. coli} has not been studied, future research revealing the chemistry of the CFE will help understand the synergy between this agent and HPP.

**CONCLUSION**

In conclusion, a new \textit{Lactobacillus} strain was isolated from a mold-ripened cheese. The strain produces a bacteriocin-like substance with an inhibitory activity against gram-positive and negative-bacteria. The CFE of \textit{Lb. casei} is potentially useful as biopreservatives in food, particularly meat. Our study demonstrated the inhibitory activity of CFE from \textit{Lb. casei} OSY-LB6A against strains of \textit{E. coli} O157. The combined use of the CFE with high pressure processing caused a significant viability loss in the strains than their single use. The synergy between CFE and HPP suggests potential application of the combination treatment in food application to control this pathogen.
REFERENCES


Smelt, J.P.P.M. 1998. Recent advances in the microbiology of high pressure processing. Trends Food Sci. Technol. 9, 152-158.


<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Media</th>
<th>Relative Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram positive bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em> Scott A</td>
<td>TSBYE</td>
<td>+++</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> 14579</td>
<td>TSBYE</td>
<td>+++</td>
</tr>
<tr>
<td><em>Pediococcus pentosaceus</em></td>
<td>MRS</td>
<td>−</td>
</tr>
<tr>
<td><strong>Gram negative bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> p220</td>
<td>TSBYE</td>
<td>++</td>
</tr>
<tr>
<td><em>E. coli</em> K12</td>
<td>TSBYE</td>
<td>++</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>TSBYE</td>
<td>++</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H12</td>
<td>TSBYE</td>
<td>++</td>
</tr>
<tr>
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<td>TSBYE</td>
<td>++</td>
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<tr>
<td><em>E. coli</em> O157-M2</td>
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<td>++</td>
</tr>
<tr>
<td><em>Salmonella</em> Enteritidis</td>
<td>TSBYE</td>
<td>+</td>
</tr>
<tr>
<td><em>Salmonella</em> Typhimurium</td>
<td>TSBYE</td>
<td>+</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>TSBYE</td>
<td>+</td>
</tr>
</tbody>
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\(^a\) TSBYE = Trypticase soy broth + 0.6 % yeast extract, MRS = de man, Rogosa and Sharpe

Table 3.1: Inhibitory spectrum of cell free extract from *Lactobacillus casei* OSY-LB6A by microtiter plate method at 37ºC.
Table 3.2: Effect of heat and enzyme treatment on activity of cell free extract from *Lactobacillus casei* OSY-LB6A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative activity (^a)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
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</tr>
<tr>
<td>Temperature</td>
<td></td>
</tr>
<tr>
<td>60°C for 10 min</td>
<td>++</td>
</tr>
<tr>
<td>70°C for 10 min</td>
<td>++</td>
</tr>
<tr>
<td>80°C for 10 min</td>
<td>++</td>
</tr>
<tr>
<td>90°C for 10 min</td>
<td>++</td>
</tr>
<tr>
<td>100°C for 10 min</td>
<td>++</td>
</tr>
<tr>
<td>Enzymes (^b)</td>
<td></td>
</tr>
<tr>
<td>Pepsin</td>
<td>++</td>
</tr>
<tr>
<td>Trypsin</td>
<td>++</td>
</tr>
<tr>
<td>Protease</td>
<td>++</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>++</td>
</tr>
<tr>
<td>Papain</td>
<td>++</td>
</tr>
<tr>
<td>Bromelin</td>
<td>++</td>
</tr>
<tr>
<td>Lipase</td>
<td>–</td>
</tr>
<tr>
<td>Pronase</td>
<td>–</td>
</tr>
<tr>
<td>Ficin</td>
<td>++</td>
</tr>
<tr>
<td>Amylase</td>
<td>++</td>
</tr>
</tbody>
</table>

