

**Antibacterial efficacy of phosvitin, carvacrol, or nisin alone or combined against
foodborne human enteric pathogens**

by

Shecoya Berell White

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Toxicology

Program of Study Committee:
Aubrey Mendonca, Co-major Professor
Ruth MacDonald, Co-major Professor
Dong Ahn
Byron Brehm-Stecher
Dennis Olson

Iowa State University
Ames, Iowa
2011

Copyright © Shecoya Berell White, 2011. All rights reserved.

TABLE OF CONTENTS

ABSTRACT	vi
CHAPTER 1. GENERAL INTRODUCTION	1
Introduction	1
Dissertation Organization	4
CHAPTER 2. LITERATURE REVIEW	6
Phosvitin	6
Composition	6
Extraction and isolation/purification	7
Structure of phosvitin	8
Stability of phosvitin	8
Metal chelation	9
Antioxidant and nutritional aspects	11
Antimicrobial properties	13
Plant derived sources of antimicrobials	14
Cavacrol	15
Antimicrobial activity	16
Mode of action	18
Nisin	20
Source	20
Composition and structure	21
Antimicrobial spectrum	22
Mechanism of action	24
Foodborne human enteric pathogens	24
<i>Escherichia coli</i>	25
Morphology and characteristics	25
<i>Escherichia coli</i> O157:H7	26

Reservoirs	27
Foodborne outbreaks	28
Characteristics of the disease	29
<i>Listeria monocytogenes</i>	30
Morphology and characteristics	30
Reservoirs	31
Foodborne outbreaks	32
Characteristics of listeriosis	33
<i>Salmonella enterica</i>	35
Morphology and characteristics	36
Reservoirs	36
Foodborne outbreaks	39
Characteristics of salmonellosis	41
<i>Staphylococcus aureus</i>	42
Morphology and characteristics	42
Reservoirs	45
Foodborne outbreaks	46
Characteristics of <i>Staphylococcus aureus</i> intoxication	47
References	48

CHAPTER 3. INFLUENCE OF PHOSVITIN, CARVACROL AND THEIR COMBINATION ON THE VIABILITY OF *SALMONELLA ENTERICA* AND *LISTERIA MONOCYTOGENES* IN ONION MUSHROOM SOUP HELD AT 12 °C OR 35 °C

Abstract	71
Introduction	71
Materials and Methods	74
Results	77
Discussion	81
References	85
Tables	92

Figures	93
---------	----

CHAPTER 4. GROWTH INHIBITION OF *STAPHYLOCOCCUS AUREUS* AND
ESCHERICHIA COLI O157:H7 AND BACKGROUND MICROFLORA BY PHOSVITIN
AND CARVACROL ADDED TO ONION MUSHROOM SOUP 102

Abstract	103
Introduction	104
Materials and Methods	106
Results	110
Discussion	114
References	116
Tables	121
Figures	122

CHAPTER 5. THE ANTIMICROBIAL EFFECT OF PHOSVITIN, NISIN AND
COMBINATION AGAINST *SALMONELLA ENTERICA*, *ESCHERICHIA COLI* O157:H7
AND *STAPHYLOCOCCUS AUREUS* IN A COMMERCIAL TYPE SOUP AT 12°C AND
35°C 131

Abstract	132
Introduction	133
Materials and Methods	135
Results	139
Discussion	142
References	143
Tables	148
Figures	149

CHAPTER 6. DEATH RATE OF FOODBORNE PATHOGENIC BACTERIA EXPOSED
TO CARVACROL INCREASES WITH RATE OF CELLULAR LEAKAGE 161

Abstract	162
Introduction	162
Materials and Methods	164
Results	166
Discussion	167
References	170
Figures	173
Tables	175
 CHAPTER 7. GENERAL CONCLUSIONS	 181
 ACKNOWLEDGMENTS	 182

ABSTRACT

Natural antimicrobials, from plant, animal or microbial sources, have good potential for use as preservative systems to improve food safety, extend shelf life and enhance the overall quality of food products, while promoting the image of “healthier” foods. The overall objective of this research was to evaluate the antimicrobial efficacy of phosvitin alone or combined with carvacrol or nisin against four human enteric foodborne pathogens. Growth inhibition of *Salmonella enterica*, *Listeria monocytogenes*, *Escherichia coli* O157:H7 or *Staphylococcus aureus* by the antimicrobials used singly or in combination in brain heart infusion (BHI) broth (35 °C, 24 hours) was evaluated using a Bioscreen C turbidometer (OD 600nm). Subsequently, selected concentrations of the antimicrobials were evaluated for their effectiveness in controlling growth of the pathogens and background microflora in onion mushroom soup at 12 °C and 35 °C. Additional experiments involving UV spectroscopy and transmission electron microscopy (TEM) were performed to investigate the antibacterial mechanism of action of carvacrol in *E. coli* O157:H7 and *L. monocytogenes* Scott A. In BHI broth the minimum inhibitory concentration (MIC) of phosvitin and carvacrol was 80 mg/ml and 0.14 mg/ml, respectively, for both *S. enterica* and *L. monocytogenes*. The MIC of phosvitin and carvacrol was greater than 100 mg/ml and 0.12 mg/ml, respectively (for *S. aureus*) and 80 mg/ml and 0.12 mg/ml, respectively, for *E. coli*. In onion mushroom soup, the combination of phosvitin (60 mg/ml) and carvacrol (0.40 mg/ml) exerted the greatest cidal effect throughout storage against all foodborne pathogens tested. Irrespective of storage temperature (12 °C or 35 °C), phosvitin combined with nisin did not offer enhanced antibacterial effect above that provided by phosvitin used alone. For all organisms, leakage

of A_{260} absorbing material from the bacterial cells increased significantly with increased carvacrol concentration and exposure time. There was a very strong correlation between the initial rate of release of A_{260} material and death rate of the pathogens ($r = 0.998$). At 0.4 mg/ml carvacrol, *E. coli* O157:H7 cells appeared collapsed and showed signs of lysis when observed by TEM. At 0.4 and 0.6 mg/ml carvacrol, *L. monocytogenes* cells showed no apparent physical damage (distortion of cell shape) despite substantial loss of viability; however, exudation of intracellular material was detected by UV spectroscopy and TEM photographs of negatively stained cells.

Based on results of the present studies it is concluded that: i) use of phosvitin and carvacrol in combination has good potential for controlling growth of foodborne pathogenic bacteria in onion mushroom soup and ensuring the microbial safety of this potentially hazardous food product, ii) phosvitin/nisin combinations are far less effective for inhibiting growth of pathogens in soup, iii) death of bacterial cells exposed to carvacrol involves damage to the cytoplasmic membrane.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Bacterial pathogens present in food and beverages can infect humans and cause foodborne illnesses. The World Health Organization estimates that 1.8 million people died in the world in 2005 due to foodborne related illnesses. The Center for Disease Control and Prevention estimated that foodborne diseases causes approximately 76 million illnesses, 325,000 hospitalizations and 5000 deaths in the United States each year (Mead et al., 1999). Based on recent epidemiological data, there has been a decrease in the incidence of foodborne disease (9.4 illnesses), hospitalizations (55,961) and deaths (1,351) annually (Scallan et al., 2011). The US cost of foodborne illness associated with *Campylobacter jejuni*, *Clostridium perfringens*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella*, *Staphylococcus aureus* and *Toxoplasma gondii* is between \$6.5 and \$34.9 billion (Buzby and Roberts, 1997). Total elimination of bacterial pathogens is virtually impossible so government, food manufacturers, processors and retailers try to ensure that foodborne diseases are minimized for consumers.

