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Antimicrobial effectiveness of Phenyllactic Acid against foodborne pathogenic bacteria and *Penicillium* and *Aspergillus* molds

by

David Kareem Manu

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Co-majors: Meat Science; Food Science and Technology

Program of Study Committee:

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Ames, Iowa

2012

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ABSTRACT

Natural antimicrobials from plant, animal, or microbial sources have the potential to increase food safety, improve shelf life and promote the idea of "natural foods." The overall objective of this research was divided into two parts. The aim of the first part was to evaluate the antimicrobial efficacy of phenyllactic acid alone or in combination with phosvitin against four human enteric pathogens. The aim of the second part was to evaluate the efficacy of phenyllactic acid (PLA) against selected mold species. Growth inhibition of *Listeria monocytogenes*, *Salmonella enterica*, *Staphylococcus aureus*, or *Escherichia coli* O157:H7 by PLA used singly or combined with phosvitin in brain heart infusion (BHI) broth (35 °C) was evaluated over a 24-hour period using a Bioscreen C Turbidometer (OD 600nm). Subsequently, selected concentrations of the antimicrobials were evaluated for their antimicrobial effectiveness in controlling growth of the pathogenic bacteria in cream of chicken soup at 12 ºC and 35 ºC. Growth inhibition of *Aspergillus ochraceus*, *Aspergillus spp*, *Penicillium roqueforti*, *Penicillium glabrum*, and *Penicillium spp*. by phenyllactic acid in brain heart infusion (BHI) broth (25 °C) was also evaluated over a 5-day period using a Bioscreen C Turbidometer (OD 600nm).

In cream of chicken soup, at both temperatures, phenyllactic acid (5 mg ml^{-1}) alone exerted the greatest bactericidal effect against all four pathogens throughout storage. At 12 °C and 35 °C, phosvitin combined with PLA (3.75 or 5 mg ml^{-1}) did not offer enhanced antibacterial effect above that provided by PLA (5 mg ml^{-1}) used alone. PLA had the greatest growth inhibitory effect on all the mold species at the lowest pH tested (pH 3.8). The minimum inhibitory concentration (MIC) of PLA at pH 3.8 against all the mold species was

15 mg ml^{-1}. Based on results of the present studies it is concluded that: i) PLA (5 mg ml^{-1}) has good potential for controlling the growth of foodborne pathogenic bacteria in cream of chicken soup and ensuring the microbial safety of this potentially hazardous food product, ii) PLA and phosvitin combinations are far less effective for inhibiting growth of pathogens in soup, iii) PLA has the potential to control *Penicillium* and *Aspergillus* molds if used in an acidic environment.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Foodborne diseases are a growing health problem worldwide. In 2005, the World Health Organization estimated that 1.8 million people died from foodborne related diseases. The Centers for Disease Control estimates that every year 48 million Americans (1 in every 6 persons) contract a foodborne illness, 128,000 are hospitalized, and 3,000 die. A majority (90%) of the annual cost of foodborne illness in the United States ($12.6 billion dollars) is attributed to 5 pathogens namely, *Salmonella enterica*, *Campylobacter* spp., *Listeria monocytogenes*, *Toxoplasma gondii*, and norovirus (Hoffman et al., 2012). While it is completely impossible to exterminate foodborne pathogens, the government, food processors and manufacturers, and retailers should take an active role in trying to ensure foodborne diseases are kept to a minimum.

Green consumerism, a consumer-driven effort to replace synthetic additives in food products with more natural ingredients, has been a topic of discussion for an extended period of time, but recently it has gained a vast amount of interest due to consumer demands for reduced use of synthetic chemical preservatives in foods. Some common examples of traditionally used "chemical" preservatives are sorbates, sulfites, nitrites and parabens. The main consumer concern is the long term adverse effects that synthetic food preservatives might have on health. Two examples of preservatives (commonly used in the cheese industry) that are being re-evaluated due to their unique problems are potassium sorbate and natamycin. Both of these compounds are antifungal agents added to cheese. Potassium sorbate is the potassium salt of sorbic acid which comes from the Rowan Berry. Although

mold growth in cheese can be effectively inhibited by potassium sorbate, certain species of *Aspergillus* and *Penicillium* can degrade potassium sorbate via decarboxylation and produce unpleasant odors (Marth et al., 1966; Sofos and Busta, 1981; Kinderlerer and Hatton, 1990). Due to the fact that *Aspergillus* and *Penicillium* can metabolize potassium sorbate, natamycin is relied on to control mold growth in cheese. Natamycin is considered as an antibiotic polyene that is quite effective against yeast and molds but not bacteria. Natamycin is produced by *Streptomyces natalensis* and has been accepted by the Food and Agriculture Organization/the World Health Organization (FAO/WHO) as a food preservative because it stimulates an unusually low level of resistance among fungi, it is rarely involved in cross-resistance among other antifungal polyenes, and DNA transfer of resistance genes between fungi does not occur to the extent as observed in some bacteria (Jay et al., 2005). The effectiveness of natamycin in cheese is limited by the fact that it has relatively poor solubility, distribution, and stability. In addition to being an antifungal in cheese, natamycin is also used to treat fungal infections including, *Candida*, *Aspergillus*, *Fusarium*, and *Penicillium*. While using potassium sorbate and natamycin does indeed inhibit mold growth in cheese, the need for natural additives as replacements for these antifungal agents is still prevalent.

An alternative replacement to synthetic preservatives in foods is natural preservatives from plant, animal, or microbial sources. Eggs from poultry contain numerous proteins that have antimicrobial properties. An egg yolk protein of interest is phosvitin. Phosvitin is a phosphoglycoprotein that has the ability to bind multivalent metals such as Fe^{2+}, Ca^{2+}, and Mg^{2+} (Grogan and Taborsky, 1987). By binding metal ions in the microorganism's

environment, phosvitin may inhibit microbial growth or by binding metal ions that stabilize the outer membrane of Gram-negative bacteria, it might be able to disrupt bacterial cells (Satter Khan et al., 2000).

Currently there are many food preservation technologies and techniques, but most of them involve the use of synthetic chemical preservatives, high amounts of energy, or substantial cost. A new emerging technique involves using lactic acid bacteria (LAB) and their metabolites to preserve food. Lactic acid bacteria have been granted Generally Recognized as Safe (GRAS) status, due to their long tradition of being consumed in dairy products and their contribution to healthy intestinal microflora in humans, so the interest in using them as a biopreservative has increased (Valerio et al., 2004). In addition to acid production, lactic acid bacteria can also produce bacteriocins. Bacteriocins are antibacterial proteins that kill or inhibit the growth of other bacteria (Cleveland et al. 2001). The most well-known bacteriocin is Nisin. Nisin produced by *Lactococcus lactis* has a broad spectrum of activity against gram-positive bacteria and is generally recognized as safe (GRAS) in the United States for use in selected pasteurized cheese spreads to control the outgrowth and toxin production of *Clostridium botulinum* (Stevens et al., 1991). Nisin is a special bacteriocin because it is effective against gram-positive organisms including, but not limited to, lactic acid bacteria and *Listeria monocytogenes.* Nisin is also nontoxic, produced by *Lactococcus lactis*, is destroyed by digestive enzymes and does not contribute to off-flavors or off-odors (Jay et al., 2005). In addition to bacteriocins, several other compounds with strong antifungal activity have been isolated from bacterial cultures. Currently, the majority of identified antifungal substances are low molecular weight compounds. Some of those

compounds are of organic acids, reuterin, hydrogen peroxide, proteinaceous compounds, hydroxyl fatty acids and phenolic compounds (Dalie et al, 2009). Research has shown that some compounds isolated from lactic acid bacteria can have an inhibitory effect on either bacteria or yeasts and molds.

The combination of natural antimicrobials could possibly lead to an enhanced inhibitory effect of the antimicrobials against foodborne pathogens as opposed to an antimicrobial being used separately. Additive or synergistic effects between the natural antimicrobials could allow the use of lower quantities of each antimicrobial and substantially reduce the cost of each treatment while still improving the efficiency of the antimicrobial to control pathogens.

Thesis Organization

This thesis is divided into five chapters. The first chapter is comprised of a general introduction. The second chapter is a literature review that contains information that is pertinent to the research performed in chapters 3 and 4. The final chapter (Chapter 5) is a general conclusion based research findings that are stated in chapters 3 and 4. All relevant tables and figures appear at the end of their respective paper, which follows a specified journal format. References are located at the end of each chapter with formatting following journal specifications. Chapter 3 is intended to be submitted to the scientific journal, Food Microbiology, and Chapter 4 is intended to also be submitted to Food Microbiology. The abstract titled “Antibacterial Effectiveness of Phenyllactic Acid against Gram-positive and Gram-negative foodborne pathogens at pH 6.0 and 7.2” was presented in a poster format at the annual meeting of the International Association for Food Protection in Providence, Rhode

Island (July 2012). The abstract titled "The Antimicrobial Efficacy of DL-3-Phenyllactic Acid against selected *Aspergillus* and *Penicillium* molds at 25 °C" was presented in a poster format at the annual meeting of the Institute of Food Technologists in Las Vegas, Nevada (June 2012).

CHAPTER 2. LITERATURE REVIEW

Phenyllactic Acid

Phenyllactic acid (PLA) is an antimicrobial compound isolated from *Geotrichum candidum* as well as from several *Lactobacillus* species. It was originally used to inhibit *L. monocytogenes* in milk (Dieuleveux and Gueguen 1998). PLA is active against Gram positive bacteria, Gram negative bacteria, and fungi. PLA has also been found in honey at concentrations ranging from 100-800 mg/kg (Mu et al. 2012). It has also been found in high concentrations in heather (820 mg/kg), ling heather (875 mg/kg), and manuka honeys (243 mg/kg) (Tan et al. 1988; Dimitrova et al. 2007). More recently the use of lactic acid bacteria (isolated from sourdough bread) to inhibit mold growth has been evaluated. This has led to the discovery that *Lactobacillus plantarum* 21B produces PLA (Lavermicocca et al., 2000; Lavermicocca et al., 2003). It has also been discovered that PLA may be present in some fermented foods (including sourdough bread) that use LAB as a starter culture (Van der Meulen et al. 2007; Ryan et al. 2009). PLA is a novel antimicrobial compound and much research still needs to be conducted to discover its full antimicrobial capabilities.

Chemical Structure and Forms

The molecular formula of PLA is $C_9H_{10}O_3$ and its molecular weight is 166 g/mol. This antimicrobial has four main forms, D-3-phenyllactic acid, L-3-phenyllactic acid, DL-3-phenyllactic acid, and 4-hydroxyphenyllactic acid, all of which are produced by specific strains of either *Geotrichum candidum* or *Lactobacillus*. Limited research has been conducted on all four forms, but DL-3-phenyllactic acid and 4-hydroxyphenyllactic acid have the greatest antifungal effect, and also have an antibacterial effect against *L. monocytogenes,*

Staphylococcus aureus, and *Escherichia coli* O157:H7 (Dalie et al, 2009; Schwenninger et al. 2008; Ohhira et al. 2004; Lavermicocca et al. 2003; Lavermicocca et al. 2000, Dieuleveux et al. 1998).

Influence of pH

DL-3-Phenyllactic acid was evaluated against *Aspergillus niger* FTDC3227 using five different pH values (2.6, 4.0, 4.5, 5.0, and 5.5). At pH 2.6, the greatest antifungal effect was observed in that mold species, whereas, in *Penicillium* and other *Aspergillus* species, the greatest effect occurred at pH 5.5. At a pH of 4.0, a value close to the pH values in several food systems, and commonly attained in the culture filtrate of lactic acid bacteria, a slight reduction in phenyllactic acid was observed (Lavermicocca et al. 2003). The activity of PLA is pH dependent, indicating that its mode of action is related to the lipophilic properties which enable the undissociated form of that organic acid to cross the microbial membrane and acidify the interior environment of microbial cells (Gould, 1996).

Inhibitory effect of PLA in the presence of other organic acids

The combined effect of PLA with other organic acids such as lactic acid and/or acetic acid was evaluated in solution against *A. niger* FTDC3227 at a PLA concentration of 5 mg ml^{-1} (Lavermicocca et al. 2003). Lactic acid alone at a concentration of 15.8 mg ml^{-1} exhibited a 33% inhibitory effect against that mold. When applied at that same concentration in combination with PLA (5 mg ml -1), lactic acid caused a 30% increase in PLA's inhibitory activity (from 53 to 82%). Acetic acid alone at a concentration of 0.67 mg ml^{-1} produced a 7% inhibitory effect against *A. niger* FTDC3227 whereas, when applied in combination with PLA, increased the antifungal activity of PLA by 18%. Those same researchers demonstrated

that when PLA, lactic acid, and acetic acid are combined, the greatest percent inhibition against *A. niger* FTDC3227 is 83%.

Antibacterial activity

The antibacterial activity of PLA was first reported by Dieuleveux et al. (1998). It was discovered that *Geotrichum candidum* produces and secretes phenyllactic acid and indolelactic acid, both of which have anti-*Listeria* activity. Phenyllactic acid in milk, cheese, and cultured medium was shown to inhibit the growth of *Listeria monocytogenes*. In ultra-high temperature (UHT) processed milk, phenyllactic acid exhibited a bacteriostatic effect on *Listeria monocytogenes* at 7 mg ml^{-1} over 5 days compared to the control (Dieuleveux and Gueguen, 1996).

Apart from its ability to inhibit *Listeria monocytogenes*, PLA can also inhibit other Gram-positive bacteria such as *Staphylococcus aureus*, *Enterococcus faecalis*, and *Bacillus cereus* as well as gram-negative bacteria, such as *Salmonella enterica*, *Escherichia coli*, *Providencia stuartii*, and *Klebsiella oxytoca* (Dieuleveux et al., 1998, Ohhira et al. 2004). The antibacterial action of PLA is has not been completely elucidated but it has been suggested that the site of action could be the bacterial cell wall. Bacterial cells exposed to PLA exhibited damaged or completely altered cell walls. Bacteria grown in 7 mg ml^{-1} PLA had broken cell wall openings along the length of the cell which allowed efflux of cellular constituents (Dieuleveux et al., 1998).

Antifungal Activity

In addition to its antibacterial action, PLA has the capability to inhibit yeast and molds as well. Schwenninger et al. (2008) demonstrated that PLA is effective against

Rhodotorula mucilaginosa, Candida pulcherrima, and *Candida parapsilosis* and Lavermicocca et al. (2000) demonstrated that PLA is effective against molds isolated from bakery products, flour, and cereal including *Aspergillus ochraceus* and *Penicillium roqueforti*. For anti-yeast activity, PLA has a relatively high minimum inhibitory concentration ranging from 50 to more than 500 mM at pH 4.0 to 6.0 and as the pH decreases the minimum inhibitory concentration decreases (Schwenninger et al., 2008). The minimum inhibitory concentration of PLA for mold at pH 4.0 is 45 mM (Strom et al., 2002).

Lavermicocca et al. (2000) first characterized and also purified PLA from the antifungal compounds produced by *Lactobacillus plantarum* 21B. The growth of mold in sourdough bread fermentation can be delayed up to seven days if *Lactobacillus plantarum* 21B is used as a starter culture (Lavermicocca et al. 2000). According to Valerio et al. (2004) the extent of the antifungal activity of lactic acid bacteria strains such as *Lactobacillus plantarum* 21B was positively related to the metabolic content of phenyllactic acid.

Phosvitin

Composition

Phosvitin, a phosphoglycoprotein isolated in 1949 from the egg yolk of hens, contains a high amount of phosphorus and accounts for 60% of total egg yolk phosphoproteins (Mecham and Olcott, 1949). Phosvitin has an amino acid composition of 50% serine, and 90% of which are phosphorylated (Mecham and Olcott, 1949; Clark, 1985). It contains 216 amino acids of which 123 are serines (Fig 1; White, 2011; Byrne et al., 1984; Clark, 1985). The molecular weight of phosvitin ranges from 36 to 60 kDa and the major component is B-phosvitin which has a molecular weight of 45 kDa (Abe et al., 1982). Phosvitin contains

12.3% nitrogen, 10% phosphorus (80% of yolk protein phosphorus), and 6.5% nitrogen (Taborsky and Mok, 1967; Xu et al., 2007; Samaraweera et al., 2011). This protein is one of the most highly phosphorylated proteins in nature, and has been used as a phosphoprotein standard (Ilg et al., 1996).

Figure 1. Amino acid sequence of hen egg yolk phosvitin. (Source: White 2011; Byrne et al. 1984; Clark 1985).

A E F G T E P D A K T S S S S S S A S S T A T S S S S S S A S S P N R K K P M D E E E N D Q V K Q
A R N K D A S S S S R S S K S S N S S K R S S S K S S N S S K R S S S S S S S S S S S S R S S S S S S S
S S S N S K S S S S S S K S S S S S S R S R S S S K S S S S S S S S S S S S S S K S S S S R S S S S S S K
S S S H H S H S H H S G H L N G S S S SS S S S S R S V S H H S H E H H S G H L E D D S S S S S S S S

Extraction, isolation, and purification

The methods described by Mecham and Olcott (1949) to extract and isolate phosvitin in 1949 are most commonly used. Generally, methods for purifying phosvitin consist of an isolation phase and a fractionation via hydrophobic interaction and ion-exchange chromatography (Castellani et al., 2003). Fractionation via chromatography helps to purify phosvitin and/or separate different phosvitin polypeptides (Mecham and Olcott, 1949; Sim and Nakai, 1994; Sundararajan et al., 1960; Joubert and Cook, 1958; Wallace and Morgan, 1986). The production methods for phosvitin are limited because most of the methods associated with preparation, extraction, and isolation use chemicals that are non-aqueous or are not food-grade (Wallace and Morgan, 1986; Castellani et al., 2003). A sodium chloride extraction method has been shown to be successful and selective in isolating phosvitin from whole egg yolk (Choi et al., 2005). The cost of phosvitin is relatively high due to the methods needed to extract and purify this protein. The cost of phosvitin is $1,225.00 per gram based on the current price listed in the Sigma-Aldrich 2012 catalog. More recently, a water/ethanol

based method, developed by researchers at Iowa State University, permits an efficient and cost effective option for extraction and purification of phosvitin from hens' egg yolk (White, 2011).

Structure

Phosvitin is largely hydrophilic and has a high number of negative charges and a low percentage of nonpolar hydrophobic side chains (Dickinson et al., 1997). Phosvitin can behave as a polyelectrolyte in liquid state due to high phosphoric acid bound to serine residues (Grizzuti and Perlmann, 1970). It is composed of 66% acidic, 16% basic, and 18% nonpolar amino acids. The amino acid sequence contains 216 amino acids composed of 123 serines (S), virtually all of which are phosphorylated, 15 lysines (K), 13 histidines (H), and 11 arginines (R) (Byrne et al., 1984). The structure of the peptide groups in the protein chain of phosvitin may be characterized as containing (a) very little α-helix conformation, (b) a minority of residues in β-sheet and/or irregular conformations, and (c) a majority of residues in an unusual conformation (Prescott et al., 1986). At near a neutral pH, phosvitin has an irregular structure, whereas at an acidic pH, phosvitin has a β-sheet structure (Taborsky, 1970; Perlmann & Grizzuti, 1971).

Stability

Phosvitin is heat resistant as first reported by Mecham and Olcott (1949). When heated for several hours at 100°C from pH 4 to 8, phosvitin solutions did not show any sign of denaturation (Mecham and Olcott, 1949). In an electropherogram, phosvitin bands were completely diffused after heating at 140°C for 10 minutes; however, at 100°C for 10 minutes no change in the electrophoretic profile was observed (Itoh et al., 1983). In addition, high

pressures up to 600 MPa do not disrupt phosvitin's stability (Castellani et al., 2004). Phosvitin is not easily digested by pepsin, trypsin, and chymotrypsin (White, 2011). After enzymatic digestion, the negative charge of the phosphate group makes the neighboring peptide bonds resistant to proteolytic action while keeping the protein core together (Goulas et al., 1996).

Antimicrobial properties

To our knowledge, there is only one published study on the antibacterial potential of phosvitin to date, In that same study, phosvitin, protease-digested phosvitin, and phosvitin-galactomannan conjugate were evaluated against *Escherichia coli* under thermal stress at 50°C (Satter Khan et al., 2000). Those authors reported that phosvitin and phosvitin-galactomanna conjugate were significantly more effective in decreasing the viability of *Escherichia coli* in L-broth at 50°C for 20 minutes, whereas, protease-digested phosvitin was not effective. The antibacterial activity of phosvitin is believed to be attributed to the chelating properties of its phosphate groups linked to removal of cations such as magnesium and calcium that stabilize the outer membrane of Gram-negative bacteria. In conjunction with thermal stress, these properties seem to induce the decrease in viability of *Escherichia coli* (Satter Khan et al., 2000).