\(^a\) Activity retained (+), activity lost (–).  
\(^b\) Enzymes were applied at a final concentration of 1 mg/ml, except lipase was added at 0.1 mg/ml.
Figure 3.1: Comparison of *Lactobacillus casei* OSY-LB6A and other lactobacilli by genetic typing using pulsed field gel electrophoresis.
Figure 3.2: Activity of cell free extract from *Lactobacillus casei* OSY-LB6A against *Escherichia coli* p220 in food extracts. (a) milk permeate, (b) cheese whey permeate, and (c) wiener exudate.
Figure 3.3: Bactericidal and bacteriostatic effect of cell free extract from *Lactobacillus casei* OSY-LB6A on *Escherichia coli* p220 in sodium phosphate buffer (20 mM, pH 7) (A), and in TSBYE (B). Culture of *E. coli* p220 was incubated with various concentrations of cell free extract, and survivors were counted. (●) Control cells (without cell free extract), (■) 32 CEAU/ml, (◆) 8 CEAU/ml, and (▲) 2 CEAU/ml.
Figure 3.4: Inactivation of *Escherichia coli* O157:H12 by cell-free extract from *Lactobacillus casei* OSY-LB6A (32 CEAU/ml, for 30 min at 25°C), high pressure processing (350 MPa for 1 to 20 min) or combination treatment in TSBYE. (a) O157:H12, (b) O157-H7, (c) O157-M1, and (d) O157-M2.
Figure 3.5: Inactivation of *Escherichia coli* O157 strains in Vienna sausages by combination of cell free extract (20 and 100 CEAU/g) and high pressure processing (500 MPa for 1 min at 25°C). (a) O157:H12, (b) O157:H7, and (c) O157-M1.
Figure 3.6: Inactivation of *Escherichia coli* O157 strains in Vienna sausages by combination of cell free extract (20 and 100 CEAU/g) and high pressure processing (400 MPa for 1 min at 25°C). (a) O157:H12, (b) O157:H7, and (c) O157-M1. a-d; different letters are significantly different from control in TSAYE media (p < 0.05). w-z; different letters are significantly different from control in MSA media (p < 0.05).
Figure 3.7: a) Thermogram of whole cells of *Escherichia coli* K12. (1) Whole cells, (2) CFE treated cells (32 CEAU/ml), (3) HPP treated cells (350 MPa for 1 min at 25°C), and (4) cells treated with combination of CFE (32 CEAU/ml) and HPP (350 MPa for 1 min at 25°C). b) Viable counts of *E. coli* K12 treated with CFE (32 CEAU/ml), HPP (350 MPa for 1 min at 25°C), or combination treatment on TSAYE and MSA. a, b; different letters are significantly different from control in TSAYE media (p < 0.05). x, y; different letters are significantly different from control in MSA media (p < 0.05).
CHAPTER 4

INACTIVATION OF LISTERIA MONOCYTOGENES IN MEAT PRODUCTS BY COMBINATIONS OF LACTOBACILLUS CASEI CELL FREE EXTRACT AND HIGH PRESSURE PROCESSING

ABSTRACT

*Lactobacillus casei* OSY-LB6A produced an intracellular bacteriocin-like substance with lethality to *Listeria monocytogenes*. Cell-free extract (CFE) of *Lb. casei* OSY-LB6A was also bactericidal to *L. monocytogenes* Scott A in a meat product. Combinations of CFE and high pressure processing (HPP) were tested against pressure-sensitive (Scott A) and pressure-resistant (V7 and OSY-8578) *L. monocytogenes* strains. Antimicrobial activity in the CFE was measured in colicin-equivalent activity units (CEAU/ml). Combination of CFE (32 CEAU/ml) and HPP (350 MPa, for 1-20 min) showed significant synergistic bactericidal effect (*p* < 0.05) and some combinations decreased pathogen’s populations >5 log_{10} CFU/ml. Synergy between CFE and HPP was most evident in the pressure-resistant strain, OSY-8578. Combinations of CFF (20 or 100 CEAU/g) and HPP (500 MPa for 1 min) were tested against *L. monocytogenes* Scott A in meat products. Pressure, combined with low-activity CFE caused considerable injury of the pathogen.
Combination of pressure and high-activity CFE caused > 5 logs reduction in the viability of *L. monocytogenes* Scott A in meat. No survivors were detected on selective and non-selective media. Synergy between CFE of *Lb. casei* OSY-LB6A and high pressure processing against *L. monocytogenes* makes this combination a feasible treatment for minimizing the risk of listeriosis resulting from consumption of ready-to-eat foods.