The increased usage of foods formulated with chemical preservatives has raised consumer health concerns and created a demand for more “natural” and minimally processed foods. As a result, there has been a great interest in naturally produced antimicrobial agents to replace chemically synthetic preservatives such as nitrites, benzoates, sulphites, sorbates, salt and ethylenediaminetetraacetic acid. The insistence for more natural antimicrobials has driven the trend towards “Green Consumerism”. “Green” antimicrobials should possess the

following attributes: 1) low mammalian toxicity/cytotoxicity, 2) low skin sensitization potential, 3) low eco-toxicity, 4) low bioaccumulation potential, 5) low emission potential during manufacture, application or post application, 6) no or low volatile organic compound (VOC) content, as applicable, and 7) complete effectiveness against the relevant target organisms (Sofos, 1998; Kruger and Mann, 2003; Shrankel, 2004; Davidson et al., 2005; Smith, et al., 2005; Rulis, 2009). Natural antimicrobials have good potential for use as preservative systems to improve food safety, extend shelf life and improve the overall quality of food products, while promoting the image of “healthier” foods. In order for the food industry to fully make the change from the use of synthetic sources to purely natural sources research must address, the limits of antimicrobial activity, the overall cost, the most effective concentration, optimization of the antimicrobial, use of hurdle technology and address regulatory concerns.

The food industry is addressing the issue by gradually incorporating natural antimicrobials from, plant, animal or microbial sources into food products to replace the more traditionally used synthetic chemical preservatives. Poultry eggs contain many proteins that have antimicrobial functions and phosvitin, a major egg yolk protein, is a highly phosphorylated protein that has the ability to bond with metal cations (Khan et al., 2000). The ability to bind metal cations deprives the environment of essential nutrients and possibly interacts with the membranes of foodborne pathogens. It is this property which could theoretically make it a candidate for uses as a natural antimicrobial. Carvacrol, an essential oil from oregano and thyme, is a phenolic compound that has been shown to have antimicrobial activity. It is believed to exert antimicrobial activity by disrupting the

cytoplasmic membrane of foodborne pathogens. In some instances the high concentration needed for it to be effective as an antimicrobial would not be organoleptically pleasing to consumers. Therefore, efforts need to be made to use plant essential oil such as carvacrol in very low amounts that would be effective in controlling microorganisms while not contributing an overwhelming flavor and/or odor notes to food products. One approach to achieve this type of application is by combining low levels of the essential oil with an antimicrobial to get the desired result. Nisin, produced by *Lactococcus lactis* spp *lactis*, is the only approved bacteriocin used in food. It is primarily active against Gram-positive bacteria by causing pores in the cell membrane, resulting in leakage of cytoplasmic constituents. Generally, food applications of nisin can be expensive based on the quantities needed for antimicrobial efficacy. In addition, nisin has little or no effect on Gram-negative bacteria except in instance when a membrane destabilizing agent such as EDTA is used in combination with this bacteriocin. It is hypothesized that the antimicrobial efficacy of nisin against Gram-negative bacteria is enhanced when this bacteriocin is combined with phosvitin.

Properties of these three sources of natural antimicrobials can be used to improve microbial safety of foods. Combining these natural antimicrobials could possibly increase the effectiveness of the antimicrobials proving to be better than if they were used alone against foodborne pathogens. Synergistic or additive effects between the antimicrobials may permit the use of relatively low amounts of each antimicrobial and thereby reduce cost of the antimicrobial treatment while improving the antimicrobial efficiency.

The first objective was to evaluate and establish the minimum inhibitory concentration (MIC) of selected antimicrobials in laboratory broth medium against various foodborne pathogens. The second objective was to determine the antimicrobial efficacy of phosvitin, carvacrol and nisin alone or phosvitin/carvacrol combination or phosvitin/nisin combination against the various human enteric pathogens *Escherichia coli* O157:H7, *Salmonella enterica*, *Listeria monocytogenes*, and *Staphylococcus aureus* in a food product. After determining the antimicrobial efficacy it was important to establish the possible mechanism of action of carvacrol correlated to death and the effect of this natural antimicrobial on the extent of sub-lethal injury in populations of foodborne human enteric pathogens that survive exposure to carvacrol.

Dissertation Organization

This dissertation is organized into seven chapters. The first chapter is a general introduction. The second chapter is a general literature review that contains information relevant to the research performed (Chapters 3-6) followed by a general conclusion. The seventh and final chapter provides a general summary of the research described. All pertinent tables, figures and graphs appear at the end their respective paper, which follows a specified journal format. References can be found at the conclusion of each chapter with formatting following journal specifications. It is the intent that Chapter 3 be submitted to the Journal of Food Protection, Chapter 4 Journal of Food Science, Chapter 5 International Journal of Food Microbiology, and Chapter 6 Journal of Food Protection. The following papers entitled “Antimicrobial efficacy of phosvitin alone or combined with nisin against

Listeria monocytogenes in a laboratory broth medium at 35 °C” and “Control of *Salmonella enterica* and *Staphylococcus aureus* in a laboratory medium and a commercial-type soup using phosvitin, carvacrol, or combinations” were presented at the annual meeting of the International Association of Food Protection in Anaheim, CA (August 2010), and Madison, WI (2011), respectively. Lastly, paper entitled “Antimicrobial efficacy of phosvitin, nisin, and combinations against *Salmonella enterica* in a laboratory medium and a commercial-type soup” will be presented at the annual meeting of the Institute of Food Technologists in New Orleans, LA (June 2011).

CHAPTER 2. LITERATURE REVIEW

Phosvitin

Composition

Phosvitin is a phosphoglycoprotein that was first isolated in 1949 by Mecham and Olcott. It is made from vitellogenin (produced in the liver) and is found exclusively in the granule fraction of chicken egg yolk (Wallace and Morgan, 1986) in which it represents 4% of egg yolk dry matter. Phosvitin has a molecular weight of 35 kDa and contains 10% phosphorus (80% of total yolk phosphorus), 12.3% nitrogen, and 6.5% carbohydrate (Renugopalakrishnan, 1985; Burley and Vadhera, 1989; Xu et al., 2007). It is the major protein component in egg yolk and is currently known as the most phosphorylated protein in nature. Phosvitin has an amino acid sequence containing 217 amino acids of which 123 are serines, with 90% of them being phosphorylated (Fig. 1; Taborsky, 1983; Byrne et al., 1984; Clark, 1985). This distinct, polyanionic, highly phosphorylated protein, contains 56% serine residues, 15% basic and 10% acidic amino acid residues (Taborsky, 1963; Taborsky and Mok, 1967; Byrne et al., 1984; Losso, 1994; Xu et al., 2007). Its unique amino acid sequence leads to the structure and properties of phosvitin.