Listeria monocytogenes

Morphology and Growth Characteristics

Listeria monocytogenes is a Gram-positive, non-sporeforming, regular rod-shaped bacterium that is catalase positive and oxidase negative. This bacterium is a psychrotrophic, facultative anaerobe that usually occurs in single or short chains and grows rapidly at 7°C-

10°C. When *Listeria monocytogenes* is grown between 20 and 25°C, flagellin is both produced and assembled at the cell surface, but at 37°C, flagellin production is reduced (Peel et al., 1988) The *Listeria* genus is composed of eight different species including *L. grayi, L. seeligeri, L.welshimeri, L. ivanovii, L. innocua, L. monocytogenes,* and the more recently identified, *L. marthii and L. rocourtiae* (Graves et al., 2010; Leclercq et al., 2010). *Listeria monocytogenes* has been categorized into several serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, and 4a, 4ab, 4b, 4c, 4d, 4e (Seeliger, 1958; Donker-Voet, 1972). Although all serotypes are capable of causing disease in humans, 95 to 98% of listeriosis cases documented in humans are attributed to serotypes 1/2a, 1/2b and 4b (Lund et al., 2000),

The optimal growth temperature for *L. monocytogenes* is 35°C to 37°C but the pathogen is also able to grow at temperatures ranging from 1.5°C to 45°C (Juntilla et al., 1988; Vasseur et al., 1999). This organism is capable of growing at a pH range of 4.5 and 9.6 (Seelinger and Jones 1986). Between pH 6.0 and 8.0 *L. monocytogenes* can reach its highest final population in the absence of growth-limiting factors (Buchanan et al., 1994; Petran and Zottola 1989; Ryser and Marth 1988). *L. monocytogenes* is also capable of tolerating salt and nitrite as well as a water activity of less than 0.93 (McClure et al., 1997; Farber et al., 1992). The lowest water activity values allowing growth to > 6.5 log CFU ml^{-1} in presence of glycerol, sucrose, NaCl, and propylene glycol were 0.90, 0.92, 0.92, and 0.97, respectively (Miller, 1992; Nolan et al., 1992) *L. monocytogenes* is destroyed by conventional thermal processing of foods at temperatures above 56°C due to irreversible cellular damage which results in the death of the organism (Bunduki et al., 1995).

Contamination sources

Listeria monocytogenes is widely located in plant, soil, silage, sewage, slaughter house waste, human and animal feces, processing environments, and catering facilities (Farber and Peterkin 1991; Beresford and others 2001). Soils containing dead or decaying vegetation and/or fertilized with animal feces or sewage sludge, can harbor *L. monocytogenes*. *Listeria monocytogenes* was not detected on standing grass but instead was detected 24 hours later on grass samples that had been cut and started to decay. Further investigation led to the interesting discovery that a natural habitat for *L.monocytogenes* is decaying grass or silage which is easily a source of listeriosis for farm animals (Fenlon et al., 1995).

Foods that have been associated with contamination of *L. monocytogenes* include: soft cheese, raw milk, ice cream/soft cream, butter, cooked chicken, turkey frankfurters, sausages, pate and rillettes, pork tongue in aspic, fish, shellfish, shrimp, smoked fish, cod roe, coleslaw salad, vegetable rennet, salted mushrooms, raw vegetables, pickled olives, rice salad, cut fruit, and cold sandwiches (Wagner and McLauchlin, 2008).

Not only can *L. monocytogenes* adhere to food and soil surfaces but it is also capable of readily adhering to the surfaces of food processing benches, machinery, and floors. Following attachment to surfaces, the pathogen can subsequently produce a biofilm matrix which increases the pathogen's resistance to adverse conditions (Blackmann and Frank, 1996). More specifically, *L. monocytogenes* serotypes 1/2a, 1/2b, and 1/2c appear to have better adapted to meat processing plants, as they have more often been isolated more often

from these particular environments (Jay, 1996; Norwood and Gilmour, 2000; Autio et al., 2002).

Listeriosis outbreaks in foods

Listeria monocytogenes was first described as a pathogen in the 1920s (Low and Donachie, 1997) and listeriosis was initially recognized as a disease of animals. It was not until the 1980s that *L. monocytogenes* was recognized as a foodborne human enteric pathogen. In 1981, an outbreak involving the consumption of coleslaw containing *L. monocytogenes* serotype 4b resulted in 41 reported cases of listeriosis (34 perinatal and 7 adult) (Farber and Peterkin, 1991). Of the 34 infected patients, 9 had stillbirths and 23 had infected infants with a 27% mortality rate and 2 live births of a well infant. In 1983, in the state of Massachusetts, pasteurized milk was contaminated with *L monocytogenes* type 4b. This contamination resulted in 49 (7 perinatal and 42 immuno-compromised individuals) cases of listeriosis and 14 deaths (Fleming et al. 1985). In 1985, a listeriosis outbreak in California was linked to contaminated Jalisco cheese due to the presence of *L. monocytogenes* type 4b. Of 142 cases, 93 were perinatal, 49 were non-pregnant, 48 were immuno-comprosed individuals and 48 individuals died (Linnan et al., 1988).

More recently listeriosis in humans was linked to fresh produce. In 2011, there was a multistate outbreak of listeriosis associated with cantaloupes from Jensen Farms located in Colorado. Illnesses totaled 147 along with 33 deaths across 28 states (CDC, 2011). The most recent multistate outbreak of listeriosis has been attributed to contamination of ricotta salata cheese from the brand Frescolina Marte. For the outbreak, 18 cases have been reported across 13 states along with 18 hospitalizations and 3 deaths (CDC, 2012).

Characteristics of listeriosis

Listeriosis occurs in a variety of animals, including humans, and the disease most often affects the uterus at pregnancy, the central nervous system, or the bloodstream (Wagner and McLauchlin, 2008). Almost all cases of human listeriosis are attributed to *L. monocytogenes* (McLauchlin, 1997). Rarely are human listeriosis infections caused by *Listeria ivanovii* and *Listeria seeligeri* (Rocourt et al., 1986; Cummins et al., 1994). Listeriosis most often affects individuals that are immuno-compromised, including the elderly, pregnant women, and unborn or newly delivered babies. Infection can be treated with antibiotics, but yet human infection has a mortality rate of 20-40% (Farber and Peterkin, 1991).

Several factors increase the risk of listeriosis such as: contamination of raw product or factory sites, cross contamination from other foods, persistence of the pathogen in the food chain, adaptation of virulent strains to certain environmental stresses, poor processing, retailing, and storage of food, and incomplete surveillance. These risks can be decreased by excluding *L. monocytogenes* from the food chain by adequate temperature and shelf-life control, environmental hygiene, increased quality of raw materials, implementation of HACCP process controls, guidelines, standards, food regulation, education of all those involved with the food chain, surveillance of clinical cases and foods, and withdrawal of contaminated foods. Those foods linked to listeriosis outbreaks generally have the following common features: i) capable of supporting the multiplication of *L.* monocytogenes, ii) processed with extended (usually refrigerated) shelf lives, iii) consumed without further cooking, and iv) contaminated with high levels of *L. monocytogenes* (McLauchlin, 1996).

Salmonella enterica

Morphology and growth characteristics

Salmonella is a rod shaped, gram-negative, non-spore-forming, facultative anaerobe belonging to the *Enterobacteriaceae* family (Hammack, 2012). Based on the most recent nomenclature, *Salmonella* can be divided into two species, *Salmonella bongori* and *Salmonella enterica* (*S. enterica*). Within *Salmonella enterica* are six subspecies: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), and *S. enterica* subsp. *houtenae* (IV), *S. enterica* subsp. *indica* (VI) (Hammack 2012). *Salmonella enterica* has over 2500 identified serovars that belong to six subspecies (Ochman and Groisman, 1994; Fierer and Guiney, 2001).

Salmonella has the ability to grow under various environmental conditions. Sodium chloride (NaCl) is not required for growth, but in the presence of 0.4 to 4% NaCl *Salmonella* is still able to grow. Most serotypes of *Salmonella* are able to grow at a temperature range of 5 to 47°C with an optimum growth temperature of 35 to 37°C. Some can even grow slowly at temperatures as low as 2 to 4°C or as high as 54°C (Gray and Fedorka-Cray, 2002) if other intrinsic and extrinsic factors such as pH, water activity, oxidation-reduction potential, and gaseous atmosphere are optimum for the pathogen. *Salmonella* is sensitive to heat and often killed at temperature of 70°C or above. This pathogen can grow at a water activity (a_w) between 0.99 and 0.94 and survive at a_w <0.2 in several dried food products. At temperatures of <7 ºC, pH <3.8, or water activity <0.94 complete inhibition is observed (Hanes, 2003; Bhunia, 2008).

Contamination sources

Salmonella is quite prevalent in the environment and this occurrence is most likely linked to several animal reservoirs of this pathogen. *Salmonella* can colonize the intestinal tracts of vertebrates, including livestock, wildlife, domestic pets, and humans (Hammock, 2012). *Salmonella serotypes* such as *S.* Typhi and *S.* Paratyphi are only found in humans (Miller and Pegues, 2005). *Salmonella* transmission has originally been linked to poultry, eggs and dairy products, but more and more it has been associated with reptiles such as pet tortoises, snakes, iguanas, frogs, and aquatic turtles (Bouchrif et al., 2009, CDC, 2003; Mermin et al. 1997). More recently, fresh produce such as fruits and vegetables have been implicated as transmission sources of *Salmonella* (Bouchrif et al., 2009). *Salmonella* contamination of fresh produce could be due to the entry of *Salmonella* through scar tissues, entrapment during embryogenesis of produce, natural uptake through root systems and transfer onto edible plant tissues during slicing. A major factor that is contributing to the prevalence of *Salmonella* in fresh produce is the increased importation of fresh fruits and vegetables from countries that have a climate that is suitable to grow these products year round. Hygienic conditions in those countries during production, harvesting, packaging, and distribution of fresh produce may not meet minimum U.S. standards. These sub-standard hygienic conditions contribute to microbial contamination of fresh produce. In addition there are several other factors that can facilitate the persistence of *Salmonella* on fresh produce. These include crops that are fertilized with untreated sewage water, washing of fresh produce with water that has been contaminated, and improper handling of produce by field workers.

Salmonellosis outbreaks in foods

Salmonella is responsible for approximately 1.4 million non-typhodial illnesses, 15,000 hospitalizations, and 400 deaths in the US every year (CDC, 2008). *Salmonella enterica* serotype Enteritidis is one of the most common *Salmonella* serotypes worldwide predominantly in developed countries. During the 1980s, an important cause of foodborne human illness was attributed to *S.* Enteritidis (Patrick et al. 2004). According to the CDC's 2011(a) "Estimates for Foodborne Illness," *Salmonella* ranked as one of the most problematic pathogen as it was responsible for an estimated 1,207,561 illnesses, 19,336 hospitalizations, and 378 deaths. Eggs and raw or undercooked poultry are foods that are most usually associated with causing *Salmonella* infections. This trend is slowly shifting: for example, from 2006 to 2012, numerous serovars of *Salmonella* have been linked to contamination of other foods including: tomatoes, peanut butter, dry pet food, pot pies, cantaloupes, Malt-o-Meal Rice/Wheat cereals, raw produce, pistachios, alfalfa sprouts, red and black pepper, Italian-style meats, frozen fruit pulp, cantaloupe, turkey burgers, imported papayas, and mangoes (CDC, 2012a).

In 2007, *Salmonella* Tennessee was involved in a large multi-state outbreak associated with peanut butter that caused 425 infections from 44 states, 71 hospitalizations, and no deaths (CDC, 2007). *Salmonella* Litchfield infected 51 people in an outbreak associated with cantaloupes imported from Honduras in 2008 (CDC, 2008a). In 2009, there was an outbreak of *Salmonella* Saintpaul associated in raw alfalfa sprouts resulting in 235 infections from 14 states and no deaths (CDC, 2009). Another notable outbreak in 2009 was attributed to *Salmonella* Typhimurium. It was responsible for a multistate outbreak in peanut

butter which resulted in 714 infections in 46 states and nine deaths (CDC, 2009a). In 2010, *Salmonella* Enteritidis was responsible for contaminated eggs in Iowa. Approximately 2000 people from 11 states suffered illness (CDC, 2010). In a more recent outbreak, *Salmonella* Typhimurium and *Salmonella* Newport are both responsible for 270 illnesses, 101 hospitalizations, and 3 deaths in a multi-state outbreak in cantaloupes (CDC, 2012b).

Characteristics of Salmonellosis

Salmonellosis is an infection caused by *Salmonella*. Elderly, infants, and those with weak immune systems are most susceptible to salmonellosis. A majority of people that are infected with *Salmonella* develop diarrhea, fever, vomiting, and abdominal cramps 12 to 72 hours after infection (CDC, 2010a). In addition, after bacterial infections, reactive arthritis (Reiters syndrome) can develop in some individuals (Dworkin et al., 2001). Reiters syndrome can last for months or years and usually causes pain and swelling that affects the ankles, knees, and feet (Colville and Berryhill, 2007). *Salmonella* infections often do not require medical treatment other than fluids taken orally and are usually resolved in 5-7 days. Antibiotics such as ampicillin, trimethoprim-sulfamethoxazole, or ciprofloxacin, are not usually necessary unless the infection spreads from the intestines (CDC, 2010). Various species of *Salmonella* have become resistant to antibiotics as a result of feeding animals, which will serve as food products, antibiotics to help promote growth. Not only is salmonellosis responsible for numerous illnesses but there is also a monetary consequence. *Salmonella* outbreaks in the United States have been estimated to range from $600 million to $3.6 billion dollars each year (Ter-Hsin et al., 2005).

Staphylococcus aureus

Morphology and Growth Characteristics

Staphylococcus aureus (S. aureus) is a member of the Micrococcaceae family. It is a Gram positive, non-spore forming facultative anaerobe and is coccus-shaped bacterium. Under microscopic examination, *S. aureus* cells are arranged in grapelike clusters. Many of the 32 *S. aureus* species and subspecies are found in foods due to environmental, human, and animal contamination (Hait, 2012). *S. aureus* is differentiated from other staphylococcal species on the basis of pigmentation of colonies, coagulase reaction (coagulase positive) and ability to ferment mannitol (Wilkinson, 1997). *Staphylococcus* species that are mostly implicated in human infections include: *S. aureus*, *S. saprophyticus*, *S. epidermidis*, *S.lugdunensis*, and *S. schleiferi* (von Eiff et al., 2002; Choi et al., 2006; Otto, 2009). In regards to human foodborne illness, *S. aureus* is the most prevalent.

Staphylococcus aureus strains can be divided into biotypes according to their human and animal origin. In 1984, Devriese developed a biotype classification system and separated *S. aureus* into human, non-β-hemolytic human, avian, bovine, ovine, and nonspecific biotypes. *Staphylococcus aureus* is capable of growing in temperatures ranging from 7 to 48.5°C with an optimum growth temperature of 30 to 37°C (Schmitt et al., 1990). In addition to being able to grow in wide range of temperatures, *S. aureus* can also grow in a wide range of pH values ranging from 4.2 to 9.3 with an optimum pH of 7 to 7.5 (Bergdoll, 1989). A unique characteristic of *S. aureus* is its ability to grow in salt concentrations as high as 15%. The optimum water activity for *S. aureus* is about 0.99 but it is also capable of growing at

water activities as low as 0.83 (Hait, 2012) if other intrinsic and extrinsic factors are optimal for growth of this pathogen

Contamination sources

Staphylococcus aureus is frequently found among the microbial flora on human skin and nasal passages (Kluytmans et al., 1997). From infected hosts, *S. aureus* has been isolated from nostrils, mouth, upper respiratory tract, mammary glands, intestinal tracts and genitourinary tracts (Murray et al., 1999). Up to 30-50% of the human population arecarriers (Le Loir, 2003). Some sources of *S. aureus* contamination are food handlers through manual contact of food products or coughing and sneezing on food products after heat treatment; however, equipment and environmental surfaces can also serve as contamination sources. In cases of human intoxication, food usually associated with an outbreak has not been kept at a refrigerated temperature of <10°C or has not been kept hot enough (>45°C) (Hait, 2012).

***Staphylococcus aureus* outbreaks in foods**

Various foods can support growth of *S. aureus* and have been implicated in staphylococcal food poisonings and outbreaks. Those foods include milk and cream, cream filled pastries, butter, ham, cheeses, sausages, canned meat, salads, cooked meals and sandwich fillings (Le Loir et al., 2003). In one instance, cheese was involved in an outbreak because it had been produced from milk contaminated after pasteurization and before inoculation with a lactic acid bacteria starter culture (Bergdoll, 1989). In 1985, chocolate milk was the source of staphylococcal poisoning due to contamination and improper storage for 4 to 5 hours (Le Loir et al., 2003). In 1989, *S. aureus* enterotoxin type A (SEA) was associated with multiple outbreaks of canned mushrooms (CDC, 1989). Individuals, across 3

states, totaling 102 contracted an illness and 12 were hospitalized. According to the Centers for Disease Control and Prevention's "Estimates of Foodborne Illness for 2011(b)," *S. aureus* was responsible for approximately 241,148 cases of foodborne illness.

Characteristics of *Staphylococcus* infection

Humans are natural carriers of *S. aureus*. Thirty to fifty percent of healthy individuals are colonized, with ten to twenty percent persistently colonized (Noble et al., 1967; Casewell and Hill, 1986). Individuals colonized with *S. aureus* are at increased risk for subsequent risk (Wenzel and Perl, 1995). *S. aureus* rates of colonization are high among individuals with type 1 diabetes (Tuazon et al., 1975), intravenous drug users (Tuazon and Sheagren, 1974), individuals undergoing hemodialysis (Yu et al., 1986), surgical patients (Weinstein, 1959; Kluytmans et al., 1995), and individuals with immunodeficiency syndrome (Weinke, 1992). *S. aureus* pathogenesis has five stages: colonization, local infection, systemic dissemination and/or sepsis, metastatic infection and toxinosis (Archer, 1998). Symptoms of staphylococcal food poisoning that appear 30 minutes – 8 hours after ingestion are abdominal cramps, nausea, vomiting, and sometimes diarrhea (diarrhea does not appear by itself) (Le Loir et al., 2003)

In addition to nosocomial (hospital-acquired) infections caused by *S. aureus*, infections by this pathogen have also been associated with many other maladies and syndromes such as: furuncle or carbuncle, impetigo bullosa, cellulitis, surgical wound infection, pyomyositis, botryomycosis, hospital-acquired bacteremia, acute or right sided endocarditis, hematogenous osteomyelitis, septic arthritis, epidural abscess, brain abscess, hospital-acquired pneumonia, empyema, septic shock, toxic shock syndrome, scalded skin

syndrome, foodborne gastroenteritis, and renal carbuncle (Archer, 1998). To prevent nosocomial *S. aureus* infections, several options are being explored. The Centers for Disease Control and Prevention established guidelines for preventing the transmission of nosocomial pathogens in an attempt to prevent the transmission of staphylococci within the hospital (Garner, 1996). In addition, the use of systemic or topical agents, to decrease nasal carriage, has been shown to reduce the incidence of *S. aureus* infections (Kauffman and Bradley, 1997).

Escherichia coli

Morphology and Growth Characteristics

Escherichia coli (E. coli) is a gram negative, facultative anaerobic, non-spore forming rod-shaped bacteria. *E. coli* has many strains and many of them are not harmful. Some are even beneficial to humans. *E. coli* normally resides among the normal microflora of the intestines. Harmful or pathogenic *E. coli* strains also are capable of residing in the intestines. *E. coli* is an indicator organism and its presence in food and water is an indication of fecal contamination. *E. coli* can be divided into six groups: 1) enteropathogenic (EPEC), 2) enterotoxigenc (ETEC),3) shiga toxin producing/enterohemorrhagic (STEC/EHEC), 4) enterinvasive (EIEC), 5) enteroaggregative (EAEC), and diffusely adherent (DAEC) (Jafari et al. 2012). The *Escherichia coli* O157:H7 used in the experiments described in the present thesis belong to the EHEC group which is known for causing hemorrhagic colitis or other serious complications in infected persons; therefore, the information in the following paragraphs will focus mainly on *E. coli* O157:H7.