**INTRODUCTION**

A new *Lactobacillus* strain with antimicrobial activity was isolated from a mold-ripened cheese and it was identified as *Lb. casei* OSY-LB6A (Chung and Yousef, 2004). The antimicrobial cell free extract (CFE) from *Lb. casei* has a bacteriocin like properties and showed broad inhibitory activity against gram positive and negative foodborne pathogenic and spoilage bacteria. Combining the CFE from *Lb. casei* with high pressure processing (HPP) synergistically inactivated *Escherichia coli* O157 strains including O157:H7 (Chung and Yousef, 2004). In the current study, the antimicrobial efficacy of CFE from *Lb. casei* was further explored against pathogenic gram positive bacteria, particularly a pressure-resistant *Listeria monocytogenes* strain.

High pressure processing (HPP) is emerging as a heat-alternative technology for food preservation. This technology has gained interest as a means of inactivating pathogenic and spoilage microorganisms and improving safety and quality in many foods (Alpas et al., 2000; Alpas and Bozoglu, 2000; Ritz et al., 2002). High pressure inactivates microbial cells by destabilizing the cytoplasmic membrane, inducing protein denaturation and inhibiting genetic mechanisms (Smelt, 1998; Mackey et al., 1994). Pressure
magnitude, pressurization time and temperature, microbial types, stages of growth, presence of antimicrobial substances, and composition of suspending media are some of the factors that contribute to the efficacy of HPP (Metrick et al., 1989; Shigehisa et al., 1991; Styles et al., 1991; Carlez et al., 1993; Mackey et al., 1995; Patterson et al., 1995b; Kalchayanand et al., 1998a, b; Benito et al., 1999).

When treated with high pressure, pathogens often exhibit a tailing behavior. Tailing phenomenon may result from the heterogeneity in the bacterial population with respect to HPP resistance (Metrick et al., 1989; Ludwig et al., 1992; Patterson et al., 1995a). Moreover, strains of some bacterial species such as *E. coli* and *L. monocytogenes* show large variability in pressure resistance (Alpas et al., 1999; Patterson, et al., 1995b; Benito et al., 1999; Tay et al., 2003). Eradication of foodborne pathogens requires application of maximum pressure or prolonged treatment, which is neither commercially acceptable nor desirable (Shimada et al., 1990; Cheftel, 1992; Mertens and Deplace, 1993). Effective preservation by HPP may be accomplished at commercially-feasible pressures by implementing the hurdle concept. Hurdle technology relies on the additive or synergistic effect of two or more suitable antimicrobial factors at moderate doses (Leistner, 1985). Combination of HPP and antimicrobial peptides showed synergistic bactericidal action, and the pressure treatment sensitized gram-negative bacteria to these peptides (Alpas and Bozoglu, 2000; García-Graells et al., 1999; Hauben et al., 1996; Kalchayanand et al., 1994, 1998a; Masschalck et al., 2000).

Consumption of food containing *L. monocytogenes* causes listeriosis, a disease that may result from low infective doses (Rocourt and Cossart, 1997). Presence of this pathogen in ready-to-eat food (e.g., meat products) recently resulted in large disease
outbreaks and wide-scale product recalls (Dalton et al., 1995; CDC, 2000; http://www.fsis.usda.gov/OA/news/newsrls.htm). In order to control this pathogen in foods, a cell free extract (CFE) from *Lb. casei*, was used in combination with HPP. The CFE has a bacteriocin like properties and showed an inhibitory effect against gram-positive and gram-negative bacteria.

The objectives of this study were (a) to assess the efficacy of CFE against *L. monocytogenes*, (b) to investigate the potential synergy between CFE of *Lb. casei* and HPP to eliminate pressure-resistant *L. monocytogenes* in meat, and (c) to evaluate effect of the treatment on cellular components of the *Listeria* using differential scanning calorimetry.