Figure 1. Amino acid sequence of hen egg yolk phosvitin. Adapted from 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 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 R S R S S S K S 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 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 and isolation/purification

Phosvitin was first isolated in 1949 by Meham and Olcott and the methods described by those researchers are most commonly used for extraction and isolation. Sigma Chemical Co. uses another method described by Sundararajan et al., 1960 for extraction and isolation of phosvitin. The current issue with previously used methods for preparation, extraction and isolation (size-exclusion, anion-exchange chromatography, hydrophobic-interaction chromatography, gel electrophoresis) is that chemicals used are not food-grade or non-aqueous (Wallace and Morgan, 1986; Castellani et al., 2003) limiting use of the methods for production of foodgrade phosvitin. Choi et al., (2005) were able to successfully purify egg yolks by a sodium chloride method. Tedious extraction and purification methods for phosvitin are largely responsible for the relatively high cost of this glycoprotein. The cost of phosvitin is \$1130.00 per gram based on current price listed in the Sigma 2011 catalog. More recently, researchers at Iowa State University developed a water/ethanol-based method that permits an efficient cost-effective way of extracting and purifying phosvitin from yolks of hens' eggs. Varying yield of final product, purity and polypeptide composition (Table 1; Castellani et al., 2003) were also a concern.

Table 1. Amino acid composition (mol %) of phosvitin sequences from the literature.

Amino Acid	Purified ^a	Seq I ^b	Seq II ^c	Amino Acid	Purified ^a	Seq I ^b	Seq II ^c
Asx	7.0	6.4	6.0	Tyr	0.6	1.6	0.5
Glx	7.2	7.6	5.0	Val	1.9	1.6	1.4
Ser	47.1	48.8	57.0	Met	0.6	0.4	0.5
Gly	3.9	3.2	2.3	Cys	0.4	0.0	0.0
His	5.5	4.0	6.0	Ile	1.1	0.4	0.9
Arg	5.5	6.3	5.1	Leu	1.6	0.8	1.4
Thr	2.3	4.0	1.8	Phe	1.0	2.0	0.5
Ala	4.1	3.2	3.2	Lys	8.7	7.1	6.9

^aCastellani et al., 2003; ^bMabuchi et al., 1996; ^cByrne et al., 1984

Structure of phosvitin

Phosvitin is a highly unfolded flexible protein consisting of two polypeptide components, (alpha and beta) (Abe et al., 1982; Itoh et al., 1983; Vogel, 1983). The main factor that influences the structure of phosvitin is pH. At a physiological pH, phosvitin exists in a random coil conformation and at acidic pH the β conformation predominates (Vogul, 1983; Yasui et al., 1989), which influences solubility of this glycoprotein (Table 2; Castellani et al., 2003). Phosvitin is extremely hydrophilic with a proportionately lower amount of hydrophobic side chains (Figure 2), and possesses a net negative charge (Sattar Khan et al., 2000). At physiological pH phosphoserine residues are exposed and can be protonated or deprotonated depending on the chemical characteristics of the solvent (Vogul, 1983). In acidic pH phosphoryl groups can shift from amide to ester linkages; whereas, at alkaline pH free amino groups become masked also causing phosvitin to release acid (Connely and Taborsky, 1961; Taborsky and Allende, 1962).

Stability of phosvitin

Native phosvitin exhibits relatively good stability to heat, pressure and enzyme action. Little or no change in structure or precipitation of this protein occurs at 100°C for several hours, concurrent with varying pH (4 to 8 pH) (Mecham and Olcott, 1949; Matsudomi et al., 2006). Phosvitin is also not affected by high pressures up to 600 MPa (Castellani et al., 2004). Phosvitin is not easily digested by pepsin, trypsin, and chymotrypsin. In fact the core of the protein remains largely intact after enzymatic digestion, due to the negative charge of the phosphate group rendering the neighboring peptide bonds

resistant to proteolytic action (Goulas et al., 1996). Dephosphorylation methods have been employed to enhance digesting phosvitin (Jiang and Mine, 2000, 2001).

Table 2. Solubility of isolated phosvitin at different pH values. Source: Castellani et al., 2003.

pH	% of solubility ^a \pm SD ^b
1.5	9.30 \pm 4.2
2.5	83.5 \pm 1.7
3.6	84.5 \pm 1.5
5.0	90.6 \pm 2.5
7.0	97.7 \pm 1.4

^aExpressed in percent of N from lyophilized isolated protein

^bStandard deviation

Figure 2. Structural schematic of hen egg yolk phosvitin.



Hen egg yolk phosvitin, amino acid composition and structure provide a variety of functional (metal chelation) and biological properties (antioxidant and antimicrobial). The primary structure of phosvitin makes it one of the strongest metal chelating glycoproteins (Grizzuti and Permann 1973; Hegenauer et al., 1979; Castellani et al., 2004; Xu et al., 2007).

Metal chelation

The capacity of phosvitin to chelate metal ions, as well as peptides resulting from enzymatic digestion of this glycoprotein are major factors related to phosvitin's influence

mineral bioavailability. Avian eggs are rich in iron and about 95% is bound by phosvitin (Greengard et al., 1964). Almost all iron, calcium, and phosphorus are contained in the yolk (Taborsky, 1983; Watkins, 1995). The numerous phosphorylated serine residues have a strong affinity for divalent metals and contribute to the chelating ability of phosvitin (Osaki et al., 1975; Grogan and Taborsky, 1987). Phosvitin contains between 3 to 6 iron atoms per molecule depending on the method of isolation from hens' eggs; however, it has a binding capacity of about 60 iron molecules (Mecham and Olcott, 1949; Taborsky, 1963; Webb et al., 1973, Albright et al., 1984). The iron binding capacity differs dependent on source of phosvitin, for example phosvitin in fish eggs have a lower iron binding capacity due to less phosphorus and serine content, and smaller size (Table 3; Guerdin-Dubiard et al., 2002). When iron binds with phosvitin an iron-phosvitin complex is formed, rendering phosvitin insoluble and the iron unavailable. Phosvitin oxidizes Fe (II) to Fe(III) and also binds substantial amounts of Fe (III) (Osaki et al., 1975).

Trypsin hydrolyzed phosvitin enhanced Ca-binding capacity and inhibited the formation of insoluble Ca phosphate (Jiang and Mine, 2001). Ishikawa et al 2004 investigated the effect of phosvitin on Fe (II)-catalyzed hydroxyl radical ($\bullet\text{OH}$) formation from H_2O_2 in the Fenton reaction system. Those authors reported that phosvitin accelerated Fe (II) autoxidation thus decreasing availability of Fe (II) participation in the $\bullet\text{OH}$ -generating Fenton reaction. Phosvitin was also found to protect DNA against oxidative damage induced by Fe (II) and H_2O_2 . These results provide insight into the mechanism of protection of the developing embryo against iron-dependent oxidative damage in ovo. Choi et al., 2005 examined the effects of phosvitin and tryptic digests of phosvitin on the efficiency of enhancing calcium absorption and accumulation in the bones of rats. Calcium absorption

was found to be greater with peptides from tryptic digestion of phosvitin; thus, increasing the bioavailability of Ca and accumulation in bone. Ishikawa et al., 2007 demonstrated that native phosvitin decreased absorption of iron, calcium and magnesium, suggesting that mineral bioavailability is influenced by the following: the type of proteins (amino acid composition), its binding strength to minerals, and resistance to proteolysis.

Table 3. Iron Binding properties of phosvitins. Source: Guerin-Dubiard et al., 2002.