Escherichia coli O157:H7

Escherichia coli is a foodborne pathogen associated with foodborne disease outbreaks all around the world. The serotype O157:H7 is responsible for the majority of *E. coli* associated foodborne outbreaks in the United States (Karmali et al., 2010). *E. coli* O157:H7 was first identified as a human pathogen in 1982, when it was implicated in two outbreaks of hemorrhagic colitis (Riley et al., 1983). The H7 type was initially isolated in 1944 from a human diarrheal sample, whereas the O157 type was first isolated and named in 1972 from swine diarrheal samples (Orskov et al., 1977). *E. coli* O157:H7 releases a shiga-like toxin which binds to endothelial cells expressing globotriaosylceramide-3 (Gb_3) which allows absorption of the toxin into the bloodstream and this toxin's distribution to other organs (Sandvig, 2001). *E. coli* strains that produce Shiga toxins are referred to as Shiga toxin-producing *E. coli* (STEC) because of the toxin they produce resembles the toxin produced by *Shigella dysenteriae* type 1 (Calderwood et al., 1996). *E. coli* O157:H7 has an optimum growth temperature of 30 to 42°C, optimum growth pH of 6 to 8, and is unable to ferment sorbitol within 24 hours (Tsai and Ingham, 1997).

E coli O157:H7 is closely related, genetically, to *E coli* O55:H7, an enteropathogenic strain that causes diarrhea among infants worldwide (Whittam et al., 1993). As with the enteropathogenic strains, *E coli* O157:H7 can adhere to epithelial cells and produce lesions (Nataro and Kaper, 1998).

Contamination sources

E. coli O157:H7 is transmitted by food and water or directly from person to person through cross contamination. Most outbreaks have been traced to foods derived from cattle

such as ground beef and raw milk (Pierard, 1992; Wall et al., 1996; Griffin, 1995). In addition to dairy products and ground beef, products such as steak tenderized by injection, kabobs, ready to eat meats, salami, venison jerky, cheese, milk, butter, yogurt, ice cream, apple juice, grapes, coleslaw, lettuce, spinach, alfalfa sprouts, and melons, have also been associated as potential hosts for *E. coli* O157:H7 (Rangel et al., 2005).

Escherichia coli **O157:H7 outbreaks in foods**

The first *E. coli* O157:H7 outbreaks in the United States occurred in Oregon and Michigan in 1982, when the pathogen was isolated from individuals suffering from bloody diarrhea and severe abdominal cramps (CDC, 1982). In 2006, a multistate outbreak occurred in fresh spinach. In this outbreak, *E. coli* O157:H7 was responsible for 199 illnesses, 102 hospitalizations, 31 cases of hemolytic-uremic syndrome (HUS), and 3 deaths across 26 states (CDC, 2006). The consumption of contaminated prepackage cookie dough in 2009 was associated with an outbreak across multiple states. There were 72 reported cases of illness, 34 hospitalizations and 10 individuals developed hemolytic uremic syndrome (HUS) (CDC, 2009b). In 2011, contaminated romaine lettuce led to 58 infections, 33 hospitalizations, and 3 individuals developed hemolytic uremic syndrome, and no deaths were reported (CDC, 2012).

Characteristics of *E. coli* O157:H7 infection

Infection due to *E. coli* O157:H7 typically occurs after ingestion of contaminated food. The minimum infectious dose of *E. coli* O157:H7 is 10 to 100 CFU compared to over one million CFU for other pathogenic *E. coli* strains (Greig et al., 2010). The fatality rate in humans is roughly 1% (Besser et al., 1999). Typically the incubation period for the onset of

disease symptoms is 3 to 4 days during which, *E. coli* O157:H7 colonizes in the intestines (Phillips et al., 2000). Illness usually begins with abdominal cramps and non-bloody diarrhea, but eventually becomes grossly bloody (Mead and Griffin, 1998; Feng, 2012). Extreme diarrhea appears to consist of blood and mucus and occurs every 15 to 30 minutes (Feng, 2012). Roughly 10-15% of individuals that are infected with *E. coli* O157:H7 develop hemolytic uremic syndrome 5-13 days after the onset of diarrhea (Tarr et al., 2005; Scheiring et al., 2009). In some extreme cases, some patients may suffer kidney damage and kidney failure, seizures, coma or ultimately, death (Martin et al., 1990; Bell et al., 1994; Boyce et al., 1995; MacDonald et al., 1998).

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CHAPTER 3

EFFICACY OF PHENYLLACTIC ACID, PHOSVITIN AND THEIR COMBINATION ON THE VIABILITY OF *LISTERIA MONOCYTOGENES*, *SALMONELLA ENTERICA, ESCHERICHIA COLI O157:H7* AND *STAPHYLOCOCCUS AUREUS* IN BHI AND CREAM OF CHICKEN SOUP STORED AT 12 °C OR 35 °C

A paper submitted to *Food Microbiology*

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Abstract

The antibacterial effectiveness of phenyllactic acid (PLA) or phosvitin alone or in combination against *Listeria monocytogenes* (LM), *Salmonella enterica* (SE), *Escherichia coli* O157:H7 (EC), and *Staphylococcus aureus* (SA) in brain heart infusion (BHI) broth and cream of chicken soup was investigated. Separate samples of broth or soup with added antimicrobials alone or combined, were inoculated with those same pathogens to obtain an initial viable count of 5.0 Log_{10} CFU ml^{-1} (broth) and 4.0 Log_{10} CFU ml^{-1} (soup) for each pathogen. Broth or soup without added antimicrobials served as control. Minimum inhibitory concentrations (MICs) and growth curves for the pathogens in BHI (35 °C, 24 hours) were obtained by using a Bioscreen C turbidometer (OD_{600nm}). Growth of the pathogens in soup (12 °C, 7 days) and at 35 °C for 6 hours was monitored by surface plating samples on appropriate selective agar. The MIC of PLA in broth was 2.5 mg ml^{-1} for all four pathogens. In control soup at 12 ºC all four pathogens grew and reached populations ranging from 5.87 to 6.47, 7.27 to 7.72 and 7.90 to 8.50 Log CFU ml^{-1} at 3, 5, and 7 days, respectively. Phosvitin (60 mg ml^{-1}) alone exhibited a significant growth inhibitory effect against LM ($P<0.05$). Compared to control, populations of viable LM were 1.53, 3.13, and 2.90 Log CFU ml^{-1} lower at 3, 5, and 7 days, respectively. In contrast, PLA at 3.75 or 5.0 mg ml^{-1} was bactericidal to all four pathogens ($P<0.05$). In soup with PLA (5.0 mg ml^{-1}) no viable LM and SE was detected at day 1 and day 5, respectively and no viable EC or SA was detected at day 7. Phosvitin was less inhibitory to LM, SE and EC in soup held at 35 ºC compared to 12 ºC, and failed to inhibit SA at 35 ºC. At 35 ºC none of the pathogens could be detected at 6 hours in soup with added PLA (5 mg ml^{-1}) by plating or enrichment. Only few viable cells of

SA were detected at 4 hours and, as early as 2 hours, none of the other three pathogens were detected. At 12 ºC or 35 ºC, combinations of PLA and phosvitin, while significantly inhibitory ($P<0.05$) compared to control, did not produce antibacterial effects greater than that exhibited by PLA alone. Based on these results PLA at 3.75 or 5.0 mg ml^{-1} has good potential for assuring the microbial safety of cream of chicken soup under temperature-abuse conditions.

1. Introduction

The rapidly growing need of time-constrained consumers for convenience foods has resulted in an explosive growth in sales in ready-to-eat (RTE) foods including a variety of soups, sandwiches and hot snacks in supermarket delicatessens and in convenience stores. Increased sales of these RTE foods may result in heightened risks of microbial contamination as food handlers prepare larger batches to meet customer demand. Proper hygienic practices of food handlers, safe hot-holding temperatures during serving and proper refrigeration during storage are important to ensuring the microbial safety of these RTE foods. More often, proper temperature control is the principal or only factor that foodservice operations can rely on to control foodborne pathogens in many potentially hazardous RTE foods. Therefore, temperature abuse of these foods can result in foodborne illness. Based on the FDA Food Code, potentially hazardous foods should be maintained at a temperature of 60 ºC or above or at 5 ºC or less (FDA Food Code, 2001) for service to consumers.

A promising approach to provide added protection of RTE foods (beyond safe hot-holding or refrigerated storage temperature) against foodborne pathogens is the use of antimicrobial ingredients. Antimicrobial ingredients may be classified as synthetic or

naturally occurring compounds. Although the toxicological safety of commonly used, approved synthetic antimicrobials is assured by regulatory authorities, health conscious consumers are increasingly opposed to the addition of synthetic chemicals to foods (Ray, 1992). In addition, those consumers are highly concerned with the long-term adverse effects to their health that might occur from eating foods containing synthetic preservatives. Due to increased consumer concern over consumption of food that has been formulated with synthetic chemical preservatives, the demand for more natural and minimally processed food has increased (Cleveland et al., 2001). Therefore, identification of novel effective ways of ensuring the microbial safety of RTE foods is vitally important to the food industry. In this regard, the use of naturally occurring antimicrobials to control foodborne human enteric pathogens may provide a solution to this problem. Natural antimicrobials can be obtained from animal, plant or microbial sources.

Phosvitin is a naturally occurring antimicrobial protein found in the yolk of hens' eggs. It is a phosphoprotein that represents 7% of the yolk proteins (Mecham and Olcott, 1949). The amino acid composition of phosvitin is 50% serine of which 90% are phosphorylated (Clark, 1985). This unique composition of phosphorylated serines makes phosvitin a very strong metal chelator of various multivalent metals such as iron, calcium, and magnesium (Grizzuti and Perlmann, 1975; Vieira, 2007; Sattar Khan et al., 2000). Phosvitin is believed to have antimicrobial properties associated with the chelation of cations, such as iron (Sattar Khan et al., 2000). Currently, the antimicrobial efficacy of phosvitin has been published in a paper by Sattar Khan et al (2000). In that study, phosvitin (0.1 mg ml^{-1}) in combination with heat stress (50 °C for 20 min) significantly inactivated *Escherichia coli* (Sattar Khan et al., 2000).

Phenyllactic acid (PLA) is an antimicrobial compound isolated from *Geotrichum candidum* as well as, more commonly, *Lactobacillus plantarum* (Dieuleveux and Gueguen, 1998; Lavermicocca et al., 2000; Lavermicocca et al., 2003). It is a naturally occurring acid that has four main forms, D-3-phenyllactic acid, L-3-phenyllactic acid, DL-3-phenyllactic acid, and 4-hydroxyphenyllactic acid all of which are produced by either *Geotrichum candidum* or *Lactobacillus plantarum* 21B. Published reports on the antibacterial effects of all four forms against foodborne pathogens are scarce. The inhibitory effects of DL-3-phenyllactic acid and D-3-phenyllactic acid against some fungal species isolated from flour, cereals and bakery products have been reported (Lavermicocca et al., 2000; Lavermicocca et al., 2003). Also, D-3-phenyllactic acid has been shown to inhibit growth of *Listeria monocytogenes* in milk, and DL-3-phenyllactic acid has been shown to inhibit *Staphylococcus aureus* and *Escherichia coli* O157:H7 in vitro (Ohhira et al. 2004; Dieuleveux et al. 1998).

To our knowledge there is no published research on the antibacterial efficacy of phosvitin against *L. monocytogenes*, *S. enterica*, *E. coli* O157:H7, and *S. aureus* or the combined use of DL-3-phenyllactic and phosvitin against those same pathogens. Accordingly, the objectives of the present study were: 1) to establish the minimum inhibitory concentration of PLA for four foodborne human enteric pathogens in brain heart infusion broth and 2) to evaluate the antimicrobial efficacy of phosvitin or PLA individually or in combination against the foodborne pathogens in cream of chicken soup stored at abusive temperatures (12 °C and 35 °C).

2. Material and methods

2.1. Bacterial cultures and culture conditions

Five strains of *Listeria monocytogenes* (Scott A NADC 2045,serotype 4b, H7969 serotype 4b , H7962 serotype 4b, H7596 serotype 4b, and H7762 serotype 4b), five serotypes of *Salmonella enterica* (Enteritidis-ATCC13076, Heidelberg, Typhimurium-ATCC 14802, Gaminara-8324, and Oranienburg-9329), five strains of *Escherichia coli* O157:H7 (FRIK125, ATCC 35150, ATCC 43894, ATCC 43895, and 93-062), and three strains of *Staphylococcus aureus* (ATCC 6538, ATCC 25923, and BAA-44) were obtained from the culture collection of the Microbial Food Safety Laboratory, Iowa State University, Ames, IA. Stock cultures were kept frozen (-70 ºC) in brain heart infusion (BHI) broth (Difco,; Becton Dickinson, Sparks, Md) supplemented with 10% (vol/vol) glycerol. Sealed vials of frozen stock cultures, thawed under cold running water, were activated in tryptic soy broth (TSB; Difco, Becton Dickinson) at pH 7.2 and incubated at 35 °C. At least two consecutive 18 to 24-h transfers of each stock culture were carried out before using the cells as inocula in each experiment.

2.2. Preparation of inoculum

Equal volumes (6 ml per culture) of each of the working cultures of *Listeria monocytogenes, Salmonella enterica, Escherichia coli* O157:H7 and *Staphylococcus aureus* were combined in a sterile centrifuge tube. The cells were harvested by centrifugation (10,000 x g, 10 min, 4 °C) using a Sorvall Super T21 centrifuge (American Laboratory Trading, Inc., East Lyme, CT) and washed once in 0.85% (wt/vol) saline. The pelleted cells

were suspended in fresh 0.85% (wt/vol) saline to obtain a final viable cell concentration of approximately10^9 CFU ml^{-1}. Viable colony counts of the washed cell suspensions were evaluated by serially diluting (10-fold) and surface plating samples on tryptic soy agar (Difco, Becton Dickinson) supplemented with 0.6% yeast extract (TSAYE). The cell suspensions were used to inoculate BHI broth or commercially sterile cream of chicken soup.

2.3. Antimicrobials

Phosvitin was supplied by Dr. Dong Ahn from the Animal Science Department at Iowa State University, Ames, IA. A commercial preparation of DL-3-Phenyllactic acid (PLA) was purchased from Sigma-Aldrich (St. Louis, Missouri)

2.4. Preparation of treatment solutions for Bioscreen C assay

BHI broth with added PLA (0, 0.312, 0.625, 1.25, or 2.5 mg ml^{-1}) was filter sterilized using 0.22 µm pore size filters (Fisher Scientific) and the pH was adjusted to 6.0 using 1M sodium hydroxide or 1M hydrochloric acid. Samples (2.5-ml) of BHI broth with added antimicrobials and the control (BHI broth with no added antimicrobial) in test tubes were each inoculated with 25 µl of diluted (1:100) *Listeria monocytogenes, Salmonella enterica, Escherichia coli* O157:H7 or *Staphylococcus aureus* cell suspensions to obtain a final cell concentration of approximately 10^5 CFU ml^{-1} of sample.

2.5 Bioscreen C assay

Inoculated samples (aliquots of 200 µl) were added in triplicate to the wells of a 100-well microtiter plate for the Bioscreen C Turbidometer (Growth Curves USA Piscataway,

NJ), an automated microbial growth analyzer and incubator. Plates were incubated in the Bioscreen C at 35 °C for 24 h and the machine took optical density (OD) measurements at 600 nm every 30 min, with shaking of samples for 10 seconds prior to each OD reading. Minimum inhibitory concentration (MIC) was defined as the lowest treatment concentration that completely inhibited (< 0.05 OD unit increase) microbial growth for 24 hr in BHI broth at 35 ºC.

2.6. Preparation and inoculation of soup

Campbell's Cream of Chicken Soup in cans was purchased from a local grocery store. The soup was reconstituted with sterile distilled water and prepared according to the directions stated on the package. Soup ingredients are shown in Table 1. After the soup was boiled, 6-ml aliquots of the soup were aseptically transferred into sterile 15 ml screw cap tubes. Separate sets of the soup in tubes were tempered to ~ 35 °C and ~ 12 °C. Phosvitin, phenyllactic acid or their combinations was added to each tube to give the following concentrations: PLA (3.75 mg ml^{-1}), PLA (5 mg ml^{-1}), phosvitin (60 mg ml^{-1}), PLA (3.75 mg ml^{-1}) + phosvitin (60 mg ml^{-1}) and PLA (5 mg ml^{-1}) + phosvitin (60 mg ml^{-1}). Both phenyllactic acid and phosvitin were added to the tubes after the addition of tempered soup. Soup without added antimicrobial served as a control. Tubes of soup with added antimicrobials were mixed via vortexing and then inoculated with 50 µl of the suspension of washed cells of the foodborne pathogens to give a final cell concentration ofapproximately10^4 CFU ml^{-1} for each pathogen. After inoculation, each tube of soup was mixed by vortexing and stored at either 12 °C or 35 °C in thermostatically controlled incubators.

2.7. Microbiological analysis

Inoculated tubes of soup stored at 12 °C were tested for numbers of viable pathogens every 2 days starting at day 1 for a total of 7 days whereas, tubes of soup held at 35 °C were tested at 1, 2, 3, 4, and 6 h. Ten-fold serial dilutions of the soup were prepared using sterile buffered peptone water. Aliquots (1.0 ml or 0.1ml) of the soup or diluted samples of soup were surface plated (in duplicate) on Modified Oxford agar (for *Listeria monocytogenes*), XLT-4 (for *Salmonella enterica*), Sorbitol MacConkey agar (for *Escherichia coli* O157:H7), and Baird Parker agar (for *Staphylococcus aureus*). Inoculated plates were incubated at 35 °C and bacterial colonies were counted at 48h. All previously mentioned selective agar media were purchased from Difco, Becton Dickinson, Sparks, Md.

2.8. Measurement of pH and water activity

Prior to inoculation, the initial pH and water activity of each soup sample including controls were measured at day 0. Measurements of pH were taken using an Orion Model 525 pH meter (Orion Research, Inc., Boston, Massachusetts) fitted with a glass electrode. Water activity measurements were taken using the Aqualab CX2 water activity meter (Decagon Services, Pullman, Washington).

2.9. Statistical analysis

Three replications of each experiment were performed. Mean numbers of viable *Listeria monocytogenes*, *Salmonella enterica*, *Escherichia coli*, and *Staphylococcus aureus* survivors were statistically analyzed using SAS statistical software version 9.3 (SAS Institute Inc., Cary, N.C.). Treatment means were evaluated for statistically significant differences

using the Waller-Duncan test. Significant differences were defined at $P < 0.05$ for all the experimental data.

3. Results

3.1 Growth inhibition by phenyllactic acid in BHI broth

The effect of PLA (0, 0.312, 0.625, 1.25 and 2.50 mg ml^{-1}) on the growth of *L. monocytogenes, S. enterica, E. coli* O157:H7, and *S. aureus* in brain heart infusion (BHI) broth at pH 6.0 is shown in figures 1-4. *L. monocytogenes, S. enterica,* and *E. coli* O157:H7 grew in control BHI broth and at about10 h reached optical density ($OD600_{nm}$) values of 0.52, 0.44, and 0.56, respectively. *S. aureus* in control broth reached a maximum OD_{600nm} of 0.44 at approximately 16 h. Compared to control, the lowest PLA concentration tested (0.312 mg ml^{-1}) inhibited growth of the Gram-positive pathogens - *L. monocytogenes* and *S. aureus* (Figs. 1 and 4) but not *S. enterica* or *E. coli* O157:H7 (Figs. 2 and 3). Growth inhibition of all four pathogens increased with increase in concentration of PLA from 0.625 to 2.50 mg ml^{-1} with the highest PLA concentration (2.5 mg ml^{-1}) completely inhibiting growth; absolutely no increase in OD was observed for 24 h at that high PLA concentration (Figs. 1-4). The minimum inhibitory concentration (MIC) of PLA for all four pathogens in BHI broth (35 ºC) was 2.5 mg ml^{-1}.

3.2. The pH and water activity of cream of chicken soup

The initial pH of the cream of chicken soup was 5.85 and the initial water activity was 0.988. The pH values for samples of soup that contained phosvitin (60 mg ml^{-1}), PLA (3.75 mg ml^{-1}) and PLA (5.0 mg ml^{-1}) were 5.33, 3.66 and 3.34, respectively. Soup that

contained combinations of the antimicrobials had pH values of 4.83 (PLA at 3.75 mg ml^{-1} + phosvitin) and 4.57 (PLA at 5.0 mg ml^{-1} + phosvitin). The water activity values for soup samples with or without antimicrobials ranged from 0.986 to 0.988.