**MATERIALS AND METHODS**

**Strains**

Strains of *Listeria monocytogenes* (Scott A, V7, and OSY-8578), and *L. innocua* ATCC 33090 were used in this study. The strains were obtained from the culture collection of the Food Safety Laboratory at the Ohio State University. Strains were grown in Trypticase Soy Broth (BBL, Sparks, MD), supplemented with 0.6% Yeast Extract (Difco Laboratories, Sparks, MD) (TSBYE) at 37°C for 15 hours. Working cultures were maintained on a slant of Trypticase Soy Agar (BBL) supplemented with 0.6% Yeast Extract (TSAYE), and stored at 4°C. Stock cultures of each strain were maintained in TSBYE with 20% glycerol and stored at –80°C.
Antimicrobial cell free extract of *Lactobacillus casei* OSY-LB6A

Isolation, identification and maintenance of *Lb. casei* OSY-LB6A were discussed in a previous report (Chung and Yousef, 2004). In brief, antimicrobial cell-free extract was prepared from *Lb. casei* OSY-LB6A culture by growing cells in MRS supplemented with 1% NaCl. Cell pellets were incubated with 5 mg/ml lysozyme (Sigma, St. Louis, MO), 0.6 M sucrose, and 5 mM MgCl$_2$ for 3 hours at 30°C. After incubation, the pellet was washed twice in 20 mM sodium phosphate buffer (pH 7), suspended in 50 ml of the same buffer, and sonicated (Torbeo ultrasonic processor, Cole Parmer, Vermon Hill, IL) at setting 5 for 5 min while maintaining the cooling. The supernatant was collected by centrifugation at 34,200 x g for 20 min, freeze-dried, and stored at –18°C until use. The freeze-dried powder (0.2 g) was resuspended in 1 ml of the same buffer; this solution was designated as cell-free extract (CFE). The extract contained variable activity as measured against *E. coli* p220, an indicator bacterium.

Measuring antimicrobial activity

The antimicrobial activity of CFE was tested in a microtiter plate assay system, as described by Jimenez-diaz et al. (1995), with modifications. Each well of the microtiter plate (Becton Dickinson, Franklin lakes, NJ) contained 25 µl 2X TSBYE medium, 25 µl CFE or its two-fold dilutions, and 10 µl indictor strain, *E. coli* p220 (10$^6$ CFU/ml). Positive and negative control wells contained 25 µl TSBYE medium, 25 µl SPB, with or without added inoculums (10 µl), respectively. The microtiter plate cultures were incubated at 37°C for 15 hours, and growth inhibition of the indicator strain was assessed by measuring Absorbance at 600 nm in a microtiter plate reader (Vmax Kinetics...
Microplate Reader, Molecular Devices, Sunnyvale, CA). The absorbance value of each dilution without the indicator was subtracted from the corresponding value of the same dilution containing the indicator microorganism. The antimicrobial activity of pure colicin against *E. coli* p220 was used as a reference for measuring the activity in the CFE as a positive control. Commercial colicin is produced by some strains of *E. coli* and the powder contains 20,000 units/mg pure proteins. One unit per ml is the minimal concentration required to cause a zone of clearing on a lawn of *E. coli* ATCC 9637 cells (Sigma). The highest dilution of the CFE causing inhibition of the indicator, compared to the positive control, was converted to colicin-equivalent activity units (CEAU).

**Antimicrobial action of cell-free extract in meat products**

Commercially sterile canned pieces of sausages (Vienna sausages, Armour®) were purchased from a local supermarket and were used as a model food. A piece of sausage (~16 g) was inoculated with *L. monocytogenes* Scott A at $10^6$ CFU/g. The inoculated product was treated with the CFE (20 CEAU/g) of *Lb. casei* prepared as described earlier. Inoculated products were held at 37°C for 4 days, sampled at 24-hour intervals, and surviving *L. monocytogenes* was counted on a selective agar medium (Oxford agar, Difco).