Phosvitin	MW phosvitin (Da)	Mol Fe/mol Phosvitin
Sigma	35,000 ^a	36.0
Laboratory	35,000 ^a	32.0
*Ling	#23,000 ^a	9.5
*Siki	#25,000 ^a	3.6

^aMW were estimated from SDS-PAGE analysis

*Ling and Siki are phosvitin from fish

Antioxidant and nutritional aspects

Phosvitin plays an important role as an antioxidant in the yolk by inhibiting iron-catalyzed lipid oxidation (Lu and Baker, 1986). Various studies have observed the antioxidant properties of phosvitin and peptides derived from phosvitin; antioxidant potential findings were both negative and positive. Phosvitin is efficient up to a Fe²⁺ to phosvitin ratio of 30:1, and pH 6 provides its maximal antioxidant activity (Guerin-Dubiard et al., 2002). Antioxidant ability did not change when phosvitin was pasteurized (61.1 °C); however, autoclaving (121.1 °C, 10 min) phosvitin decreased inhibitory capacity on iron catalysis (Lu and Baker, 1986). Native phosvitin has been considered nutritionally negative, in that it resists proteolytic actions (Goulas et al., 1996) and lowers bioavailability of iron (Morris and Greene, 1972) due to its highly phosphorylated structure. It has been

demonstrated via both *in vitro* and *in vivo* experiments that yolk protein intake resulted in decreased mineral absorption and digestibility of the phosvitin in rats (Ishikawa et al., 2007).

To by-pass the hurdle of low bioavailability of iron, phosvitin has been hydrolyzed to form smaller peptides (1-3kDa) following dephosphorylation (Jiang and Mine, 2000, 2001). The smaller peptides consist of about 35% of the phosphorus content of native phosvitin, indicating that the peptides would be individually less active than native phosvitin (Jiang and Mine, 2001). Ishikawa et al., (2004) found phosvitin and digested phosvitin were more efficient antioxidants in inhibiting $\bullet\text{OH}$ formation compared to metal-binding proteins (ferritin, transferrin, lactoferrin, ovotransferrin, and bovine serum albumen), iron chelators (DFO-deferoxamine mesylate, NTA-nitriloacetic acid, EDTA-ethylenediamine tetraacetate, citrate, ADP, phytate) and hydroxyl radical scavengers (mannitol, thiourea, ethanol, DMSO). The tryptic digested phosvitin was as efficient an antioxidant as phosvitin towards inhibiting Fe (II) catalyzed $\bullet\text{OH}$ formation, indicating that both forms have iron-binding ability. Xu et al., 2007 also observed the antioxidant activities of intact phosvitin, and tryptic digests of phosvitin. Those same researchers reported that digested phosvitin was more effective than intact phosvitin at inhibiting lipid oxidation in a linoleic acid system. In addition, the digested form of phosvitin was efficient in radical scavenging activity on 2,2-diphenyl-1-picrylhydrazyl induced free radicals; however, its chelating capabilities were relatively less effective. Antioxidant activity of phosvitin has been demonstrated in egg yolk emulsion as well as meat model systems (Lu and Baker, 1986; Lee et al., 2002). Also phosvitin inhibited lipid peroxidation induced by UV irradiation in a dose-dependent manner in the presence of excess iron (Ishikawa et al., 2005). These results suggest that phosvitin and peptides derived

from phosvitin might be useful for prevention of other iron-mediated oxidative stress related diseases, such as colorectal cancer (Katayama et al., 2006; Ishikawa et al., 2009).

The mechanism of action of digested phosvitin is less understood, and is possibly influenced by peptide composition (histidine, methionine, and tyrosine) (Yamashoji et al., 1979) or structure and not solely on the phosphorus content (native phosvitin postulation) (Hegenauer et al., 1979; Xu et al., 2007). The aforementioned properties, illustrate the possibility of its commercial uses as a natural antioxidant.

Antimicrobial properties

The iron binding capabilities contribute to the antimicrobial properties of phosvitin. Iron is an essential element for all living organisms and interestingly, ninety-five percent of iron in hen's egg yolk is bound by phosvitin in a very strong and stable conformation to avoid being used by microbes (Greengard et al., 1964). To our knowledge there has only been one published study on the antimicrobial potential of phosvitin. Sattar Khan et al., 2000 tested native phosvitin, a protease digested phosvitin fraction and phosvitin-galactomannan conjugate against *Escherichia coli* under thermal stress. These authors reported that at 50 °C for 20 minutes in L-broth, both native phosvitin and phosvitin-galactomannan conjugate were significantly more effective than the control in decreasing the viability of the target organism; whereas, the digested fraction was not effective. It was noted the antimicrobial properties of phosvitin could be reversed by the addition of Ca^{2+} (Table 4) indicating that divalent cations may occupy sites on phosphate groups of phosvitin to prevent further binding of cations important in meeting the nutritional needs of the organism. Antimicrobial activity of intact phosvitin is believed to be due to chelating abilities and surface activity

(affinity for outer membrane). These activities in conjunction with thermal stress seem to contribute to the loss of viability of *E. coli* (Sattar Khan et al., 2000).

Table 4. Determination of Calcium Binding Capacity of 0.1% Native and Modified Phosvitins. Source: Sattar Khan et al., 2000.

Samples	bound calcium mol/mol of phosvitin)	
	at pH 3.6	at pH 7.0
Native phosvitin ^a	0	0
Native phosvitin	20	148
Phosvitin-galactomannan conjugate	27	151
α -chymotrypsin-digested phosvitin	17	118

^aPhosvitin without calcium

Plant-derived sources of antimicrobials

The growing number of foodborne illness-related outbreaks due to human enteric pathogens is an increasing concern to regulatory agencies, the food industry, and consumers. While effective control of foodborne pathogens is crucial to ensuring microbial food safety, methods of control involving synthetic chemical preservatives are becoming less desirable to consumers. This consumer attitude is part of a growing trend towards “Green Consumerism” emphasizing the reduction or elimination of preservative agents such as nitrite and sodium chloride from foods (Burt, 2004) and encouraging the use of herbs and spices with antimicrobial properties to preserve foods, promote healthier foods and improve food safety.

Herbs and spices have been used for centuries specifically for medicinal purposes, flavor, fragrances, and as natural preservative methods for a variety of foods (Achinewhu et al., 1995; Cowan, 1999). The majority of essential oils are classified as Generally

Recognized As Safe (GRAS). From a food safety standpoint, the antimicrobial properties possessed by the essential oil components of herbs and spices have only been scientifically confirmed within the last 30 years (Olasupo et al., 2004). The phenolic compounds in the essential oil fraction of the plant possess antimicrobial properties (Beuchat, 1994). There have been numerous studies published addressing the antimicrobial properties of a wide variety of essential oils (Remmal et al., 1993; Hammer et al., 1999; Dorman and Deans, 2000; Frieman et al., 2002; Burt, 2004). Limitations to applications of essential oils as antimicrobial agents are mainly due to intense flavors and odors that effective antimicrobial concentrations may add to foods. One approach that has been deemed useful is to determine the minimum inhibitory concentration (MIC) and apply the essential oil in combination with other antimicrobials or preservative methods to control foodborne pathogens. Such an approach aims to take advantage of additive or synergistic effects of antimicrobial interventions.

Carvacrol

Source and structure

Carvacrol, an essential oil, is found in the leaves and flowering plant of both thyme (*Thymus vulgaris*) and oregano (*Origanum vulgare*) (Burt, 2004; Oussalah, 2007). The percent composition of carvacrol in oregano ranges from trace amounts to 82% and 2 to 45% in thyme (Lagouri et al., 1993; Arrebola et al., 1994; Burt, 2004). Carvacrol [2-methyl-5-(1-methylethyl) phenol] (Figure 3) is biosynthensized from p-cymene and g-terpinene (Poulouse and Croteau, 1978). The hydrophobic phenolic compound in its structure is thought to be the

main contributor to its antimicrobial properties; however, other components of its structure may be also important in this regard (Veldhuizen et al., 2006).