3.3 Growth inhibition by phenyllactic acid and phosvitin in cream of chicken soup at 12 ºC

Viable counts of all four pathogens in cream of chicken soup (12°C) are shown in Tables 2-5. The initial viable count for each of the four pathogens was 4.0 Log_{10} CFU ml^{-1}. *L. monocytogenes* grew in control soup and reached 8.5 Log_{10} CFU ml^{-1} at day 7 (Table 2). Phosvitin alone inhibited growth of the pathogen throughout storage; viable counts in soup with added phosvitin were 1.53, 2.13, and 2.90 Log cycles lower than those of control at 3, 5, and 7 days, respectively. Initial populations of *L. monocytogenes* in soup with added PLA (3.75 or 5.0 mg ml^{-1}) declined throughout storage at 12 C. PLA at 3.75 mg ml^{-1} reduced initial numbers of *L. monocytogenes* by 0.37, 1.47, 2.95 and 3.43 Log cycles at 1, 3, 5, and 7 days, respectively. PLA (5 mgml^{-1}) inactivated initial numbers of the pathogen by 2.04 and 3.23 Log cycles at 1 and 3 days, respectively; however, no viable *L. monocytogenes* was detected by plating or enrichment at 5 or 7 days of storage (Table 2). Combinations of PLA and phosvitin significantly inhibited growth of *L. monocytogenes* compared to control ($P<0.05$); however, those combinations were less effective in inhibiting growth of the pathogen compared to PLA (3.75 or 5.0 mg ml^{-1}) used alone (Table 2).

Viable populations of *S. enterica* in control soup reached 8.0 Log_{10} CFUml^{-1} at day 7 (Table 3). Throughout storage there were no significant differences in viable counts of *S. enterica* in soup with added phosvitin compared with those of control ($P>0.05$). The higher concentration of PLA (5.0 mg ml^{-1}) was the most effective of all treatments tested; at day 1

and throughout the remainder of storage, no viable salmonellae were detected by plating or enrichment. PLA (5.0 mg ml^{-1}) and phosvitin combined significantly ($P<0.05$) inhibited growth of *S. enterica* compared to control but was less effective in inhibiting growth of the pathogen compared to PLA (3.75 or 5.0 mg ml^{-1}) used alone (Table 3).

In the control soup the viable count of *Escherichia coli* O157:H7 increased during storage and at 7 days numbers of the pathogen were 8.30 Log_{10} CFU ml^{-1} (Table 4). As observed with *S. enterica*, there were no significant differences in viable counts of *E. coli* O157:H7 in soup with added phosvitin compared with those of control ($P>0.05$). Both concentrations of PLA in the soup were bactericidal to *E. coli* O157:H7; PLA at 3.75 and 5.0 mg ml^{-1} reduced initial numbers of the pathogen by 0.3 and 1.07 Log respectively, at day 1. In soup with added PLA (5 mg ml^{-1}), reductions in viable counts were 2.57, 3.80, and 4.00 Log CFU ml^{-1} at 3, 5, and 7 days, respectively. No viable *E. coli* O157:H7 was detected by plating or enrichment at 7 days of storage. Both PLA and phosvitin combinations significantly inhibited *E. coli* O157:H7 growth compared to control ($P<0.05$). Those two combination treatments were not significantly different from each other with respect inhibiting growth of *E. coli* O157:H7 ($P>0.05$) and were less effective in inhibiting growth of the pathogen compared to PLA at 3.75 or 5.0 mg ml^{-1} (Table 4).

The initial population of *S. aureus* in control soup increased steadily and attained a level of 7.90 Log_{10} CFU ml^{-1} at 7 days of storage (Table 5). Compared to control, phosvitin exhibited a significant growth inhibitory effect against *S. aureus* at 3 and 5 days ($P<0.05$); Log differences in viable count between control soup and soup with added phosvitin were 1.07 (day 3) and 0.93 (day 5). PLA at 3.75 mg ml^{-1} reduced initial counts of the pathogen by 1.23 and 1.47 Log cycles at day 1 and day 3, respectively. No viable *S. aureus* was detected

by plating or enrichment at 5 and 7 days (Table 5). At 1, 3, and 5 days, PLA at 5 mg ml^{-1} reduced counts of the pathogen by 2.57, 2.97, and 3.77 Log cycles, respectively; no viable *S. aureus* was detected at day 7 (Table 5). As previously mentioned for *L. monocytogenes*, *S. enterica* and *E. coli* O157:H7, combinations of PLA and phosvitin in soup significantly inhibited growth of *S. aureus* compared to control or phosvitin ($P<0.05$); however, those combinations were relatively less inhibitory to the pathogen compared to PLA at 3.75 or 5.0 mg ml^{-1} (Table 5).

3.3 Growth inhibition by phenyllactic acid and phosvitin in cream of chicken soup at 35 ºC

Viable counts of all four pathogens in the cream of chicken soup (35°C) are shown in Tables 6-9. The average initial viable counts of the pathogens ranged from 3.73 to 4.5 Log_{10} CFU ml^{-1}. The initial viable count of *L. monocytogenes* in control soup increased to 6.03 Log_{10} CFU ml^{-1} in 6 hours (Table 6). For up to 4 hours there were no significant differences in viable counts of the pathogen in soup with added phosvitin alone compared to control soup ($P>0.05$). At 6 hours the viable counts of *L. monocytogenes* in soup with phosvitin were 5.07 Log_{10} CFU ml^{-1}, a difference of 0.96 Log cycles lower than control ($P<0.05$). In 1 hour, PLA at 3.75 and 5.0 mg ml^{-1} reduced initial counts of the pathogen by 2.90 and 3.83 Log cycles, respectively ($P<0.05$). No viable *L. monocytogenes* in soup with added PLA was detected by plating or enrichment as early as 2 hours (5.0 mg PLA ml^{-1}) or 3 hours (3.75 mg ml^{-1}). At 3, 4, and 6 hours, viable counts of the pathogen in soup with PLA (3.75 mg ml^{-1}) and phosvitin combined were significantly lower (0.80, 1.37 and 2.23 Log cycles, respectively) than counts in control soup ($P<0.05$). Soup with the higher PLA concentration (5.0 mg ml^{-1}) combined

with phosvitin had numbers of viable *L. monocytogenes* that were significantly lower than those of soup with PLA (3.75) combined with phosvitin ($P<0.05$).

The initial viable count of *S. enterica* in control soup at 35 ℃ reached 7.10 Log_{10} CFU ml^{-1} in 6 hours (Table 7). Phosvitin alone in soup at that temperature did not significantly affect growth of *S. enterica* compared to control ($P>0.05$). PLA at 3.75 mg ml^{-1} reduced initial counts of *S. enterica* by 2.70, 2.93 and 3.03 Log cycles at 2, 3, and 4 hours, respectively ($P<0.05$); no viable *S. enteric* was detected by plating or enrichment at 6 hours. In contrast, PLA at 5.0 mg ml^{-1} reduced the initial viable count of *S. enterica* by 3.53 Log cycles in 1 hour and completely inactivated the pathogen within 2 hours; no viable counts were detected in the soup by plating or enrichment at 2 to 6 hours. Combinations of PLA and phosvitin in soup significantly inhibited growth of *S. enterica* compared to control ($P<0.05$). No significant differences in viable counts of the pathogen was observed between the two PLA/phosvitin combinations at 1 to 4 hours ($P>0.05$). At 6 hours viable counts (Log_{10} CFU ml^{-1}) of *S. enterica* in soup with PLA and phosvitin combined were 4.00 (3.75 mg PLA + phosvitin) and 1.70 (5.0 mg PLA + phosvitin).

The initial viable count of *E. coli* O157:H7 in soup at 35 ℃ increased to 7.90 Log_{10} CFU ml^{-1} within 6 hours (Table 8). At 1 to 4 hours, there no significant differences in *E. coli* O157:H7 survivors in control soup compared to soup with added phosvitin alone ($P>0.05$); however, at 6 hours viable counts of the pathogen increased to 7.90 Log_{10} CFU ml^{-1} compared to 6.50 Log_{10} CFU ml^{-1} in soup with added phosvitin alone ($P<0.05$). At 1 and 2 hours, PLA at 3.75 mg ml^{-1} reduced initial numbers of the pathogen in soup by 1.0 and 2.87 Log cycles, respectively; no viable cells of the pathogen were detected at 3 to 6 hours (Table 8). In soup with added PLA (5 mg ml^{-1}), the initial counts of the pathogen was reduced by

2.68 Log_{10} CFU ml^{-1} in 1 hour and no viable cells could be recovered from the soup at 2 through 6 hours. Compared to control, soup with PLA/phosvitin combinations significantly inhibited growth of *E. coli* O157:H7 ($P<0.05$) with a greater decline in cell viability occurring in soup that contained 5.0 mg PLA ml^{-1}. At 3, 4, and 6 hours, viable counts of the pathogen in soup with the higher concentration of PLA (5.0 mg ml^{-1}) combined with phosvitin were lower than those of soup with PLA (3.75 mg ml^{-1}) plus phosvitin ($P<0.05$). From 2 to 6 hours viable counts *E. coli* O157:H7 in soup with PLA/phosvitin combinations, irrespective of the PLA concentration, were lower than those in soup with added phosvitin alone ($P<0.05$).

At 35 ºC the *S. aureus* in control soup attained a maximum concentration of 5.90 Log_{10} CFU ml^{-1} at 4 hours (Table 9). Contrary to our observations of soup held at 12 ºC, phosvitin in soup at 35 ºC did not significantly inhibit growth of *S. aureus* compared to control ($P>0.05$). The *S. aureus* exhibited a heightened sensitivity to PLA in the soup at 35 ºC with about 3.84 to 4.0 Log reduction in initial viable counts occurring as early as 1 hour. At 2 to 6 hours Log reductions of *S. aureus* in soup with PLA at 3.75 mg ml^{-1} and 5.0 mg ml^{-1} ranged from 3.75 to 4.00 and 2.74 to 4.00, respectively. Compared to control and soup with phosvitin alone, soup with PLA and phosvitin added as combined treatments had significantly lower viable counts of the pathogen ($P<0.05$); however, those same combinations were not as lethal to the pathogen compared to PLA at 3.75 or 5.0 mg ml^{-1} (Table 9).

4. Discussion

Based on the results on growth of the four pathogens in BHI broth with added PLA, the observed growth inhibition of both Gram negative and Gram positive pathogens by PLA suggests that PLA might have a broad spectrum of inhibitory activity for various types of foodborne bacteria. Our observation agreed with those of a previous study in which the agar-well diffusion assay was used to evaluate the antibacterial effect of PLA (Dieuleveux et al., 1998). Those same researchers reported that PLA (20 mg ml^{-1}) inhibited growth of several Gram negative and Gram positive bacteria isolated from human sources (Dieuleveux et al., 1998).

Generally, it is well recognized that antimicrobial concentrations that are effective microbial inhibitors in in laboratory media may not be the same in actual food systems because the food matrix is complex and different in composition when compared to microbiological media. Since the published report on the antibacterial effects of phosvitin describe results obtained from *E. coli* in microbiological media (Sattar Khan et al., 2000) and only one study reported the effects of PLA against *L. monocytogenes* in milk and cheese (Dieuleveux and Gueguen, 1998), more studies on the antibacterial effects of PLA and phosvitin against human enteric pathogens in foods are warranted.

In the present study concentrations (3.75 mg ml^{-1} or 5 mg ml^{-1}) of PLA were evaluated alone or combined with phosvitin (60 mg ml^{-1}) to control pathogens in cream of chicken soup at abusive temperatures. The selection of phosvitin (60mg ml^{-1}) for use in the present study was based on our preliminary studies which demonstrated that phosvitin at 60 mg ml^{-1}strongly inhibited the growth of the four pathogens in BHI broth at 35 °C (data not shown). Data from the present study suggest that phosvitin alone is more inhibitory to the

growth of the four pathogens in cream of mushroom soup at 12 ºC compared to 35 ºC. The temperature of 35 ºC was used to represent temperature abuse of the soup; however, that temperature is optimum for growth of the pathogens used in the present study. It seems that it is more difficult for some antimicrobial agents to inhibit growth of pathogens at the pathogens' optimum growth temperature. It is well known that bacteria are adapted to function optimally in their normal physiological environments. In this regard, any environmental change from their optimum environmental conditions imposes a stress on bacteria. The extent of the environmental change such as a reduction in growth temperature to 12 ºC, can influence whether the bacteria exhibit an increased lag phase, grow slower, stop growing or become inactivated (Ray, 1986; Russell et al., 1995). Therefore, it is likely that the observed difference in antibacterial activity of phosvitin was due to stress imposed by both phosvitin and the sub-optimum growth temperature (12 °C) used in the present study.

In cream of chicken soup at 35 °C, PLA at 3.75 or 5.0 mg ml^{-1} exerted a bactericidal effect on all four pathogens. We observed that PLA exhibited a greater antimicrobial effect against the Gram-positive pathogens (*L. monocytogenes* and *S. aureus*) compared to the Gram negative pathogens (*E. coli* O157:H7 and *S. enterica*). In fact, within 2 hours, the lower concentration of PLA (3.75 mg ml^{-1}) in soup at 35 °C reduced initial populations of *L. monocytogenes* and *S. aureus* by 3.52 and 3.80 Log cycles respectively whereas, under those same conditions, initial polulations of *S. enterica* and *E. coli* O157:H7 were reduced to a lesser extent (2.70 and 2.87 Log cycles, respectively) (Tables 6-8). Our observations are in agreement with the results of a previous study which revealed that Gram positive bacteria isolated from humans were more sensitive to PLA than the Gram negative isolates at 37 °C based on a zone inhibition tests using the agar-well diffusion assay (Dieuleveux et al., 1998).

This reasons for this observed difference remains speculative and warrants further investigation.

Contrary to our expectation, combinations of PLA and phosvitin added to cream of chicken soup were not more inhibitory that PLA alone. For all four pathogens the antibacterial effect of PLA in the soup was reduced when that antimicrobial was used in combination with phosvitin. Generally, the efficacy of many natural antimicrobials for inhibiting growth of microorganisms may be reduced by certain components of foods (Glass and Johnson, 1994). Increased concentrations of proteins or fats have been supposedly linked to the protection of microorganisms from inactivation by natural antimicrobials (Aureli et al., 1992; Pandit and Shelef, 1994; Tassou et al., 1995). Phosvitin is a glycoprotein and it is likely that its addition to the soup protected the pathogens against the killing effect of PLA because of an increase in protein content in the soup. Also, lipophilic organic acids such as lactic, sorbic and acetic acid exhibit greater antimicrobial effect at low pH values which favor more of the undissociated form of those acids (Davidson, 2001). Preliminary studies in our laboratory revealed increased antibacterial activity of PLA with decreases in phosvitin concentration and vice versa (data not shown). Also, the higher pH of soup with the PLA/phosvitin combination compared to that of soup of soup with PLA alone might have contributed to the decreased antibacterial effect of PLA because PLA has been reported to exert stronger antimicrobial effects at acidic pH (Ohhira et al., 2004).

A major objective of the present study was to evaluate the antimicrobial efficacy of phosvitin or phenyllactic acid alone or in combination against foodborne pathogens in cream of chicken at 12 °C and 35 °C. Cream of chicken soup was selected for use in the present study because chicken soup is a very popular soup consumed in homes, soup kitchens, salad

bars, and cafeterias. Also, cream of chicken soup, unlike bullion-type soup, is a more complex food product that contains ingredients such as dehydrated cooked chicken, chicken stock, chicken fat, milk cream and food starch (Table 1) that make it challenging for antimicrobial agents to destroy contaminating microorganisms. The soup was intentionally held at 12 °C and 35 °C to simulate temperature abuse conditions that can compromise the microbial safety of potentially hazardous foods. The temperatures of 12 °C and 35 °C both reside in the temperature danger zone (5 ºC to 60 ºC) in which pathogenic bacteria can multiply rapidly and reach large populations thus increasing the risk of foodborne disease. At both temperatures PLA by itself was more effective at controlling all pathogens as opposed to a combination of PLA and phosvitin. Based on these findings PLA has good potential for enhancing the microbial safety of cream of chicken soup and reducing the risk of foodborne infection or intoxication in soup that might be inadvertently exposed to abusive temperatures that are suitable for the growth of foodborne pathogens. Further research is needed to determine the influence of PLA on the sensory characteristics and quality of food.

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TABLE 1. [a]Ingredients in cream of chicken soup
Chicken stock
Water
Vegetable Oil
Modified Food Starch
Wheat Flour
Cream (Milk)
Salt
Dehydrated Cooked Chicken
Soy Protein Concentrate
Monosodium Glutamate
Chicken Fat
Yeast Extract
Flavoring
Beta Carotene for Color
Chicken Flavor
Partially Hydrogenated Soybean
Partially Cottonseed Oil
Butter (Milk)

*Sodium 750 mg - 31%

[a] Ingredients are listed in the same order as they appear on the label of the package

TABLE 2. Antibacterial effectiveness of phenyllactic acid (3.75 mg ml^{-1} and 5 mg ml^{-1}) alone or combined with phosvitin (60 mg ml^{-1}) against *Listeria monocytogenes* in cream of chicken soup held at 12 °C for 7 days.

	Viable count (Log_{10} CFU ml^{-1})[x]			
Treatment (mg ml^{-1})	1d	3d	5d	7d
Control	4.10 ± 0.56a	6.20 ± 0.62a	7.53 ± 0.21a	8.50 ± 0.46a
Phenyllactic acid (3.75)	3.63 ± 0.06a	2.53 ± 0.42c	1.05 ± 1.16d	0.57 ± 0.98d
Phenyllactic acid (5)	1.96 ± 1.03b	0.77 ± 1.33d	0.00 ± 0.00e	0.00 ± 0.00d
Phosvitin (60)	4.40 ± 0.20a	4.67 ± 0.32b	5.40 ± 0.44b	5.60 ± 0.44b
PLA + Phos (3.75 + 60)	3.50 ± 0.69a	4.10 ± 0.10b	4.33 ± 0.25c	4.47 ± 0.06c
PLA + Phos (5 + 60)	3.47 ± 0.46a	4.27 ± 0.31b	4.27 ± 0.25c	4.33 ± 0.31c

[x]Each reported value for viable count represents the mean (standard deviation) of three replications of the experiment

[a,b,c,d]Means with a different letter within a column differ significantly ($P<0.05$)

TABLE 3. Antibacterial effectiveness of phenyllactic acid (3.75 mg ml^{-1}and 5 mg ml^{-1}) alone or combined with phosvitin (60 mg ml^{-1}) against *Salmonella enterica* in cream of chicken soup held at 12 °C for 7 days.

	Viable count (Log$_{10}$ CFU ml^{-1})^{x}			
Treatment (mg ml^{-1})	1d	3d	5d	7d
Control	4.37 ± 0.29a	5.87 ± 0.61a	7.27 ± 0.66a	8.00 ± 0.44a
Phenyllactic acid (3.75)	2.17 ± 1.07c	0.70 ± 1.21c	0.00 ± 0.00c	0.00 ± 0.00d
Phenyllactic acid (5)	0.00 ± 0.00d	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00d
Phosvitin (60)	4.17 ± 0.15ab	5.40 ± 0.52a	6.63 ± 0.55a	7.50 ± 0.56a
PLA + Phos (3.75 + 60)	3.33 ± 0.15b	4.87 ± 0.72ab	4.70 ± 0.60b	4.23 ± 0.23b
PLA + Phos (5 + 60)	3.27 ± 0.55b	4.07 ± 0.15b	4.07 ± 0.11b	3.63 ± 0.06c

[x]Each reported value for viable count represents the mean (standard deviation) of three replications of the experiment

[a,b,c,d]Means with a different letter within a column differ significantly ($P<0.05$)

TABLE 4. Antibacterial effectiveness of phenyllactic acid (3.75 mg ml^{-1} and 5 mg ml^{-1}) alone or combined with phosvitin (60 mg ml^{-1}) against *Escherichia coli* O157:H7 in cream of chicken soup held at 12 °C for 7 days.