**Treatment with combinations of cell-free extract and high pressure**

Cell suspensions of *L. monocytogenes* were treated with CFE, HPP, or their combinations. One mL aliquots of *L. monocytogenes* cultures were placed in sterile stomacher bags (polyethylene, 4”x6”, Fisher Scientific International Inc., Pittsburgh, PA)
and were treated with 0.1ml CFE for 30 min at 25°C. Control treatment was prepared similarly, but without addition of CFE. For HPP, 1ml aliquots of *L. monocytogenes* culture were dispensed in sterile plastic bags; these were first heat sealed, double sealed in a bag containing Clorox solution for safety reason, and placed in water/ice bath until pressure treated. The sealed bags were high pressure processed at 350 MPa for 1-20 min, using a hydrostatic food processor (Quintus QFP6, Flow Pressure Systems, Kent, WA), containing a water/propylene glycol (Houghto-Safe 620-TY, Hougton International, Inc., Valley Forge, PA) mixture (1:1, v/v) as the pressure transmitting fluid. Adiabatic heating due to compression was taken into account by maintaining the samples at appropriate initial temperatures such that the final temperature during the pressurization was 30°C. The holding time at the desired pressure ranged from 1 to 20 min. Samples not treated with pressure acted as controls and these were used to estimate the initial counts. For combination treatments, 1 ml aliquots of *L. monocytogenes* culture were treated with CFE and high pressure, sequentially. The sequence of applying CFE and high pressure was reversed and inactivation of the pathogen was assessed. Treated and non-treated samples were enumerated by spread-plating on tryptose agar.

Selected meat products were treated with CFE, HPP, or their combinations. Cocktail wieners (Hillshire Farm) and canned sausage (Vienna sausage, Armour®) were purchased from a local supermarket and were used as model foods. Each cocktail wiener link was placed in a bag, weighed, and inoculated with *L. monocytogenes* culture so that the product contained approximately $10^7$ CFU/g. A similar procedure was applied to a piece of Vienna sausage, but a lower inoculum ($10^5$ CFU/g) was used. The inoculum was
distributed evenly by hand-mixing bag contents for 5 min. The bags were refrigerated for 30 min to allow attachment of the culture onto the sausage. Cell-free extract containing 20 or 100 CEAU/g was added to the inoculated product and the bags were refrigerated (~4°C) for 30 min. The sausage, mixed with *Listeria* culture and CFE, was high-pressure processed at 500 MPa for 1 min. *Listeria monocytogenes* in treated and non-treated samples was enumerated by spread-plating onto Tryptose agar and Oxford agar; therefore, total and uninjured populations of the pathogen were determined, respectively.

**Thermal analysis by differential scanning calorimetry (DSC)**

Cultures of *L. innocua* were grown in TSBYE for 13 hours at 37°C. Seventy ml of *L. innocua* was treated with *Lb. casei* CFE (32 CEAU/ml for 30 min), HPP (350 MPa, 1 min) or combination of the treatments. Conditions for HPP treatment are similar to that described earlier. Cells were centrifuged at 10,000 x g for 10 min and the pellets were washed for differential scanning calorimetry (DSC) as described by Lee and Kaletunc (2002). Cell pellets (~60 mg wet weight) were transferred into DSC pans. Reference sample was filled with water approximately 80% of sample weight (~48mg). Cell pellets in pans were heated in differential scanning calorimeter (DSC 111, Setaram, France) from 1 to 140°C with 4°C/min heating rate.

**Statistical analysis**

Decreases in log_{10} CFU/ml or log_{10} CFU/g (inactivation values) were analyzed using MINITAB statistical program (Minitab Inc., State College, PA). One-way analysis of variance was performed for the effect of high pressure and combination treatment with
CFE against *Listeria*. When treatment factors were significant, Tukey’s range test was used for multiple comparisons of means. Variables compared include bacterial counts on Tryptose agar and Oxford agar.

**RESULTS AND DISCUSSION**

**Antimicrobial action of cell-free extract in meat products**

A previous study demonstrated the antimicrobial efficacy of *Lb. casei* cell-free extract against gram-positive and gram-negative bacteria (Chung and Yousef, 2004). The product has bacteriocin-like properties. In this study, gram positive foodborne pathogen *Listeria monocytogenes* was targeted.

Vienna sausage is a cooked and cured canned meat product. The product is sterile, therefore, it is suitable for inoculation and testing antimicrobial efficacy. Survivors are counted in treated product by plating on selective agar media. The growth of *L. monocytogenes* inoculated on Vienna sausage was examined in the presence of *Lb. casei* CFE (20 CEAU/g) (Figure 4.1). The CFE exhibited bactericidal mode of action and inactivated *L. monocytogenes* considerably.