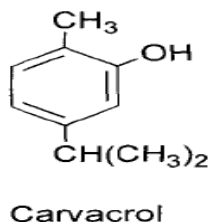


Figure 3. Structure of carvacrol - 1 [2-methyl-5-(1-methylethyl) phenol].

Antimicrobial activity

Several studies have demonstrated that carvacrol has both bacteriostatic and bactericidal activity against microorganisms: *Listeria monocytogenes* (Pol and Smid, 1999; Karatzas et al., 2001, Friedman et al., 2002; Periago et al., 2004; Gaysinsky et al., 2005; Oussalah et al., 2006, Veldhuizen et al., 2007; Solomakos et al., 2008; Perez-Conesa et al., 2011), *Bacillus cereus* (Pol 1999; Ultee et al., 1998, 1999, 2000 a, b; Ultee and Smid, 2001), *Escherichia coli* O157:H7 (Helander et al., 1998; Friedman et al., 2002, 2004; Lacroix et al., 2004; Gaysinsky et al., 2005; Kisko and Roller, 2005; Oussalah et al., 2006; Juneja and Friedman 2008; Xu, 2008; Obaidat and Frank, 2009; Pei et al., 2009; Perez-Conessa et al., 2011), *Staphylococcus aureus* (Knowles et al., 2005; Lambert et al., 2001; Oussalah et al., 2006, 2007), *Salmonella enterica* (Helander, 1998, Lacroix, 2004, Friedman et al., 2004; Knowles et al., 2005; Nazer et al., 2005; Zhou et al., 2007; Ravishankar et al., 2010), *Campylobacter jejuni* (Friedman et al., 2002), *Clostridium perfringens* (Juneja and Friedman, 2007), *Pseudomonas fluorescens* (Lambert et al., 2001, Arfa et al., 2006; Di Pasqua et al.,

2006), *Lactobacillus plantarum* (Arfa et al., 2006), *Saccharomyces cerevisiae* (Chami et al., 2005, Arfa et al., 2006), *Botrytis cinerea* (Arfa et al., 2006), *Shigella spp* (Bagamboula et al., 2004) and *Candida albicans* (Chami et al., 2005). The minimum inhibitory concentrations of carvacrol for some foodborne pathogenic bacteria are shown in Table 5.

Table 5. Minimum inhibitory concentration of carvacrol tested in vitro against selected foodborne pathogens. Adapted from Burt, 2004.

Foodborne Pathogen	Minimum Inhibitory Concentration (MIC) range (ppm)	References
<i>Escherichia coli</i>	225 – 5000	Kim et al, 1995a; Cosentino et al 1999; Pei et al., 2009
<i>Salmonella typhimurium</i>	150 – 250	Kim et al., 1995a; Cosentino et al., 1999; Nazer et al 2005
<i>Staphylococcus aureus</i>	175 – 450	Cosentino et al., 1999, Lambert et al., 2001
<i>Listeria monocytogenes</i>	375 – 5000	Kim et al., 1995a; Cosentino et al 1999; Pol and Smid, 1999
<i>Bacillus cereus</i>	187.5 – 900	Cosentino et al., 1999; Pol and Smid, 1999

Carvacrol has been studied extensively in broth model systems as well as the following foods: cheese (Smith-Palmer et al., 2001), apple cider (Friedman et al., 2004, Kisko and Roller 2005), fruit (kiwi and melon) (Roller and Seedhar, 2002), carrot broth (Valero and Salmeron, 2003), salad (Koutsoumanis et al., 1999), lettuce and spinach leaves (Obaidat and Frank, 2009), rice (Ultee et al., 2000b), turkey (Juneja and Friedman, 2007), meat (Veldhuizen et al., 2007, Juneja and Friedman, 2008; Solomakos et al., 2008, Friedman et al., 2009), fish/seafood (Kim et al., 1995; Meljholm and Dalgaard, 2002; Ravishankar et al.,

2010). Like other antimicrobials there are several factors that influence the efficacy of carvacrol as a natural antimicrobial with concentration being the most important factor. When carvacrol is present at non-lethal concentrations (0.4mM) *Bacillus cereus* can adapt to carvacrol at 30°C in growth medium (Ultee et al., 2000a).

Carvacrol has been combined with other preservatives or preservative methods to control foodborne microorganisms. It has been shown to have stronger antimicrobial effect with increased temperature and storage time, and at a lower pH (Burt 2004, Friedman et al., 2004). Some antimicrobials that have been combined with carvacrol to control foodborne microorganisms include p-cymene (Periago et al., 2004; Kisko and Roller, 2005), nisin (Pol and Smid, 1999; Olasupo 2004; Solomakos, 2008), thymol (Nazer et al., 2005, Zhou et al., 2007; Pei et al., 2009), eugenol (Nazar et al., 2005; Pei et al., 2009), citral (Nazer et al., 2005), cinnamaldehyde (Zhou, 2007b) and EDTA (Zhou, 2007a). Encapsulated carvacrol in surfactant micelles has been proven effective in inhibiting growth of *Escherichia coli* O157:H7 and *Listeria monocytogenes* (Gaysinsky et al., 2005; Perez-Conessa et al., 2011). Karatzas et al., 2001 reported that carvacrol and high hydrostatic pressure (HPP) acted synergistically in inactivating *Listeria monocytogenes*.

Mode of action

The effectiveness of carvacrol as a natural antimicrobial is well established; however, the mechanism of action is less understood and is believed to be associated with damage to the cell membrane. The phenolic component of carvacrol has prompted research focused on its effect on structural and functional damage to cellular membranes (Sikkema et al., 1995, Sivropoulou et al., 1996, Ultee et al., 1999). Permeability of the cell membrane is dependent

on the hydrophobicity of the solutes that have to cross the membrane and the composition (Sikkema et al., 1995). Several methods have been used to investigate the antibacterial mechanism of action of carvacrol: spectrofluorometry (Ultee et al., 1999, 2002; Lambert, 2001), scanning electron microscopy (Kwon et al., 2003; Di Pasqua et al., 2007), confocal laser scanning microscopy (Gill and Holly, 2006) and flow cytometry (Xu et al., 2008). Results of those studies suggest that the antimicrobial mechanism of action of carvacrol is linked to alterations of cellular membranes.

Lambert et al. (2001) determined that the addition of low levels of carvacrol (6.7 mM) against *Pseudomonas aeruginosa* and *Staphylococcus aureus* increased permeability of cells to nuclear stain ethylene bromide, dissipated pH gradients and caused leakage of inorganic ions. Arfa et al., 2006 investigated the relationship between the chemical structure of carvacrol, and carvacrol derivatives (carvacrol methyl ether and carvacryl acetate) against *Escherichia coli*, *Pseudomonas fluorescens*, *Staphylococcus aureus*, *Lactobacillus plantarum*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Botrytis cinerea*. The carvacrol derivatives were not effective antimicrobials and indicated the importance of the free hydroxyl group in antimicrobial activity of carvacrol. Carvacrol's hydrogen bonding ability and proton releasing ability caused cell death due to membrane modification; however, this was not seen with carvacrol derivatives. Carvacrol caused a rapid decline in cellular ATP and corresponding increase in extracellular ATP of glucose energized *Bacillus cereus* (Ultee et al 1999), *Escherichia coli* (Helander et al., 1998, Fitzgerald et al., 2004), *Listeria innocua* (Fitzgerald et al., 2004) and *Salmonella typhimurium*. Ultee et al. (2002) proposed that carvacrol also acts as a proton exchanger, by exchanging its hydroxyl proton for another ion such as potassium. Xu et al. (2008) concluded that carvacrol damages the cellular membrane