	Viable count (Log_{10} CFU ml^{-1})[x]			
Treatment (mg ml^{-1})	1d	3d	5d	7d
Control	4.47 ± 0.32ab	6.03 ± 0.68a	7.53 ± 0.70a	8.30 ± 0.30a
Phenyllactic acid (3.75)	3.70 ± 0.35bcd	2.53 ± 0.40c	1.03 ± 0.31c	0.10 ± 0.17c
Phenyllactic acid (5)	2.93 ± 0.50d	1.43 ± 1.24c	0.20 ± 0.35d	0.00 ± 0.00c
Phosvitin (60)	4.57 ± 0.06a	5.60 ± 0.61a	6.93 ± 0.38a	7.93 ± 0.29a
PLA + Phos (3.75 + 60)	3.40 ± 0.78cd	4.37 ± 0.15b	4.37 ± 0.06b	4.00 ± 0.50b
PLA + Phos (5 + 60)	3.80 ± 0.40abc	4.20 ± 0.10b	4.07 ± 0.23b	3.93 ± 0.25b

[x]Each reported value for viable count represents the mean (standard deviation) of three replications of the experiment

[a,b,c,d]Means with a different letter within a column differ significantly ($P<0.05$)

TABLE 5. Antibacterial effectiveness of phenyllactic acid (3.75 mg ml^{-1} and 5 mg ml^{-1}) alone or combined with phosvitin (60 mg ml^{-1}) against *Staphylococcus aureus* in cream of chicken soup held at 12 °C for 7 days.

	Viable count (Log_{10} CFU ml^{-1})[x]			
Treatment (mg ml^{-1})	1d	3d	5d	7d
Control	4.70 ± 0.10a	6.47 ± 0.42a	7.70 ± 0.10ay	7.90 ± 0.10a
Phenyllactic acid (3.75)	2.77 ± 0.15b	2.53 ± 0.31d	0.00 ± 0.00d	0.00 ± 0.00c
Phenyllactic acid (5)	1.43 ± 1.25c	1.03 ± 0.48e	0.23 ± 0.40d	0.00 ± 0.00c
Phosvitin (60)	4.60 ± 0.00a	5.10 ± 0.79b	6.77 ± 0.68b	7.57 ± 0.25a
PLA + Phos (3.75 + 60)	3.50 ± 0.10b	4.17 ± 0.06c	4.37 ± 0.15c	3.87 ± 0.15b
PLA + Phos (5 + 60)	3.47 ± 0.15b	4.67 ± 0.42bc	4.00 ± 0.00c	3.63 ± 0.57b

[x]Each reported value for viable count represents the mean (standard deviation) of three replications of the experiment

[a,b,c]Means with a different letter within a column differ significantly ($P<0.05$)

TABLE 6. Antibacterial effectiveness of phenyllactic acid (3.75 mg ml^{-1} and 5 mg ml^{-1}) alone or combined with phosvitin (60 mg ml^{-1}) against *Listeria monocytogenes* in cream of chicken soup held at 35 °C for 6 hours.

	Viable count (Log_{10} CFU ml^{-1})[x]				
Treatment (mg ml^{-1})	Hour 1	Hour 2	Hour 3	Hour 4	Hour 6
Control	4.50 ± 0.17a	4.50 ± 0.26a	4.83 ± 0.51a	5.37 ± 0.38a	6.03 ± 0.32a
Phenyllactic acid (3.75)	1.10 ± 1.91b	0.48 ± 0.44c	0.00 ± 0.00c	0.00 ± 0.00d	0.00 ± 0.00d
Phenyllactic acid (5)	0.17 ± 0.29b	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00d	0.00 ± 0.00d
Phosvitin (60)	3.93 ± 0.74a	4.43 ± 0.25a	4.33 ± 0.51a	4.63 ± 0.45ab	5.07 ± 0.55b
PLA + Phos (3.75 + 60)	4.10 ± 0.10a	3.97 ± 0.12a	4.03 ± 0.32a	4.00 ± 0.52b	3.80 ± 0.35c
PLA + Phos (5 + 60)	3.40 ± 1.22a	3.23 ± 0.74b	1.47 ± 1.37b	0.93 ± 0.86c	0.30 ± 0.52d

[x]Each reported value for viable count represents the mean (standard deviation) of three replications of the experiment

[a,b,c,d]Means with a different letter within a column differ significantly ($P<0.05$)

TABLE 7. Antibacterial effectiveness of phenyllactic acid (3.75 mg ml^{-1} and 5 mg ml^{-1}) alone or combined with phosvitin (60 mg ml^{-1}) against *Salmonella enterica* in cream of chicken soup held at 35 °C for 6 hours.

	Viable count (Log_{10} CFU ml^{-1})^x				
Treatment (mg ml^{-1})	Hour 1	Hour 2	Hour 3	Hour 4	Hour 6
Control	4.53 ± 0.15a	4.70 ± 0.17a	5.50 ± 0.20a	5.97 ± 0.32a	7.10 ± 0.40a
Phenyllactic acid (3.75)	3.23 ± 0.85b	1.30 ± 2.25b	1.07 ± 1.85d	0.97 ± 1.67d	0.00 ± 0.00d
Phenyllactic acid (5)	0.47 ± 0.81c	0.00 ± 0.00b	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00d
Phosvitin (60)	4.50 ± 0.36a	4.83 ± 0.20a	5.10 ± 0.36ab	5.53 ± 0.06ab	5.93 ± 0.67a
PLA + Phos (3.75 + 60)	4.30 ± 0.10a	4.10 ± 0.00a	4.03 ± 0.15bc	4.30 ± 0.10bc	4.00 ± 0.00b
PLA + Phos (5 + 60)	3.80 ± 0.26ab	3.97 ± 0.06a	3.63 ± 0.29c	3.53 ± 0.76c	1.70 ± 1.48c

[x]Each reported value for viable count represents the mean (standard deviation) of three replications of the experiment

[a,b,c,d]Means with a different letter within a column differ significantly ($P<0.05$)

TABLE 8. Antibacterial effectiveness of phenyllactic acid (3.75 mg ml^{-1} and 5 mg ml^{-1}) alone or combined with phosvitin (60 mg ml^{-1}) against *Escherichia coli* O157:H7 in cream of chicken soup held at 35 °C for 6 hours.

	Viable count (Log_{10} CFU ml^{-1})[x]				
Treatment (mg ml^{-1})	Hour 1	Hour 2	Hour 3	Hour 4	Hour 6
Control	4.87 ± 0.06a	5.03 ± 0.12a	6.00 ± 0.10a	6.80 ± 0.10a	7.90 ± 0.00a
Phenyllactic acid (3.75)	3.00 ± 0.56c	1.13 ± 0.21c	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00e
Phenyllactic acid (5)	1.32 ± 0.33d	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00e
Phosvitin (60)	4.87 ± 0.21a	5.10 ± 0.10a	5.87 ± 0.25a	6.17 ± 0.06a	6.50 ± 0.00b
PLA + Phos (3.75 + 60)	4.37 ± 0.12ab	4.20 ± 0.10b	4.17 ± 0.06b	4.00 ± 0.00b	4.00 ± 0.00c
PLA + Phos (5 + 60)	4.00 ± 0.10b	3.67 ± 0.84b	2.57 ± 0.76c	2.30 ± 0.87c	2.37 ± 1.23d

[x]Each reported value for viable count represents the mean (standard deviation) of three replications of the experiment

[a,b,c,d,e]Means with a different letter within a column differ significantly ($P<0.05$)

TABLE 9. Antibacterial effectiveness of phenyllactic acid (3.75 mg ml^{-1} and 5 mg ml^{-1}) alone or combined with phosvitin (60 mg ml^{-1}) against *Staphylococcus aureus* in cream of chicken soup held at 35 °C for 6 hours.

	Viable count (Log_{10} CFU ml^{-1})^{x}				
Treatment (mg ml^{-1})	Hour 1	Hour 2	Hour 3	Hour 4	Hour 6
Control	3.73 ± 0.57a	4.23 ± 0.57a	4.80 ± 0.10a	5.90 ± 0.20a	5.80 ± 0.10a
Phenyllactic acid (3.75)	0.00 ± 0.00c	0.20 ± 0.17c	0.00 ± 0.00d	0.60 ± 1.04c	0.49 ± 0.50c
Phenyllactic acid (5)	0.16 ± 0.28c	1.26 ± 1.12c	0.00 ± 0.00d	0.70 ± 0.96c	0.00 ± 0.00c
Phosvitin (60)	4.27 ± 0.06a	4.67 ± 0.06a	4.90 ± 0.00a	5.53 ± 0.31a	5.20 ± 0.62a
PLA + Phos (3.75 + 60)	2.43 ± 0.15b	2.97 ± 0.42b	3.63 ± 0.31b	3.03 ± 0.57b	2.33 ± 0.38b
PLA + Phos (5 + 60)	2.77 ± 0.59b	2.77 ± 0.87b	3.20 ± 0.10c	1.60 ± 1.00c	1.85 ± 0.88b

[x]Each reported value for viable count represents the mean (standard deviation) of three independent replications of the experiment

[a,b,c,d]Means with a different letter within a column differ significantly ($P<0.05$)

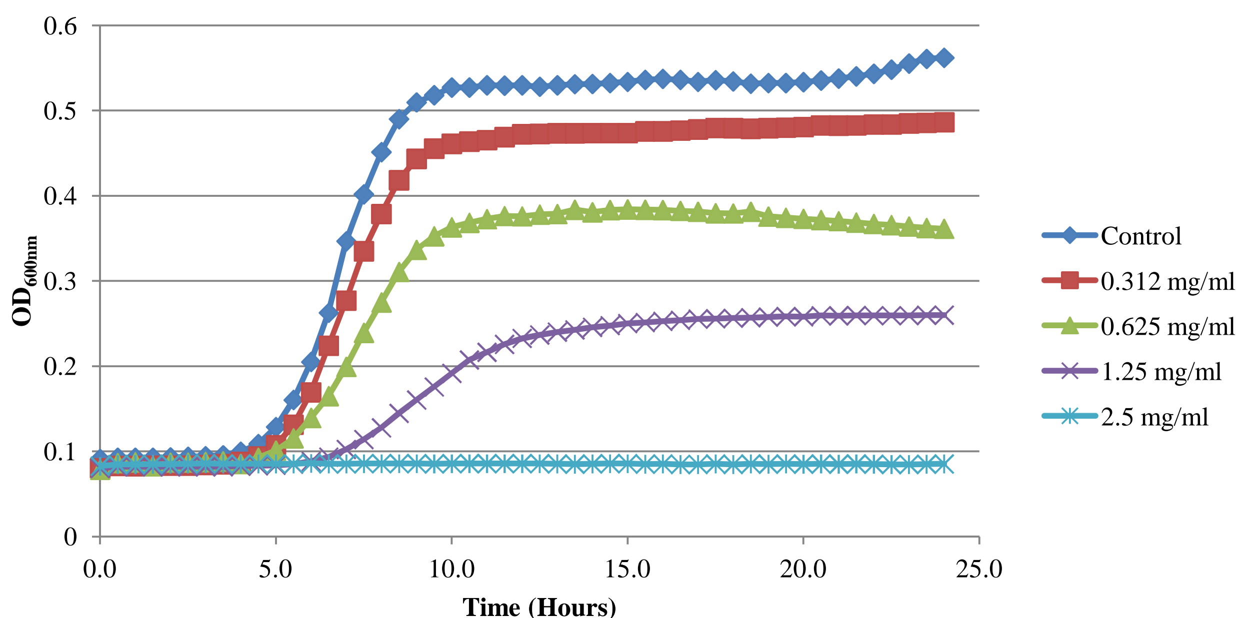

Figure 1. Growth of *Listeria monocytogenes* in BHI broth (35 °C) at pH 6.0 supplemented with various concentrations of phenyllactic acid.

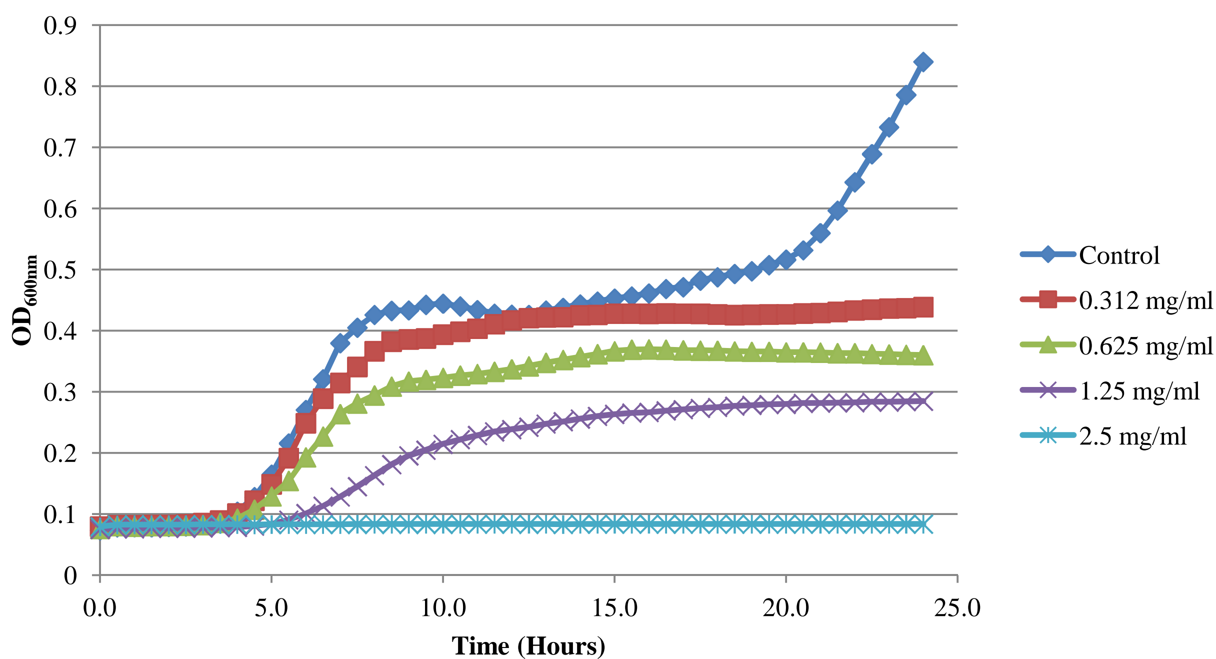

Figure 2. Growth of *Salmonella enterica* in BHI broth (35 °C) at pH 6.0 supplemented with various concentrations of phenyllactic acid.

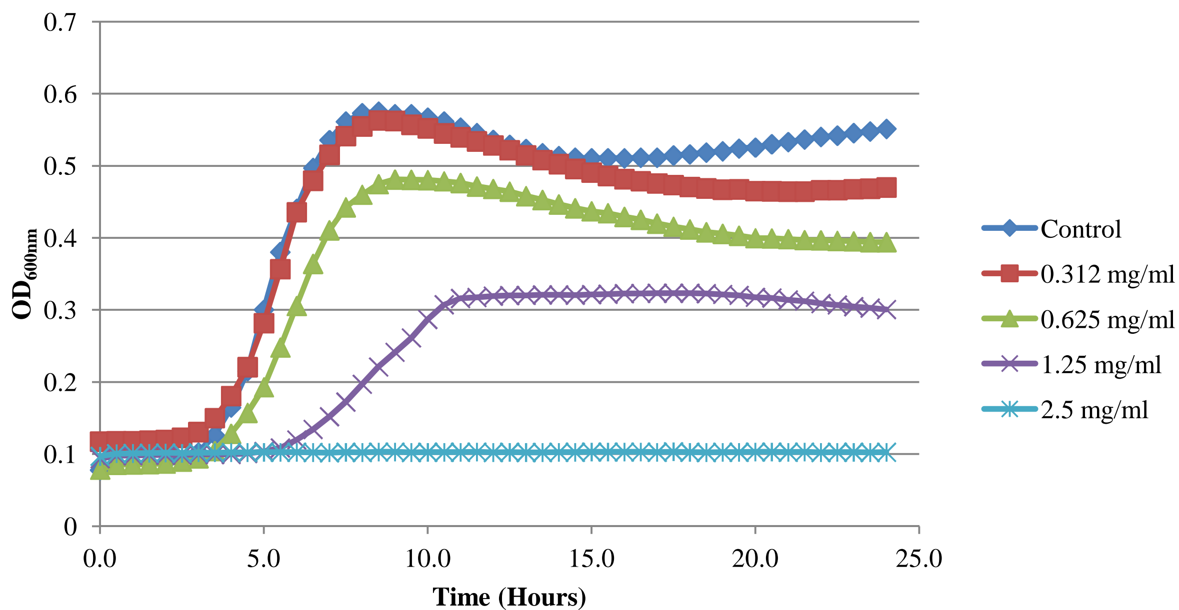

Figure 3. Growth of *Escherichia coli* O157:H7 in BHI broth (35 °C) at pH 6.0 supplemented with various concentrations of phenyllactic acid.

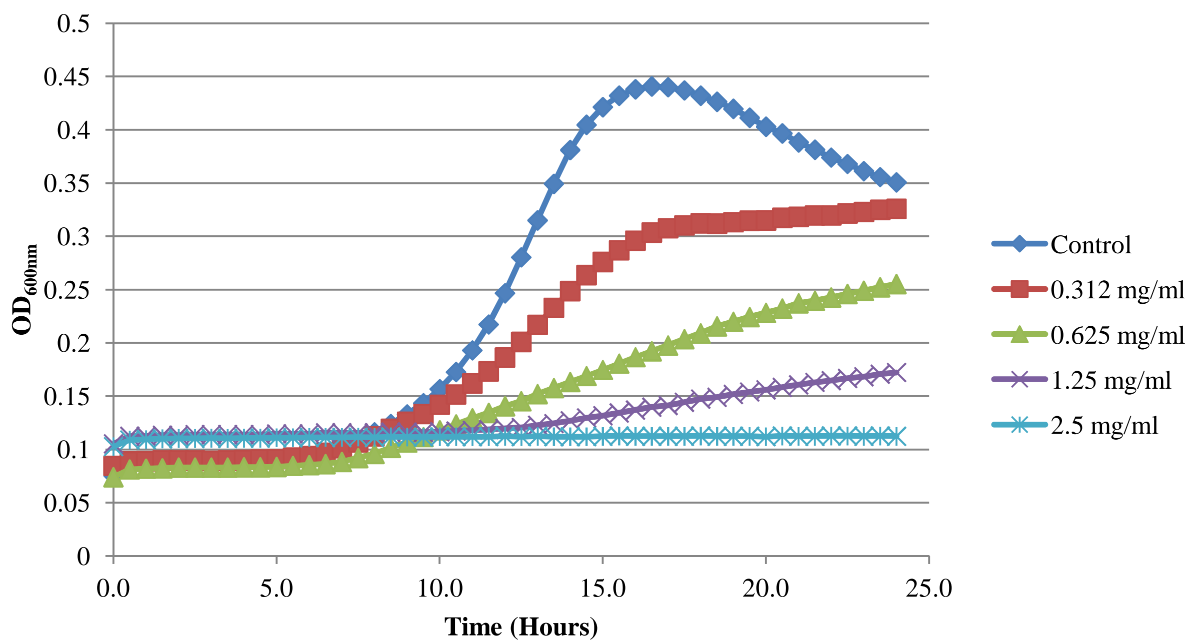

Figure 4. Growth of *Staphylococcus aureus* in BHI broth (35 °C) at pH 6.0 supplemented with various concentrations of phenyllactic acid.

CHAPTER 4

AN IN VITRO STUDY ON THE ANTIMICROBIAL EFFICACY OF DL-3-PHENYLLACTIC ACID AGAINST SELECTED *ASPERGILLUS* AND *PENICILLIUM* MOLDS AT 25 °C

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Key words: *Aspergillus, Penicillium*, phenyllactic acid, antifungal activity

Abstract

The objective of this research was to determine the effectiveness of phenyllactic acid (PLA) at three different pH values against *Aspergillus ochraceus*, *Aspergillus spp*, *Penicillium roqueforti*, *Penicillium glabrum*, and *Penicillium spp* in brain heart infusion (BHI) broth. Broth with added PLA (2.5 – 15 mg/ml) was inoculated with *A. ochraceus*, *Aspergillus spp*, *P.roqueforti*, *P. glabrum* or *Penicillium spp* at 10^6 conidia/ml. Broth without added PLA served as a control. Growth curves for molds (25 °C, 5 days) were developed from data obtained using a Bioscreen C turbidometer (OD 600 nm) which recorded optical density (OD) measurements every 12 hours. . At pH 3.8, PLA at 5.0 to 15 mg/ml inhibited growth of all five molds with stronger growth inhibition at occurring with increased PLA concentrations. Compared to the aspergilli, the penicillia exhibited increased sensitivity to PLA at pH 3.8. PLA at 10 or 15 mg/ml completely inhibited growth of penicillia, whereas only PLA at 15 mg/ml completely inhibited growth of aspergilli. At pH 5.0 or 5.3, growth inhibition of molds by PLA was markedly decreased. Generally, the rate of increase in OD of the mold cultures was similar at all PLA concentrations tested. Based on the results of this study, PLA is more effective in inhibiting the growth of molds at a lower pH (3.80). The capability of PLA to effectively control the growth of several foodborne fungi at low pH offers new perspectives for its application as an antifungal ingredient in acidic foods that are prone to spoilage by molds.