**Inactivation of *Listeria monocytogenes* culture by cell-free extract and high pressure**

*Lactobacillus casei* CFE and HPP were tested against *L. monocytogenes* Scott-A, V7, and OSY-8578 (Figure 4.2). These strains showed differences in pressure resistance (Tay et al., 2003; Lado and Yousef, 2003). The strain OSY-8578 was the most resistant
and therefore considered a good target for the combination treatments in this study. Compared to the control that received no treatment, exposure of *Listeria* cultures to CFE (32 CEAU/ml) for 30 min caused less than one log reduction in pathogens’s population (Figure 4.2). HPP treatment at 350 MPa for 1 min also inactivated < log CFU/ml. Differences in sensitivity to pressure among the strains were most obvious at intermediate treatment times. Treating *L. monocytogenes* with high pressure alone for up to 20 min resulted in sigmoidal (S-shaped) survivor plots.

Concave survivor plots, however, were obtained when the pathogen was treated with a combination of HPP and CFE. Therefore, presence of CFE eliminated the resistance of the pathogen when it was exposed to short HPP treatments. Pathogen’s tailing behavior was apparent when the samples were pressurized for extended treatment times. Presence of CFE did not eliminate the tailing effect, but this combined treatment decreased the viability of pathogen in the tailing region of survivor’s plots. Interestingly, inactivation by the combined treatment was most pronounced in the pressure-resistant OSY-8578 strains. It is noticeable that the inactivation of OSY-8578 by the combination treatment is significantly different from control during every treatment periods (p < 0.05).

Efficacy of combination treatment was investigated when the sequence of applying CFE and HPP was reversed. Results show that viability of cells decreased < 1 log CFU/ml when HPP was applied before the CFE treatment (Data not shown).

**Inactivation of *Listeria monocytogenes* in meat products by cell-free extracts and high pressure** Cocktail wieners were inoculated to contain $10^7$ CFU/g *L. monocytogenes* Scott A, treated with CFE for 30 min and pressure-processed at 500 MPa for 1 min.
Efficacy of individual preservation factors, or their combination, against *L. monocytogenes* was smaller when the pathogen was on wiener surfaces than in cell suspension (Figure 4.3a). This limited inactivation may be due to a protective effect of product ingredients (Yuste et al., 1998) or its natural microbiota. Natural microbial load on cocktail wieners was > 10^7 CFU/g. The cocktail wieners contained 25% fat and the baroprotective effect of fat on *Listeria* spp. has been reported (Gervilla et al., 1997; Patterson and Kilpatrick, 1998; García-Graells et al., 1999). Count of *L. monocytogenes* decreased only 1.5 log CFU/ml in UHT milk, compared to > 7 log inactivation in phosphate buffer at 340 MPa for 15 min at 20ºC, suggesting the protective effect of fat (Styles et al., 1991). Additional studies proved that cell destruction by pressurization was greater in phosphate buffer than in food (Patterson et al., 1995b; Garcia-Risco et al., 1998; Patterson and Kilpatrick, 1998). Reduced water activity and complex food matrices like meat were also believed to protect bacteria from HPP inactivation (Metrick et al., 1989; Styles et al., 1991; Oxen and Knorr, 1993). In contrary, Styles et al. reported that *Vibrio parahaemolyticus* was more sensitive in clam juice than in phosphate buffer (1991).

Cocktail wieners were treated with UV irradiation at 1.54 mW/cm² for 30 min before they were inoculated with *Listeria* culture. The UV treatment decreased the natural microbiota by one log CFU/ml (data not shown). After UV irradiation, wieners were inoculated with *L. monocytogenes* Scott A (10^5 CFU/g), and pressure treated at 500 MPa for 1 min. This pressure treatment inactivated 3.2 log CFU/g, compared with < 2 log CFU/g when the wieners were not treated with UV (Figure 4.3b).
Combining CFE with HPP had no additional antimicrobial efficacy, compared with the inactivation caused by the HPP alone. Interestingly, CFE seems to increase the recovery of pressure-injured population (Figure 4.3b).