and reported that flow cytometry could be used to determine other targets (sensitive sites) to elucidate the true underlying antimicrobial effect of carvacrol. *Saccharomyces cerevisiae* membrane was damaged by oregano essential oil which contains carvacrol (Chami et al., 2005). Damage to the cell membrane of that yeast was shown by the release of substances absorbing at 260 nm, as well as by scanning electron microscopy. Gill and Holly (2006) demonstrated that carvacrol inhibited motility, disrupted the cytoplasmic membrane and inhibited ATPase activity in both *Escherichia coli* and *Listeria monocytogenes*. They also suggested that membrane disruption may be a secondary effect rather than the primary effect. Analysis of lipid profiles of bacterial cells (*Escherichia coli* O157:H7, *Staphylococcus aureus*, *Salmonella* Typhimurium, *Pseudomonas fluorescens*, and *Brochothrix thermosphacta*) by gas chromatography showed a decrease of unsaturated fatty acids, particularly C18:2 trans and C18:3 cis after being exposed to carvacrol (Di Pasqua et al., 2007). Those same authors attributed that decrease in unsaturated fatty acids to membrane alterations noticed in scanning electron microscope images.

Nisin

There are several published reviews on nisin (Hurst, 1981; Ray, 1992; Hurst and Hoover, 1993; Delves-Broughton and Gasson, 1994; Delves-Broughton et al., 1996; Wessels et al., 1998; Thomas et al. 2000; Abee and Delves-Broughton, 2003; Juncioni de Arouz et al., 2009; Chu et al., 2010). Accordingly, this writing is not intended to be a comprehensive review on nisin but will briefly discuss source, composition and structure, antimicrobial activity, and mode of action of this bacteriocin.

Source

Nisin, a bacteriocin, was first discovered in England 1928 by Rogers and Whittier. It is produced as a defense response to some lactic acid bacteria (LAB), specifically *Lactococcus lactis* subspecies *lactis* (Juncioni de Arouz et al., 2009). The use of nisin as a suitable food preservative was first demonstrated by Hirsch et al. in 1951. To date, nisin is the only Generally Recognized As Safe (GRAS) commercially available bacteriocin approved for use as a preservative additive in over fifty countries (FAO/WHO, 1969; FDA, 1988; Jay, 2000). Nisin can be made synthetically (Fukase et al., 1988) or naturally produced from culture fluids or cells of the originating organism during fermentation of a modified milk medium (Cheeseman and Berridge, 1957; Bailey and Hurst, 1971; Lee and Kim, 1985). Nisaplin® (produced by Danisco) and Chrisin are commercial fermentates prepared by using wild-type isolates of *Lac. lactis* subsp. *lactis*.

Composition and structure

Nisin is categorized as a Class I lantibiotic. It is a small cationic, amphiphilic polypeptide containing lanthionine and methyllanthione groups (Klaenhammer, 1993). It is very flexible in structure and normally present in the more stable dimer form. The monomer of nisin has a molecular weight of 3.5 kDa and contains 34 amino acids residues. There are two forms of nisin produced, A (Figure 4) and Z. The two forms differ only by the number of histidines present; form A has two and Z has one. In variant Z the histidine at position 27 is exchanged for asparagine, thus increasing its solubility and diffusion characteristics of nisin (Mulders et al., 1991; De Vos et al., 1993; Laridie et al., 2003). The unusual amino acids are thought to be responsible for the functional properties of nisin (ex. acid tolerance,

thermostability at low pH, antimicrobial activity, and bactericidal mode of action) (Sahl, 1991; De Vuyst and Vandamme, 1992).

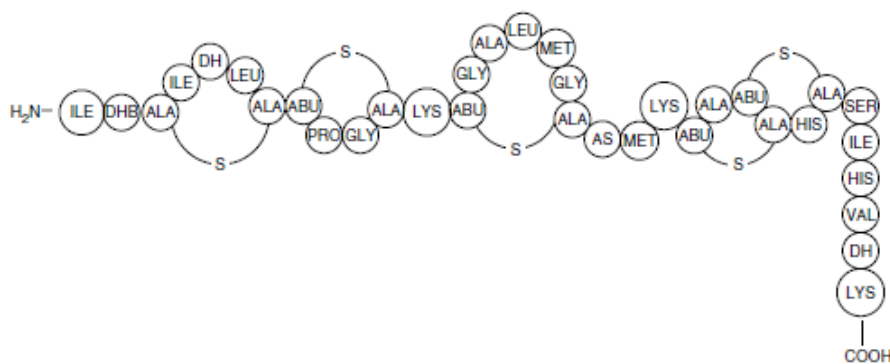


Figure 4. Structure of nisin form A. ABU, aminobutyric acid; DHA, dehydroalanine; DHB, dehydrobutyrine (β -methyldehydroalanine); ALA-S-ALA, lanthionine; ABU-S-ALA, β -methyllanthionine. Source: Gross and Morrell, 1971.

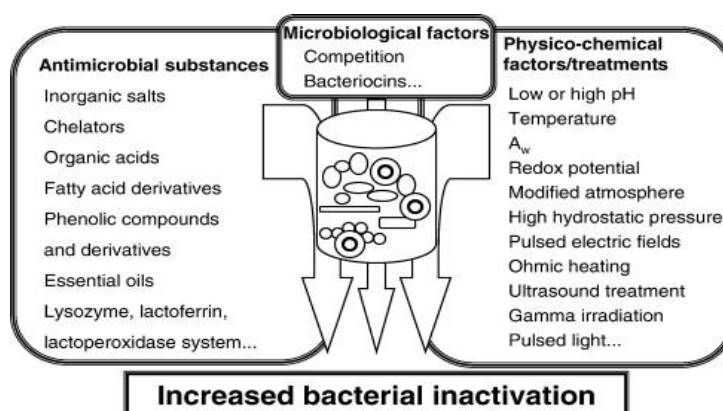
Antimicrobial spectrum

Nisin has been used as a natural antimicrobial peptide and is effective against gram positive bacteria, including its producer organism. The sensitivity of vegetative cells and spores to nisin may vary between strains within the same species of the following Genera of bacteria: *Alicyclobaccillus* (Komitopoulou et al., 1999), *Bacillus* (Jaquette and Beuchat, 1998), *Brochothrix* (Cutter and Siragusa, 1996), *Clostridium* (Okereke and Montville, 1992), *Enterococcus* (Laukova, 1995), *Lactobacillus* (Chun and Hancock, 2000) *Listeria* (Knight et al., 1999), *Micrococcus* (Dutreux et al., 2000), and *Staphylococcus* (Thomas and Wimpenny, 1996). Gram-positive strains that are resistant to nisin have the ability to synthesize the enzyme nisinase which degrades nisin (Abee et al., 1995). Nisin is unable to penetrate the cell wall of yeast. Gram-negative bacteria are resistant to nisin; the antimicrobial is unable to

penetrate the complex cell wall, particularly the outer lipopolysaccharide membrane, to reach the cytoplasmic membrane (Kordel et al., 1989; Boziaris and Adams, 1999).