Introduction

A topic of discussion that is gaining a vast amount of interest is Green Consumerism which is a consumer-driven effort to replace synthetic additives in food products with more natural ingredients. This topic has gained interest because consumer demand for reduced use of chemical preservatives in foods has increased. Long term adverse health effects are the major consumer concern regarding eating food with synthetic chemical preservatives. Nitrites, parabens, sulfites, and sorbates are examples of chemical preservatives that consumers perceive as not being natural. Natural alternative replacements to synthetic chemical preservatives can be isolated from plant, animal, and microbial sources.

Mold spoilage has been a persistent problem faced by the food industry. Molds can grow over a wide pH range from below pH 2.0 to above pH 9.0 (Beuchat and Cousin, 2001) and therefore can grow in a wide variety of food products including acidic sauces, salad dressings and cheeses. In order to combat mold spoilage, food preservatives such as potassium sorbate or sodium benzoate are used to control fungal growth in many acidic condiments. Also sorbate or natamycin is use to inhibit mold growth in cheese.

Both sodium or potassium sorbate and natamycin are antifungal agents but they also have unique problems. Certain species of *Aspergillus* and *Penicillium* can degrade potassium sorbate and produce unpleasant odors (Marth et al., 1966; Sofos and Busta, 1981; Kinderlerer and Hatton, 1990). On the other hand, natamycin stimulates low level resistance among fungi (Jay et al., 2005), and has relatively poor solubility, distribution, and stability. Also the name “natamycin” may give consumers the impression that an antibiotic has been added to the cheese as a preservative. Considering the previously mentioned limitations in using sorbate

or natamycin in cheese in addition to negative consumer perception, natural alternatives for control of foodborne fungi are needed.

An alternative emerging new preservation technique utilizes lactic acid bacteria (LAB) and their metabolites. The interest in using LAB, as biopreservatives, has increased because LAB are Generally Recognized as Safe (GRAS) due their contribution to the healthy microflora of human intestinal mucosa (Valerio et al., 2004) and their long history being safely consumed in fermented foods. Lactic acid bacteria are capable of producing antimicrobial compounds such as bacteriocins which are proteins with antibacterial activity that inhibit or kill bacteria (Cleveland et al., 2001). A well-known bacteriocin, Nisin, is produced by *Lactococcus lactis* and has a broad spectrum of antibacterial activity against gram-positive bacteria as well as GRAS status because of its ability to control the outgrowth and toxin production of *Clostridium botulinum* in pasteurized cheese spreads (Stevens et al., 1991). The majority of identified antifungal compounds are low molecular weight compounds comprised of organic acids, reuterin, hydrogen peroxide, proteinaceous compounds, hydroxyl fatty acids and phenolic compounds (Dalie et al, 2009) but research has indicated that certain compounds isolated from lactic acid bacteria can have an inhibitory effect on either bacteria or yeasts and molds.

An emerging antimicrobial substance isolated from *Geotrichum candidum* as well as several *Lactobacillus* species is phenyllactic acid (PLA). This natural antimicrobial was originally used to inhibit *Listeria monocytogenes* in milk (Dieuleveux and Gueguen, 1998). More recently, lactic acid bacteria have been used to inhibit mold growth in sourdough bread. This has led to the discovery that *Lactobacillus plantarum* 21B produces PLA (Lavermicocca et al., 2000; Lavermicocca et al., 2003). Phenyllactic acid has four different forms: D-3-

phenyllactic acid, L-3-phenyllactic acid, DL-3-phenyllactic acid, and 4-hydroxyphenyllactic acid, all of which are produced by either *Geotrichum candidum* or *Lactobacillus plantarum* 21D and certain other lactic acid producing bacteria (Mu et al., 2012). Limited research has been conducted on all four forms, but it has been found DL-3-phenyllactic acid and 4-hydroxyphenyllactic acid have the greatest antifungal effect (Dalie et al, 2009; Lavermicocca et al. 2003; Lavermicocca et al. 2000). To discover the full potential of PLA, much research still needs to be conducted to determine its antifungal capabilities.

The antifungal activity of PLA may be used to improve microbial safety of foods as well as increase shelf life. The knowledge of natural antimicrobials is steadily increasing, but for this particular antimicrobial (PLA) only few published reports are available on its antimicrobial effectiveness against bacteria and fungi at various levels of pH. The objective of the present study was to evaluate the antifungal efficacy of PLA in BHI broth (25 °C) at three different pH values.

Materials and Methods

Fungal cultures and culture conditions. *Aspergillus spp*, *Penicillium roqueforti* (Blue cheese isolate), and *Penicillium spp*. were obtained from the culture collection of the Microbial Food Safety Laboratory, Iowa State University, Ames, IA. *Penicillium glabrum* (ATCC 11080) and *Aspergillus ochraceus* (ATCC 58722) were obtained from the American Type Culture Collection (Manassas, Virgina). Stock cultures were kept from (-70 °C) in acidified potato dextrose agar (Difco, Becton Dickinson, Sparks, Md) slants. Stock cultures were activated by placing 5 ml of sterile distilled water in stock cultures. Cultures were then

mixed by vortexing then surface-plated on acidified potato dextrose agar. Cultures were incubated at 25 °C for 7 days to induce the production of mold conidia.

Preparation of inoculum. Sterile distilled water (6.0 ml) was added to the lawn growth of mold. A sterile stainless steel bent rod was used to make suspensions of mold conidia by mixing the conidia with the water added to the lawn of mold on agar surface. The suspensions of conidia were used as inocula for BHI broth. The conidial suspensions were serially diluted (1:10) in 0.85% (w/v) NaCl (saline) and plated on acidified potato dextrose agar to determine concentration (colony forming units per ml).

Antimicrobial. A commercial preparation of DL-3-Phenyllactic acid was purchased from Sigma-Aldrich (P7251 Aldrich, St. Louis, Missouri).

Preparation of treatment solutions for Bioscreen C assay. BHI broth with added PLA (0, 2.5, 3.75, 5, 7.5, 10, or 15 mg/ml) was filter-sterilized using 0.22 µm pore size filters (Fisher Scientific, Pittsburgh, PA) and the pH was adjusted to 3.8, 5.0, and 5.3 using 1M sodium hydroxide (NaOH) or 1M hydrochloric acid (HCl). Treatment solutions (2.5-ml samples of BHI broth) and the control (BHI with no added antimicrobial) were each inoculated with 25 µl of diluted (1:100) *Aspergillus ochraceus*, *Aspergillus spp*, *Penicillium roqueforti*, *Penicillium glabrum*, or *Penicillium spp*. conidia to obtain a final concentration of 10^6 conidia/broth sample. Inoculated BHI broth was distributed in wells of a microtiter plate for the Bioscreen C Turbidometer.

Bioscreen C assay. Inoculated samples (aliquots of 200 µl) were added in triplicate to the wells of a 100-well microtiter plate for the Bioscreen C Turbidometer (Growth Curves USA

Piscataway, NJ), an automated microbial growth analyzer and incubator. Microtiter plates were incubated in the Bioscreen C at 25 °C for 7 days and the machine took optical density (OD) measurements at 600 nm every 12 hours, with shaking of samples for 10 seconds prior to each OD reading.

Results and Discussion

Inhibitory effect of PLA in BHI broth at pH 3.8. The effect of PLA on the growth of *Aspergillus ochraceus*, *Aspergillus spp*, *Penicillium roqueforti*, *Penicillium glabrum*, and *Penicillium spp*. in BHI broth at pH 3.8 is shown in figures 1-5. At pH 3.8, PLA at 2.5 and 3.75 mg/ml was ineffective in inhibiting the growth of *Aspergillus* and *Penicillium* molds. PLA concentrations ranging from 5.0 to 15 mg/ml were the most effective mold inhibitors; growth inhibition increased with increasing PLA concentration. Compared to the aspergilli, the penicillia exhibited increased sensitivity to PLA. PLA at 10 or 15 mg/ml completely inhibited growth of penicillia based on no increase in OD during 120 hours whereas, growth of aspergilli was completely inhibited by PLA at 15 mg/ml.

Molds can grow over a very wide pH range (Beuchat and Cousin, 2001) and therefore can spoil a variety of food products. Also, their ability to grow at low pH gives these organisms the advantage in being the dominant spoilage organisms in acidic foods that inhibit bacterial growth. For the present study, the pH of 3.8 was chosen because it is within the pH range (3.68 to 4.44) of several commercial sauces and dressings including mayonnaise-mustard sauce (pH 3.68), thousand island dressing (pH 3.76), seafood sauce (pH 4.38) and blue cheese dressing (pH 4.44) (Weagant et al., 1994). Mold growth in dressings

has been reported especially in dressings with large head spaces in the container or where improperly fitted caps increase the amount of air (Smittle, 2000).

Our results indicate that at pH 3.8, PLA exerts a strong growth inhibitory effect against the five molds (*Aspergillus* spp and *Penicillum* spp) used in the present study. These results are relevant to shelf-life extension of acidic foods that are susceptible to spoilage by fungi (yeast and molds). PLA activity (pK 3.46) has been shown to be dependent on pH, indicating that PLA's mode of action is somewhat related to the lipophilic properties which enable the undissociated form of that antimicrobial to cross microbial membranes (Gould, 1996). Considering the fact that the pK of PLA is 3.46, our use of pH 3.8 would permit a greater availability of the undissociated form of PLA to exert an antimicrobial action against the molds.

Inhibitory effect of PLA in BHI broth at pH 5.0 or 5.3. Figures 6-10 and 11-15 show the effect of phenyllactic acid on *Aspergillus ochraceus*, *Aspergillus spp*, *Penicillium roqueforti*, *Penicillium glabrum*, and *Penicillium spp*. at pH 5.0 and 5.3, respectively. The two pH values (5.0 and 5.3) used in the present study were chosen because they are within the pH range for several types of cheeses (pH 5.0 to 5.7). At pH 5.0 or 5.3, the inhibitory action of PLA against the molds was diminished in BHI broth. We observed that at those pH values, the rate of increase in OD values of the mold cultures was generally similar for almost all PLA concentrations and control. This observed clustering of growth curves of the molds indicated that at pH 5.0 or 5.3, the ODs of mold cultures in treatment broth easily approached ODs of control cultures. Schwenninger et al. (2008) reported that at pH 4.0 to 6.0, PLA has a relatively high minimum inhibitory concentration (MIC; 50 to greater than

500 mM) for antifungal activity but the MIC decreased with decreasing pH. Based on the results on PLA's antifungal activity at pH 5.0 or 5.3, higher concentrations of PLA might be required to achieve a substantial growth inhibitory effect against molds in certain cheeses with pH at about 5.1 to 5.7. Also, to circumvent the use of larger amounts of PLA and associated costs, consideration should be given to the combined application of lower concentrations of PLA with another natural antimicrobial or a "clean" technology such as high hydrostatic pressure to achieve microbial control.

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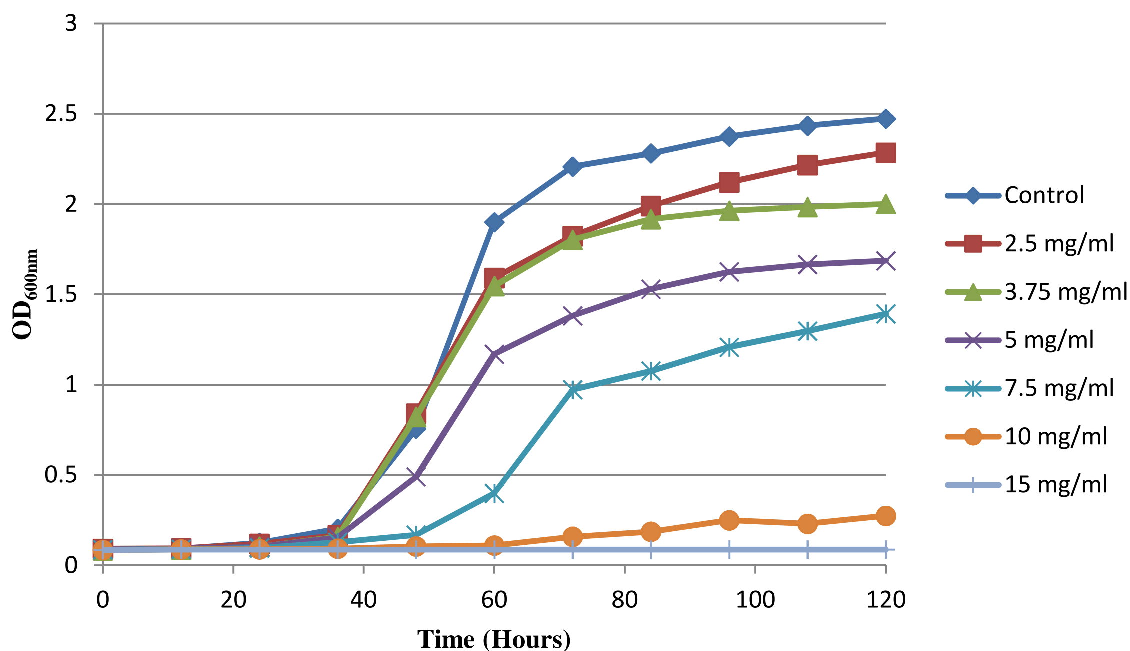

Figure 1. Growth of *Aspergillus ochraceus* in BHI broth (25 °C) at pH 3.8 supplemented with various concentrations of phenyllactic acid.

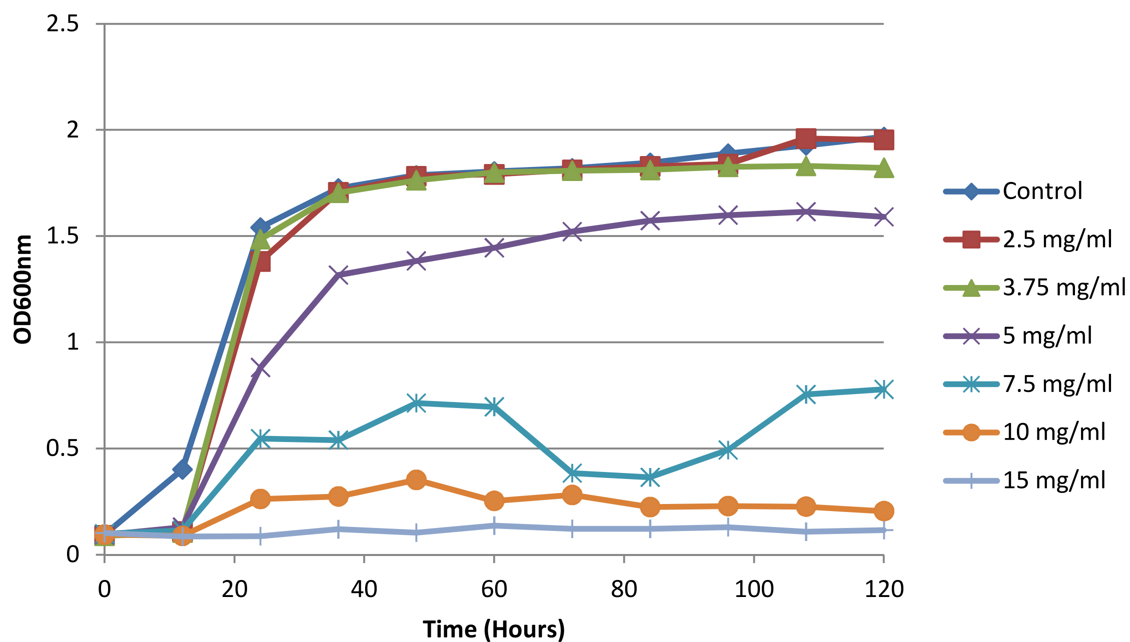

Figure 2. Growth of *Aspergillus spp.* in BHI broth (25 °C) at pH 3.8 supplemented with various concentrations of phenyllactic acid.

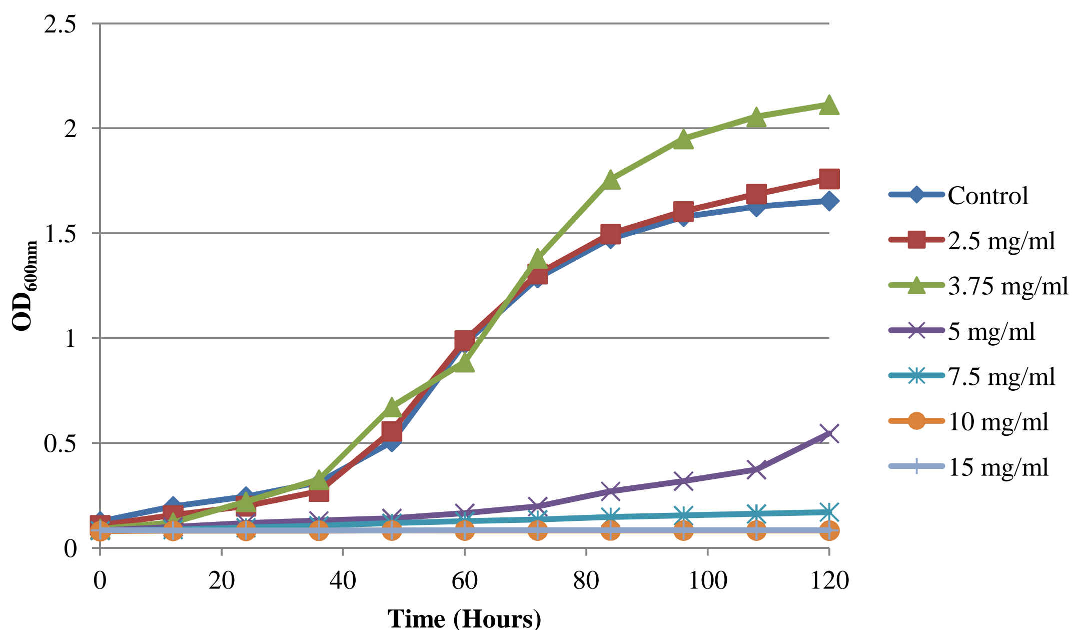

Figure 3. Growth of *Penicillium roqueforti* in BHI broth (25 °C) at pH 3.8 supplemented with various concentrations of phenyllactic acid.

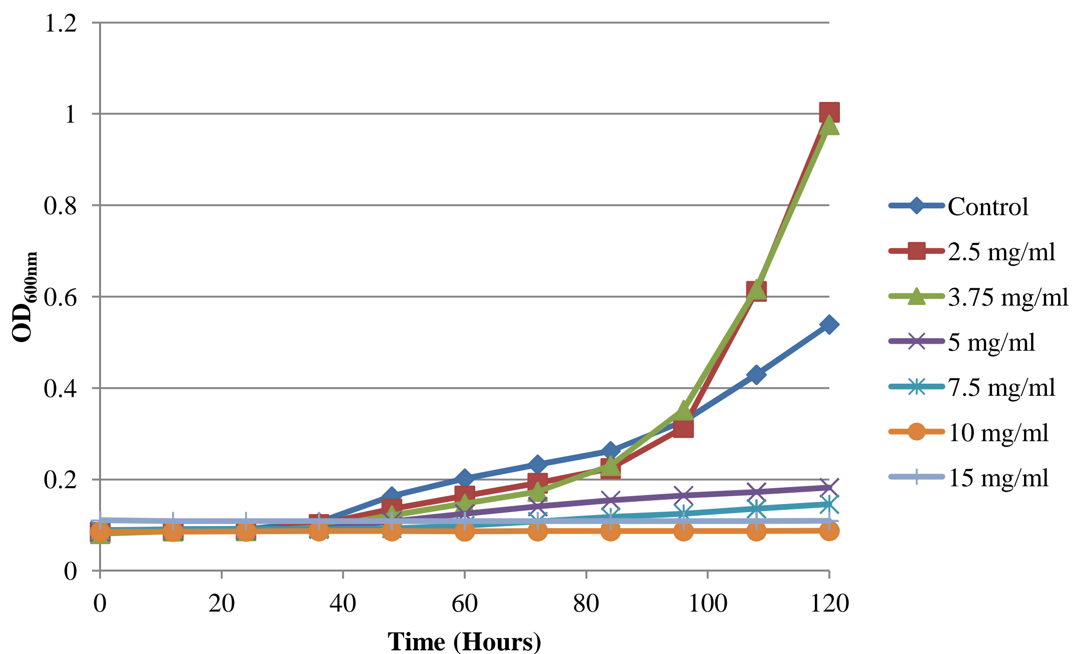

Figure 4. Growth of *Penicillium glabrum* in BHI broth (25 °C) at pH 3.8 supplemented with various concentrations of phenyllactic acid.