The cocktail wieners were washed repeatedly (4 times) in sterile water (1:10 ratio of wiener: water). The washing procedure reduced the natural microbiota on wieners to below detectable level (data not shown). After washing, the wieners were inoculated with $10^5$ CFU/g *Listeria* and treated with CFE, HPP and the combination. Pressure treatment decreased *Listeria* population 3.6 log CFU/g. Although there was no obvious difference in viability loss by the combination treatment, compared to the HPP alone, the increase in cell injury due to the combination treatment indicates a potential synergism between the CFE and HPP against *L. monocytogenes*. Injury caused by CFE was concentration dependent; higher magnitude of injury occurred in the presence of higher concentration of CFE (Figure 4.3c).

Efficacy of HPP-CFE combination against *L. monocytogenes* Scott-A was verified using commercially sterile canned sausage (Vienna sausages). The trend of inactivation of *Listeria* by HPP, CFE or their combination, was similar to that in the washed cocktail wieners (Figure 4.3c), with greater inactivation of the pathogen in Vienna sausages (Figure 4.3d). Compared to the control, HPP alone, or the combination treatments showed significant inactivation of *Listeria* cells ($p < 0.05$). Samples pressurized in the presence of high concentration of CFE (100 CEAU/g) showed > 5 logs reduction in the viability of *Listeria* population, compared to the untreated control. The combination treatment also resulted in significant increase of injury in the pathogen’s population ($p < 0.05$). When inoculated pieces of Vienna sausage were pressurized in the
presence of high concentration of CFE (100 CEAU/g), the pathogen’s population was not
detected by plating on selective or non-selective agar media (Figure 4.3d). The increased
injury and complete inactivation of the pathogen by HPP in the presence of CFE of Lb.
casei suggests the possibility of using CFE to control L. monocytogenes Scott A in food
application. The CFE showed capability to maintain the survivors after pressurization
below the detection limit. Similar result was observed with nisin (Garriga et al., 2002).

**Thermal analysis by differential scanning calorimetry (DSC)**

DSC is a technique that detects and monitors thermally induced transitional
changes as a function of temperature. DSC has been used as a thermal analysis method
for analyzing foodborne bacteria, as its thermogram of whole cells can display changes in
the transitions of the microorganisms in response to stresses (Stephens and Jones, 1993;
Mohacsi-Farkas et al., 1994; Niven et al., 1999; Lee and Kaletunc, 2002). The
effectiveness of CFE, HPP or the combination treatment on cellular components of L.
innocua was determined by whole cell DSC and plate counting (Figure 4.4). When the
bacterial cells were heated, endothermic transitions, which corresponded to denaturation
of cell structures such as ribosome (peak a, 70°C), and DNA (peak b, 90°C) were
observed (curve 1). Exposure of cells to CFE for 30 min had no effect on the cells
viability. No apparent changes in the peak area in the DSC curve were observed after
addition of the CFE (curve 2). HPP at 350 MPa for 1 min reduced the cells’ population
by 2 logs. A decrease in the peak associated with ribosome denaturation (peak a) was
shown in the DSC thermogram after HPP treatment (curve 3). When L. innocua was
pressurized in the presence of CFE, reduction of 8 logs CFU/ml was observed in viable
counts (Figure 4.4b). The combination treatment resulted in the absence of peaks associated with cellular components in the thermogram (curve 4), while the ribosome and DNA peaks are still apparent in the thermogram of pressurized cells (curve 3). The DSC data suggest addition of CFE caused irreversible damage to cellular components during high pressure treatment. In addition, the decrease in total enthalpy (J/g) associated with the damage of the cellular components in DSC curve shows correlation with the loss of cell viability (data not shown).

One of the targets of high pressure treatment is the cell membrane (MacDonald, 1984). The pressure treatment does not mechanically destroy cells (von Butz et al., 1990). Tholozan et al., (2000) reported that disruption of the membrane was not observed in *L. monocytogenes* and *Salmonella Typhimurium* at 600 MPa. However, mild to high pressurization results in several physiological changes such as cell permeabilization, and decrease in membrane potential, intracellular potassium, and ATP contents. Cells eventually die when the membrane is extensively permeabilized (Macdonald 1984; Smelt, 1998; Wouters et al., 1988; Tholozan et al., 2000; Ritz et al., 2002).