Combinations of nisin and other antimicrobial have been reported to have substantial inhibitory or killing effects against bacteria. Nisin has been successfully combined with other bacteriocins such as pediocin (Cabo et al., 2009) and leucocin F10 (Parente et al., 1998), the plant essential oils carvacrol (Pol and Smid, 1999) and thymol (Solomakos et al., 2008), lysozyme (Chun and Hancock, 2000; Mangalassary et al., 2007), EDTA (Branen and Davidson 2004; Ukuku et al., 2009), heat (Beard et al., 1999), pulsed electric fields (Dutreux et al., 2000), and high pressure treatment (Yuste et al., 1998). Nisin alone is not effective against gram negative bacteria. When nisin is combined with hurdle technology (Figure 5) its antimicrobial efficiency is enhanced by either synergistic or additive effects against both gram negative and gram positive microorganisms. Hurdle technology also offers the opportunity to provide an additional barrier and avoid the development of resistant strains.

Figure. 5. Application of bacteriocins as part of hurdle technology. Source: Galvez et al., 2007.



Mechanism of action

Numerous studies have addressed the mechanism of action of nisin (Ramsier, 1960; Gao et al., 1991; Bruno and Montville, 1993; Mol et al., 1996, 1997; Hasper et al., 2006). In susceptible vegetative cells the primary site of action by both A and Z forms is the cytoplasmic membrane (Abee et al., 1995; Kuwano et al., 2005). Nisin has the ability to bind the membrane and form pores that destroy the membrane integrity. Pore formation leads to leakage of K⁺ ions and ATP, depletion of the proton motive force, and depolarization of transmembrane potential, resulting in cell death (Sahl, 1991; Bruno and Montville, 1993; Millette et al., 2004). It has also been reported that nisin interferes with cell wall biosynthesis by binding with lipid II, a peptidoglycan precursor (Mantovani and Russell, 2001; Bauer and Dicks, 2005; Kruijff and Breukink). Cells with low levels of lipid II have been found to be less sensitive to nisin (Brotzel et al., 1998).

Foodborne human enteric pathogens

More than 250 known diseases are transmitted through food and foodborne diseases are a widespread and growing public health problem, both in developed and developing countries. The Center for Disease Control reports that foodborne diseases previously caused approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths in the United States each year (Mead et al., 1999). Known pathogens account for an estimated 14 million illnesses, 60,000 hospitalizations and 1,800 deaths (Mead et al., 1999). Recent data reports that foodborne disease related illnesses has declined annually causing approximately 9.4 million episodes, 55,961 hospitalizations, and 1,351 deaths (Scallan et al., 2011). Bacteria are a common cause of foodborne illness. In the past, typical examples of food transmission

vehicles were raw meats, chicken, eggs, juices, milk, and ready-to-eat meat etc. More recently other types of food products including vegetables, peanut butter, and spices have been incriminated in disease outbreaks (Natvig et al., 2002). The following human enteric pathogens among those that are of great concern to federal health agencies, food processors and consumers are *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* and *Staphylococcus aureus* (Kotula et al., 2000).

Escherichia coli

Morphology and characteristics

Escherichia coli is a part of the *Enterobacteriaceae* family. It is morphologically classified as a Gram-negative, non spore-forming, motile or nonmotile straight rod (1-4µm). It is a mesophilic, facultative anaerobe that is a normal inhabitant of intestines of humans and other warm-blooded animals (mammals and birds). *E. coli* is an indicator organism and its presence in food or water is generally indicative of fecal contamination. There are several pathogenic strains of *E. coli* that can be placed into 6 groups: 1) enterotoxigenic (ETEC), 2) enteroinvasive (EIEC), 3) enteroaggregative (EAEC), enteropathogenic (EPEC), diffusely adherent (DAEC), and enterohemorrhagic (EHEC) (Buchanan and Doyle, 1997; Ray, 2004). The *Escherichia coli* O157:H7 used in experiments described in the present dissertation belong to the EHEC group which is known to cause hemorrhagic colitis in infected persons. Apart from *E. coli* O157:H7 there are other major non-O157:H7 EHEC serotypes including O157:NM, O111:H8, and O26:H11 (Meng et al., 2001); however, the information in the following paragraphs will mainly focus on *E. coli* O157:H7 because it is the most common

serotype in the EHEC group and there are far more published reports on this serotype than other EHEC serotypes.

***Escherichia coli* O157:H7**

In 1982 *Escherichia coli* O157:H7 was first associated with two outbreaks of hemorrhagic colitis (Wells et al., 1983). Although other *E. coli* serotypes such as O111, O26, and O126:NM (a sorbitol fermenting serotype) have been since linked to cases of hemorrhagic colitis, *E. coli* O157:H7 is the major cause of EHEC-associated illness in the United States and several countries (Meng et al., 2001). Just like all other EHEC serotypes, this pathogen produces Shiga-like toxin (Stx) or verotoxin (VT) (Johnson et al., 1983; Karmali et al., 1985). *Escherichia coli* strains that produce Shiga toxin are called Shiga toxin-producing *E. coli* (STEC) because of the strong similarity between the toxin they produce and the toxin produced by *Shigella dysenteriae* type 1 (Calderwood et al., 1996). Over 200 non O157:H7 serotypes of STEC were isolated from humans; however, only those isolates that cause hemorrhagic colitis are classified as EHEC. Other characteristics of *E. coli* O157:H7 include inability to ferment sorbitol within 24 hours, optimum growth within 30 to 42°C and pH 6 to 8, and acid tolerance although optimal pH for growth is between 6 and 8 (Tsai and Ingham, 1997). In addition, this pathogen is unable to grow well, if it does, at temperatures > 44.5 C in *E. coli* broth. It does not produce β -glucuronidase (GUD), therefore it is unable to hydrolyze 4-methylumbelliferyl-D-glucoronide (MUG), a fluorogenic substrate that is hydrolyzed by *E. coli* to produce the fluorescent 4-methylumbelliferyl moiety that fluoresces under long wave ultra violet light. This reaction helps in differentiation of *E. coli* from other organisms although a few salmonellae, shigellae

and some corynebacteria are GUD positive. *Escherichia coli* O157:H7 has an attaching and effacing (eae) gene and possesses a 60-MDa plasmid (Griffin, 1995). This pathogen can easily be destroyed by pasteurization or cooking (Williams and Ingham, 1997) therefore, undercooking and/or post-cook contamination of certain foods such as ground beef are factors that drastically increase the risk of foodborne infection by this pathogen.

Reservoirs

Cattle are a major reservoir for *E. coli* O157:H7. Orskov et al. (1987) reported the first isolation of *E. coli* O157:H7 from a calf (less than three weeks old) with coccobacillosis in Argentina. Generally, the prevalence of *E. coli* O157:H7 in cattle is about 10 to 25%. Interestingly, the isolation rates are much lower for non- O157 STEC. Based on results of two major surveys conducted in the United States, 31 (3.2%) of 965 dairy calves and 191 (1.6%) of 11,881 cattle on feedlots tested positive for *E. coli* O157:H7 (Zhao et al., 1995). Adult cattle seem to carry less *E. coli* O157:H7 than young cattle. In the feces of calves, populations of this pathogen range from detectable via enrichment culture ($< 10^2$ CFU/g) to 10^5 colony forming units (CFU)/g.