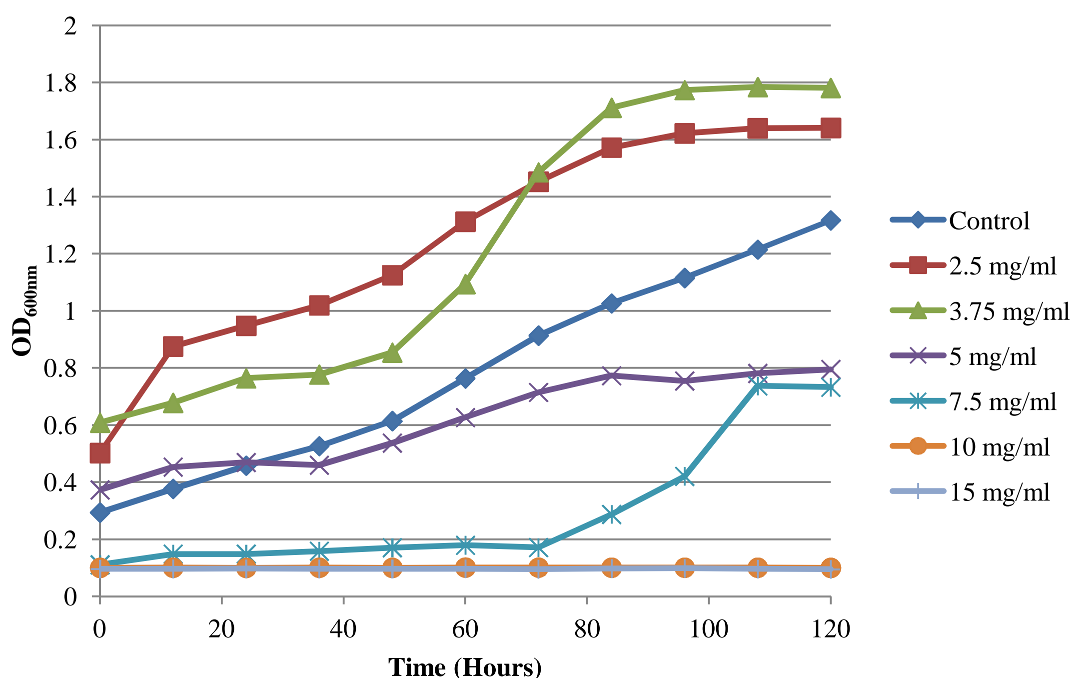

Figure 5. Growth of *Penicillium spp.* in BHI broth (25 °C) at pH 3.8 supplemented with various concentrations of phenyllactic acid.

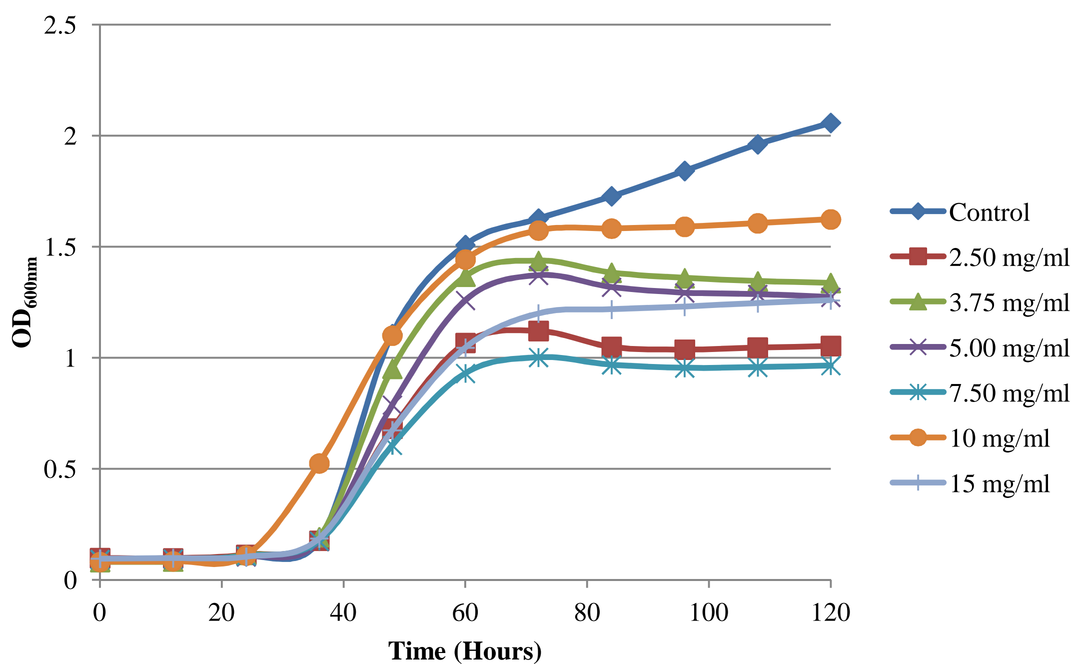

Figure 6. Growth of *Aspergillus ochraceus* in BHI broth (25 °C) at pH 5.0 supplemented with various concentrations of phenyllactic acid.

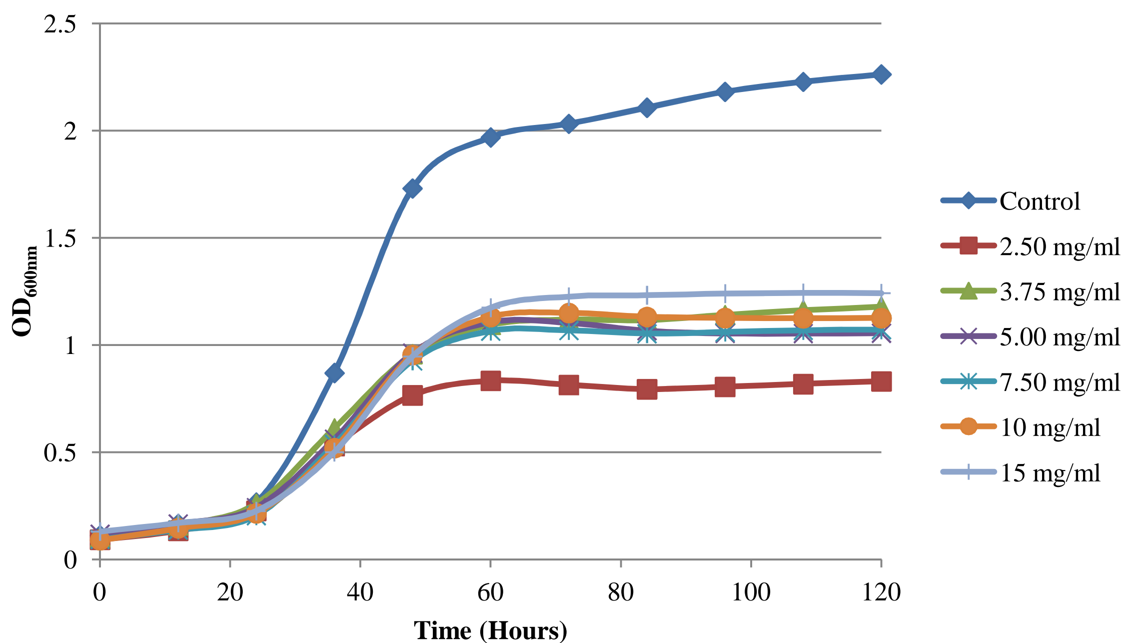

Figure 7. Growth of *Aspergillus spp.* in BHI broth (25 °C) at pH 5.0 supplemented with various concentrations of phenyllactic acid.

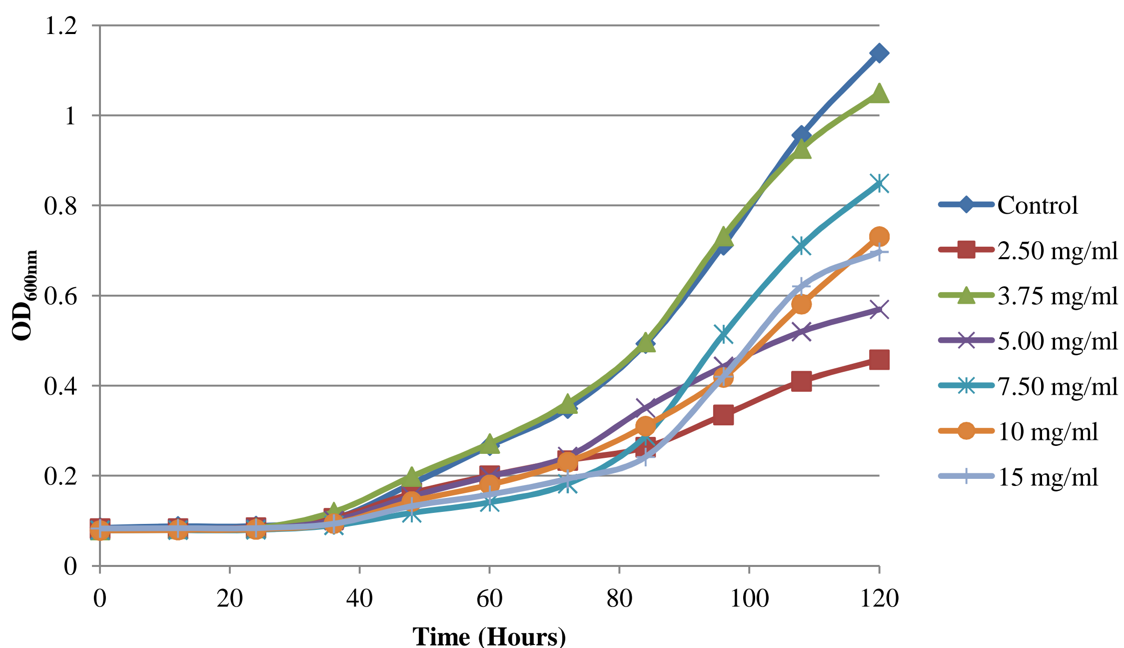

Figure 8. Growth of *Penicillium roqueforti* in BHI broth (25 °C) at pH 5.0 supplemented with various concentrations of phenyllactic acid.

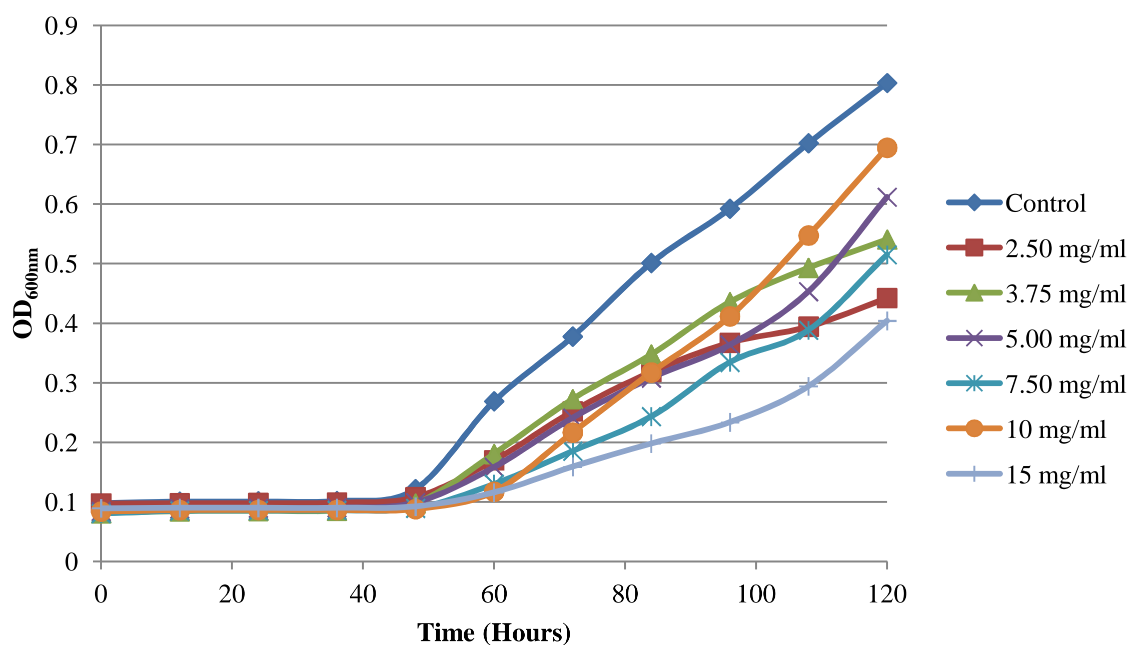

Figure 9. Growth of *Penicillium glabrum* in BHI broth (25 °C) at pH 5.0 supplemented with various concentrations of phenyllactic acid.

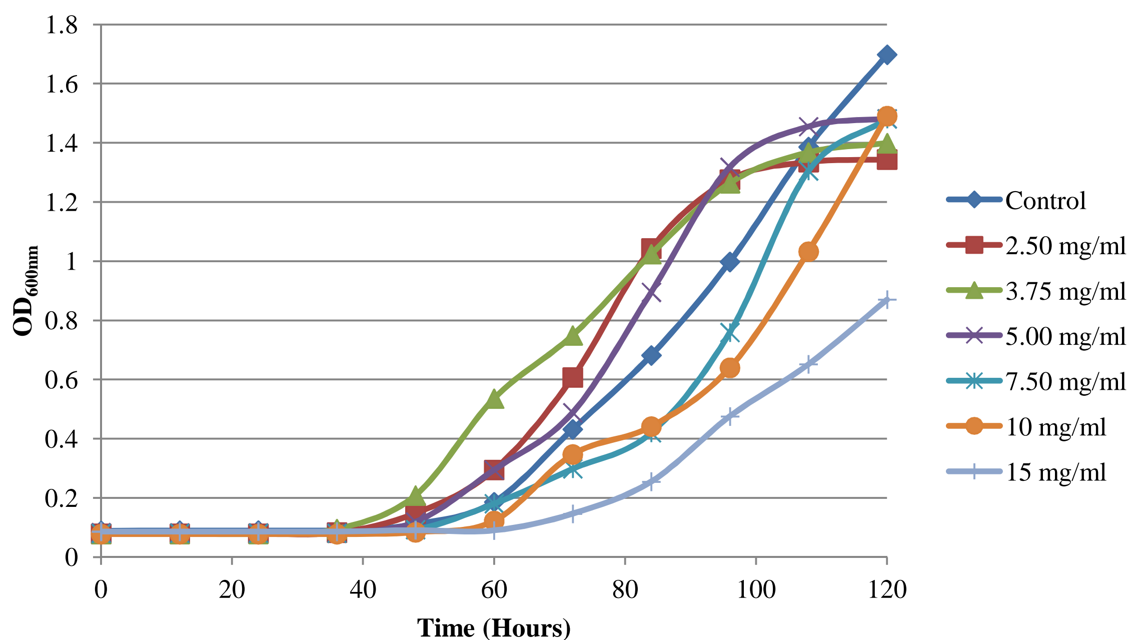

Figure 10. Growth of *Penicillium spp.* in BHI broth (25 °C) at pH 5.0 supplemented with various concentrations of phenyllactic acid.

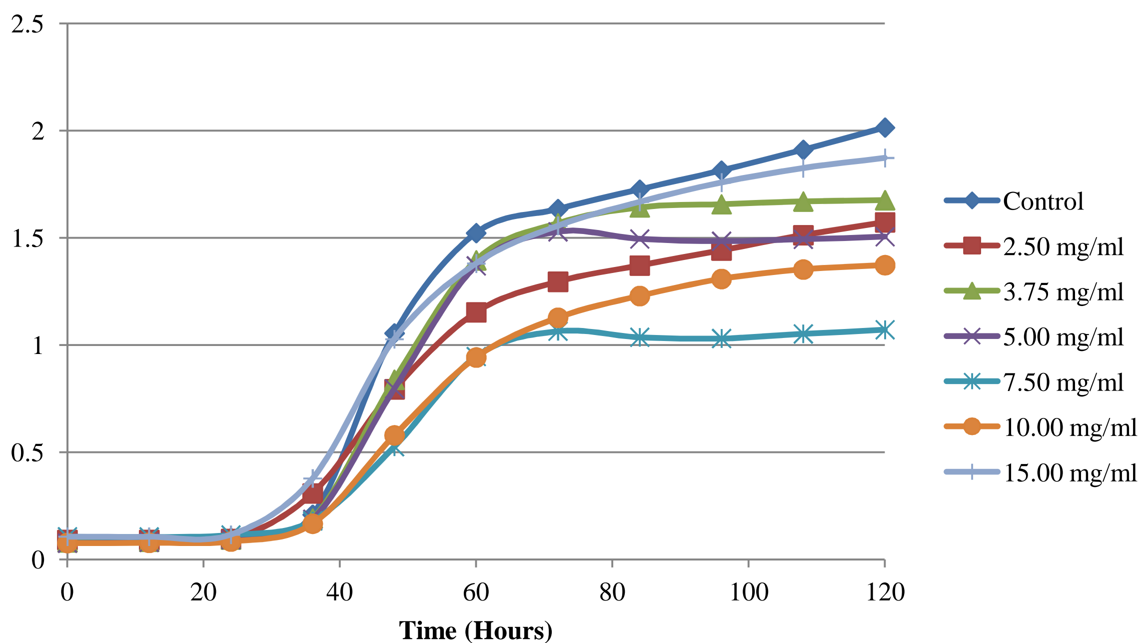

Figure 11. Growth of *Aspergillus ochraceus* in BHI broth (25 °C) at pH 5.3 supplemented with various concentrations of phenyllactic acid.

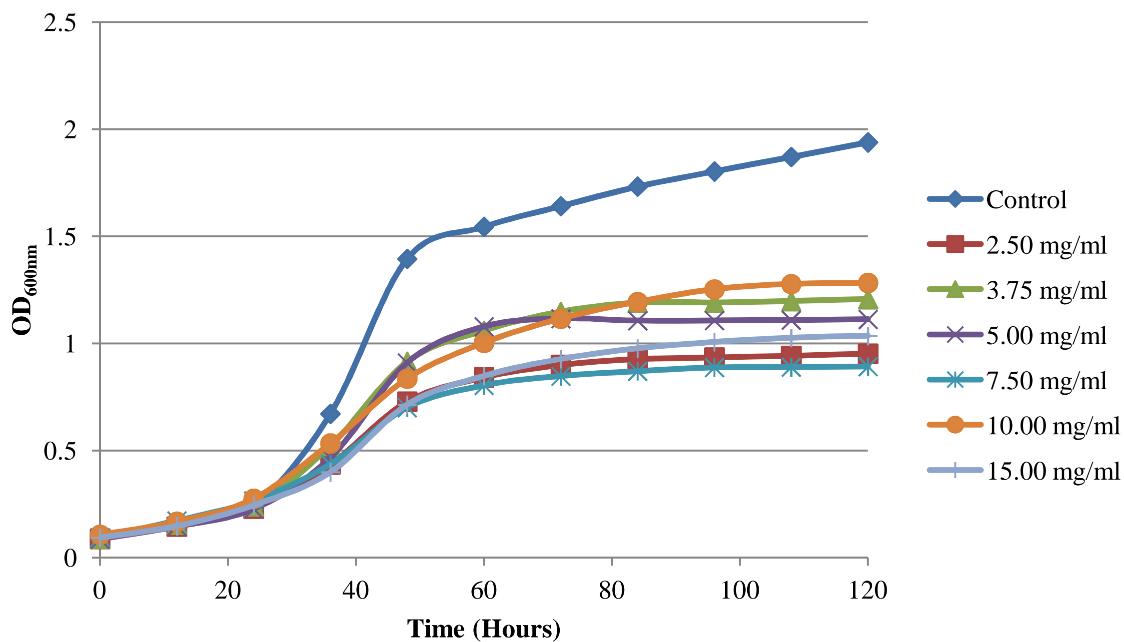

Figure 12. Growth of *Aspergillus spp.* in BHI broth (25 °C) at pH 5.3 supplemented with various concentrations of phenyllactic acid.