Combination of antimicrobial agents like bacteriocins or bacteriocin-like substances and high pressure treatment increases the inactivation of the pathogens as cells surviving pressurization become sublethally injured and are then killed by bacteriocins (Kalchayanand et al., 1998a). Damage to cytoplasmic membranes due to high pressure treatment may facilitate uptake of the antimicrobial agent into the cells. These changes may suppress cell’s ability to maintain homeostasis, repair injuries, or synthesize biological materials required for membrane integrity, and thus contributing to cell death.
This study demonstrated that combination of cell free extract from *Lb. casei* OSY-LB6A with high pressure processing is effective against diverse strains of *L. monocytogenes*. The synergy between CFE and HPP against a pressure-resistant strain of *L. monocytogenes* suggests the potential to control this pathogen in meat products via a combined treatment.

**REFERENCES**


Smelt, J.P.P.M. 1998. Recent advances in the microbiology of high pressure processing. Trends Food Sci. Technol. 9, 152-158.


Figure 4.1: Inhibition of *Listeria monocytogenes* Scott A, on Vienna sausage, by CFE of *Lactobacillus casei* during incubation at 37°C.
Figure 4.2: Inactivation of *Listeria monocytogenes* by high pressure processing (350 MPa for 1 to 20 min at 25°C) or combination of high-pressure and cell-free extract from *Lactobacillus casei* OSY-LB6A (32 CEAU/ml, for 30 min at 25°C) in tryptose broth. (a) Scott A, (b) V7, (c) OSY-8578.
Figure 4.3: Viability loss of *Listeria monocytogenes* Scott A by combination of cell free extract at 20 and 100 colicin-equivalent activity units (CEAU)/g, and high pressure processing (500 MPa for 1 min at 25°C). (a) cocktail wieners-untreated, (b) cocktail wieners-UV treated (1.54 mW/cm² for 30 min), (c) cocktail wieners-serially washed in sterile water, and (d) Vienna sausages. a-c; different letters are significantly different from control in TA media (p < 0.05). x-z; different letters are significantly different from control in Oxford media (p < 0.05).
Figure 4.4: a) Thermogram of whole cells of *Listeria innocua*. (1) Whole cells, (2) cells treated with cell-free extract (CFE) at 32 colicin-equivalent activity units (CEAU)/ml, (3) cells treated with high pressure (350 MPa for 1 min at 25°C), and (4) cells treated with combination of CFE (32 CEAU/ml) and high pressure (350 MPa for 1 min at 25°C). b) Viable counts of *L. innocua* treated with CFE (32 CEAU/ml), HPP (350 MPa for 1 min at 25°C), or combination treatment. a-c; different letters are significantly different from control in TA media (p < 0.05).
LIST OF REFERENCES


Beveridge, T. J. 1995. The periplasmic space and the concept of the periplasm in gram positive and gram negative bacteria. ASM News. 61, 125-130.


A, a bacteriocin produced by Lactobacillus bavaricus M1410. Appl. Microbiol. Letts. 17,
132-134.

acid bacteria isolated from sour doughs: purification and characterization of bavaricin A,

Laukova, A. 1992. The effect of culture medium on bacteriocin production in some
bacterial strains. Veterinary medicine (Praha). 37, 661-666.


Lazdunski, C., D. Baty, V. Geli, D. Cavard, J. Morlon, R. Lloubes, P. Howard, M.
The membrane channel forming colicin A: synthesis, secretion, structure, action and

Mheen. 1999. Purification and characterization of a bacteriocin produced by Lactococcus
lactis subsp. lactis H-559 isolated from Kimchi. J. Biosci. Bioeng. 88, (2) 153-159.

heat inactivation of Escherichia coli and Lactobacillus plantarum. Appl. Environ.
Microbiol. 68, 5379-5386.

Leisner, J. J., G. G. Greer, M. E. Stile. 1996. Control of beef spoilage by a sulfide-
producing I strain with bacteriocinogenic Leuconostoc gelidum UAL 187 during

Leistner, L. 1985. Hurdle technology applied to meat products of the shelf stable product
(Eds.), Properties of Water in Foods in Relation to Quality and Stability, Martinus

Food Sci. Technol. 6, 41-46.

artificially made biofilm of a nisin producing strain of Lactococcus lactis. 51, 169-182.


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Smelt, J. P. P. M. 1998. Recent advances in the microbiology of high pressure processing. Trends Food Sci. Technol. 9, 152-158.