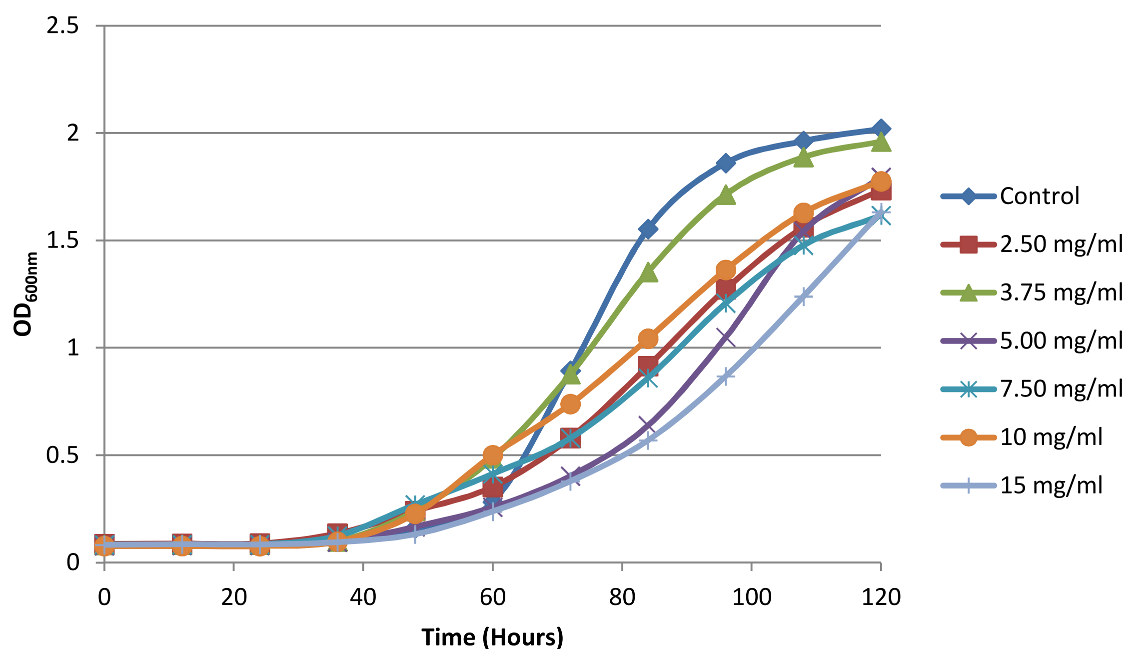

Figure 13. Growth of *Penicillium roqueforti* in BHI broth (25 °C) at pH 5.3 supplemented with various concentrations of phenyllactic acid.

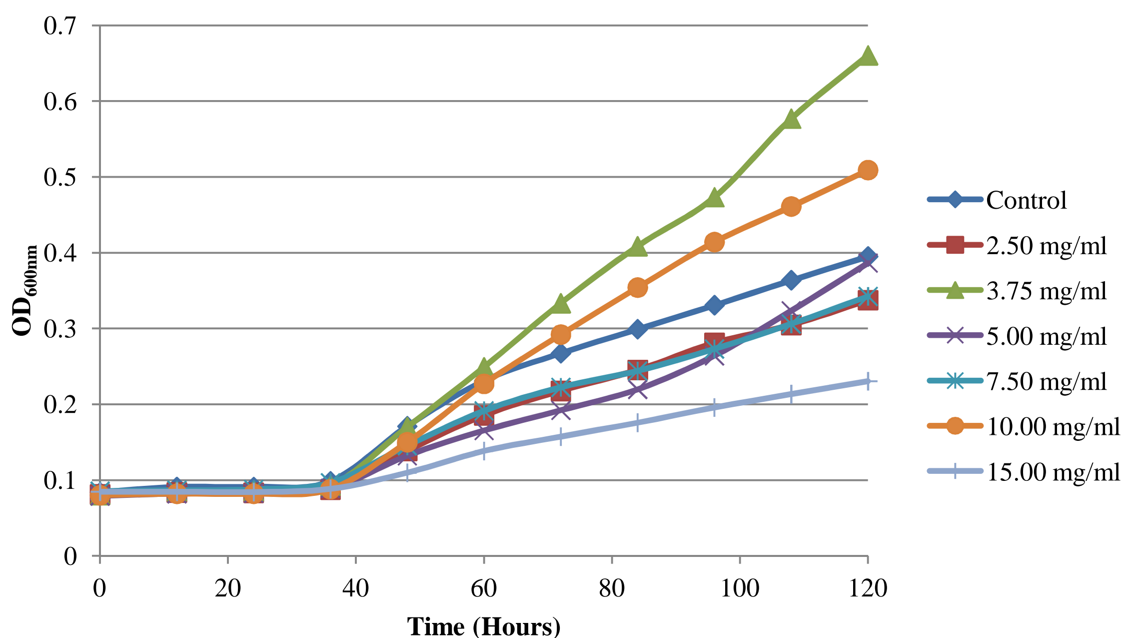

Figure 14. Growth of *Penicillium glabrum* in BHI broth (25 °C) at pH 5.3 supplemented with various concentrations of phenyllactic acid.

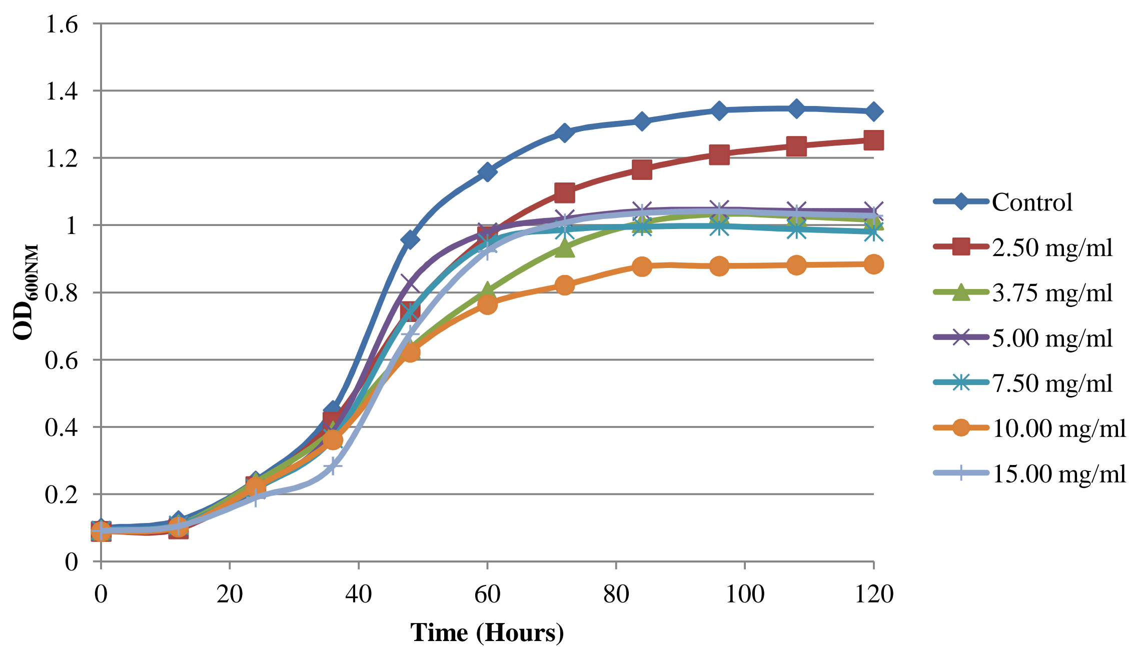

Figure 15. Growth of *Penicillium spp.* in BHI broth (25 °C) at pH 5.3 supplemented with various concentrations of phenyllactic acid.

CHAPTER 5. GENERAL CONCLUSIONS

Bacterial pathogens and molds in food and beverage products pose a huge food safety risk to consumers because they can cause foodborne illnesses due to infection or intoxication. Antimicrobials derived from natural sources such as plant, animal, or microbial sources are gaining much consumer interest because of the increased demand for more "natural" ingredients in food products.

The present research indicated that the naturally produced antimicrobial phenyllactic acid alone is strongly inhibitory to *Listeria monocytogenes*, *Salmonella enterica*, *Staphylococcus aureus*, and *Escherichia coli* O157:H7 in laboratory broth and in cream of chicken soup that was held under temperature-abuse conditions (12 °C and 35 °C). Phenyllactic acid (5 mg ml^{-1}) proved to be the most effective treatment at controlling the bacterial pathogens used in this study as it exhibited a bactericidal effect on all pathogens tested in the present study.

Phenyllactic acid combined with phosvitin (60 mg ml^{-1}) does not offer any significant effect compared to phenyllactic acid used alone against Gram-positive and Gram negative bacterial pathogens in cream of chicken soup stored at 12 °C or 35 °C. Phenyllactic acid combined with phosvitin (60 mg ml^{-1}) had a very minimal antimicrobial effect against foodborne pathogens. Phenyllactic acid in laboratory broth has a greater effect against molds in acidic pH (pH 3.8) compared to pH 5.0 or 5.3. Further research is needed to address the possibility of combining phenyllactic acid with other hurdle technology techniques such as high hydrostatic pressure or other natural antimicrobials for control of foodborne pathogens

and spoilage microorganisms. Also the impact of phenyllactic acid on the sensory characteristics of food products warrants investigation.

APPENDIX A.

GRAPHS: EFFECT OF PLA ON PATHOGEN GROWTH IN BHI BROTH AT PH 7.2

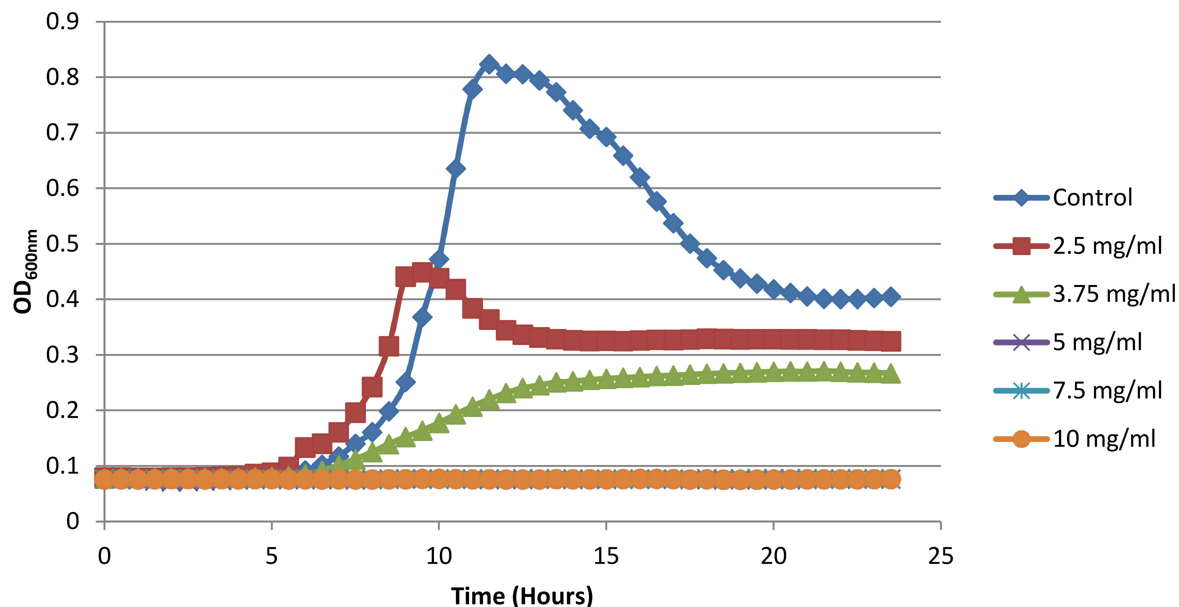

Figure 5. Growth of *Listeria monocytogenes* in BHI broth (35 °C) at pH 7.2 supplemented with various concentrations of phenyllactic acid.

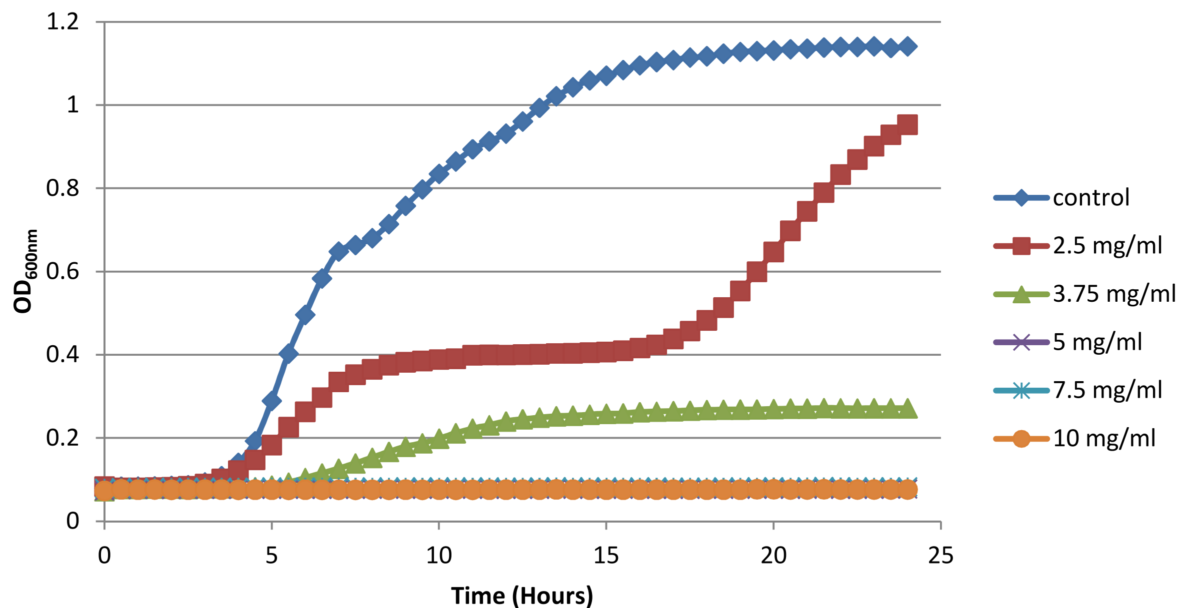

Figure 6. Growth of *Salmonella enterica* in BHI broth (35 °C) at pH 7.2 supplemented with various concentrations of phenyllactic acid.

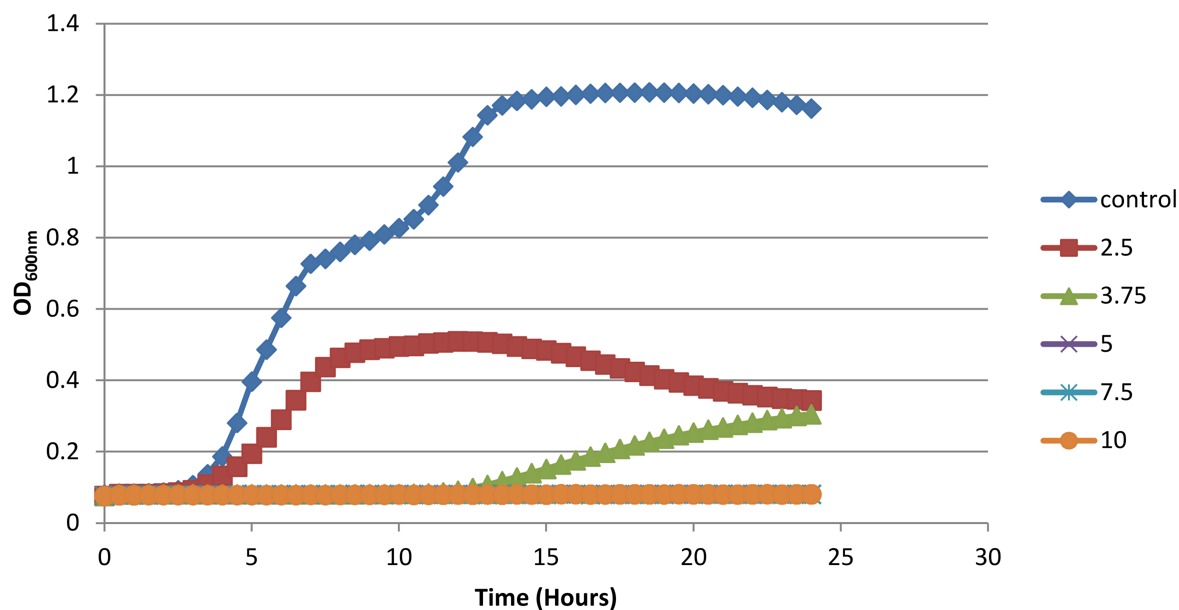

Figure 7. Growth of *Escherichia coli* O157:H7 in BHI broth (35 °C) at pH 7.2 supplemented with various concentrations of phenyllactic acid.

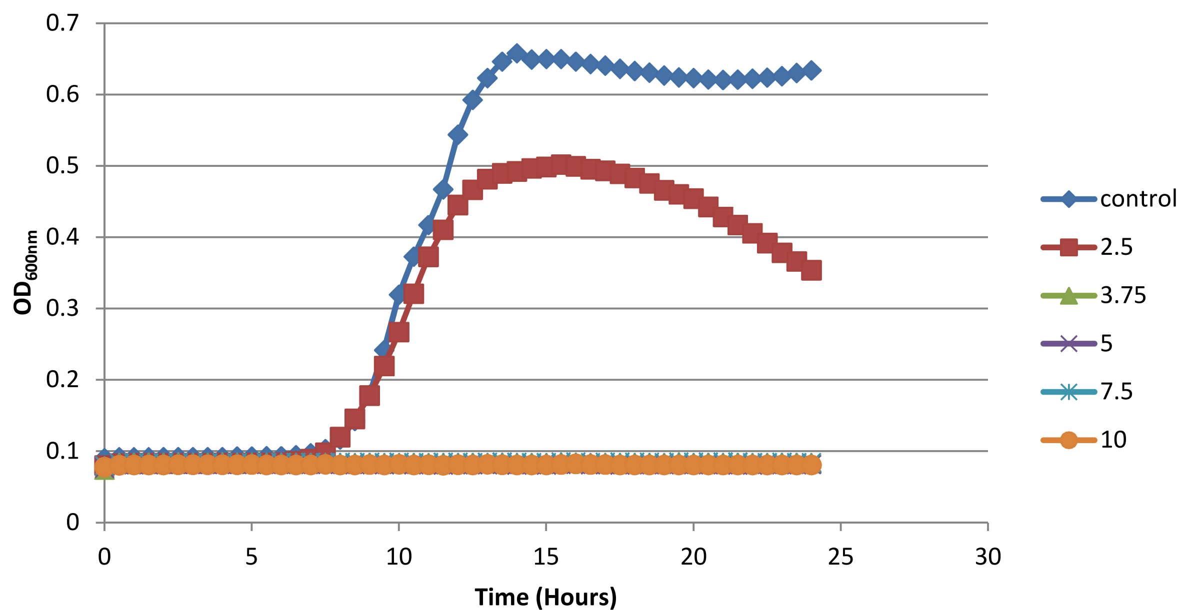

Figure 8. Growth of *Staphylococcus aureus* in BHI broth (35 °C) at pH 7.2 supplemented with various concentrations of phenyllactic acid.

APPENDIX B.

TABLE: INITIAL PH AND WATER ACTIVITY VALUES OF THE SOUP

TABLE 10. Initial pH and water activity of cream of chicken soup (23 ± 1 °C) with or without added antimicrobials.

Treatment code	Treatment Description	pH	Water Activity (a_w)
CTRL	Control (no treatments added)	5.85 ± 0.14a	0.988 ± 0.00
PLA 3.75	Phenyllactic acid (3.75 mg ml^{-1})	3.66 ± 0.17f	0.987 ± 0.00
PLA 5	Phenyllactic acid (5.0 mg ml^{-1})	3.34 ± 0.04e	0.987 ± 0.00
PHOS	Phosvitin (60 mg ml^{-1})	5.33 ± 0.11b	0.988 ± 0.00
PLA 3.75 + PHOS	Phenyllactic acid (3.75 mg ml^{-1}) + Phosvitin (60 mg ml^{-1})	4.83 ± 0.06c	0.986 ± 0.00
PLA 5.0 + PHOS	Phenyllactic acid (5.0 mg ml^{-1}) + Phosvitin (60 mg ml^{-1})	4.57 ± 0.03d	0.986 ± 0.00

[x]Each reported value for viable count represents the mean (standard deviation) of three replications of the experiment

[a,b,c,d,e,f]Means with a different letter within a column differ significantly ($P<0.05$)

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