During the warmer months of the year there is a higher prevalence of the pathogen in cattle that correlates with the seasonal variation in *E. coli* O157:H7 infections in humans. In England, 38% of cattle delivered for slaughter in the spring tested positive for *E. coli* O157:H7 whereas, only 4.8% of cattle were positive for this pathogen during the winter season. Similarly a study in the United States revealed a high prevalence of the pathogen in cattle delivered for slaughter during July to August 1999 (Elder et al., 2000). *Escherichia coli* O157:H7 is not a pathogen of calves or adult cattle; therefore, cattle that harbor this

pathogen are not ill. Although, in some instances *E. coli* O157:H7 was linked to diarrhea and attaching and effacing lesions in new born calves (Cray et al., 1995; Dean-Nystrom et al., 1997).

Apart from cattle several domestic animals and wildlife including dogs, cats, horses, sheep, goats, pigs and deer, have been found to harbor *E. coli* O157:H7 and other STEC. These microorganisms were also found in rats and seagulls (Cizek et al., 1999; Wallace et al., 1997). Compared to other animals, sheep seem to have the highest prevalence of *E. coli* O157:H7 and STEC. Results of a survey of seven animal species in Germany revealed that STEC were most frequently isolated from sheep (66.6%), goats (56.1%), and cattle (21.1%). A much lower frequency of isolation of the pathogen was reported for cats (13.8%); pigs (7.5%), dogs (4.8%) and chickens (0.1%) (Beutin et al., 1993).

Humans can carry and shed *E. coli* O157:H7 for a substantial amount of time. In patients with hemorrhagic colitis or hemolytic uremic syndrome (HUS) the fecal shedding of this pathogen typically lasts for approximately 13 to 21 days after the onset of symptoms. Karch et al. (1995) reported that a child who was infected during an *E. coli* O157:H7 outbreak at a day care center continued to shed the pathogen for 62 days after the onset of diarrhea. To date no asymptomatic long term carriage of the pathogen in humans has been reported. Fecal carriage of the *E. coli* O157:H7 in humans is important because of the potential for person-to-person spread of this pathogen which has a low infectious dose (< 100 cells).

Foodborne Outbreaks

In the late 1800s *E. coli* was considered to be relatively harmless. An outbreak that caused

infants to have diarrhea in the mid 1940s resulted in some strains of *E. coli* being designated as enteropathogenic. This organism has been implicated as the contaminant in a variety of foods related to food recalls and foodborne illness. In 1982, *E. coli* O157:H7 was first recognized as a foodborne pathogen after an outbreak of hemorrhagic colitis associated with the consumption of hamburgers (Riley, et al., 1983). In 1993 consumption of contaminated hamburgers from restaurant chains caused 700 illnesses and 4 deaths across 4 states, the largest documented *E. coli* outbreak in the United States (Wachsmuth et al., 1997). United States Department of Agriculture division of Food Safety and Inspection Service identified *E. coli* O157:H7 as an adulterant in ground beef and instituted a sampling program to test for the pathogen in federally inspected establishments and retail stores in October 1994 (Griffin et al., 1994). The following food vehicles have been linked to *E. coli* O157 contamination: ground beef (Firstenberg-Eden and Sullivan, 1997), venison, sausage, dried uncooked salami, unpasteurized milk and cheese, unpasteurized apple juice and cider (Cody et al., 1999), orange juice, vegetables (alfalfa, parsley, radish sprouts, lettuce, cabbage, and spinach) (Breuer et al., 2001), fruits and berries, nuts and cookie dough. Until recently any outbreak of *E. coli* O157:H7 was assumed to be linked to raw or undercooked meat, specifically beef.

Characteristics of the disease

Escherichia coli O157:H7 infection occurs after ingestion of food contaminated with the pathogen and can result in diarrhea (non-bloody), hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and thrombocytopenic purpura (TTP). The infectious dose (< 50 cells) to cause severe clinical syndromes is very low compared to that of several other foodborne pathogens (Bell, 2002; Ray, 2005). The incubation period is usually 3 to 4 days

with a range of 2 to 12 days. During this time the pathogen colonizes the large intestine and binds tightly to cells in the intestinal lining. During the first two days of illness infected persons experience non-bloody diarrhea and abdominal cramps that progresses to bloody diarrhea that may last for four to ten days (Besser et al., 1999). Usually, symptoms regress and stop after about one week; however, approximately 6% of infected individuals develop HUS with half of them requiring dialysis and 75% requiring blood transfusion (Cohen, 1996; Ref 32, Frontiers). Some patients endure kidney damage and occasionally kidney failure, seizures, coma and may die (Martin et al., 1990; Bell et al., 1994; Boyce et al., 1995; MacDonald et al., 1998). The fatality rate due to human infection with this pathogen is about 1% (Besser et al., 1999)

Listeria monocytogenes

Morphology and characteristics

Listeria monocytogenes is Gram-positive, non-spore-forming, short, rounded rod-shaped (0.5 x 1 µm) bacterium which is motile (at 28 °C) by means of peritrichous flagella. This organism is a psychrotrophic, facultative anaerobe, occurring singly or in short chains and belongs to the *Listeriaceae* family. The genus *Listeria* is separated into the following six species based on DNA relatedness: 1) *Listeria monocytogenes* – human pathogen, 2) *Listeria ivanovii* – pathogen in sheep, 3) *Listeria innocua* – non pathogenic, 4) *Listeria welshimeri*, 5) *Listeria seeligeri*, and 6) *Listeria grayi*. *Listeria monocytogenes* has several serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, and 4a, 4ab, 4b, 4c, 4d, 4e, and 7. While all serotypes are capable of causing disease in humans, serotypes 1/2a, 1/2b and 4b are responsible for about 95 to 98% of documented human listeriosis cases (Lund et al., 2000).

Listeria monocytogenes has the ability to adapt and survive exposure to many environmental stresses. This pathogen can survive and grow in a wide temperature range - 1.5 to 45°C (Juntilla et al., 1988; Vasseur et al., 1999); with optimal growth at 35 to 37 °C. Refrigeration temperature leads to delayed growth (Rosenow and Marth, 1986). This pathogen can also tolerate reduced water activity (< 0.93) by freezing or drying (Farber et al., 1992; Mena *et al.*, 2004), high salt concentrations (up to 30%), acidic pH (below 5.0) (Bacon and Sofos, 2003), and nitrite concentrations currently approved for use in foods (ICMSF, 1996; Murray et al., 1999). These characteristics contribute to its survival under conditions usually adequate to control the growth of pathogens in food. *Listeria monocytogenes* is readily killed by conventional thermal processing of foods with prolonged exposure to elevated temperatures (above 56 °C) causing irreversible cellular damage resulting in death of the organism (Bunduki et al., 1995).

Reservoirs

Listeria is ubiquitous in the natural environment and therefore there are several environmental reservoirs of this pathogen. It has been isolated from soil, sewage, water, dead vegetation, human and animal feces, food processing facilities and animal feed (Beresford et al., 2001; Teixeira et al., 2008). Soils with decaying vegetation and/or fertilized with animal excreta or sewage sludge are highly likely to harbor *L. monocytogenes*. Interestingly, the natural habitat of this pathogen seems to be decaying vegetation such as aerobically spoiled silage, which supports prolific growth of this pathogen and serves as a source of listeriosis in farm animals (Fenlon, 1996). Due to *L. monocytogenes*'s widespread occurrence in nature, many feral and domestic animals carry this pathogen in their lymph

nodes and/or in their intestinal tract. About 11 to 52% of animals are carriers of this pathogen; pigs (45%) and cattle (24%) were found to carry the pathogen in their tonsils and pharyngeal lymph nodes, respectively (Doyle et al., 2007). Approximately 5 to 10% of humans exhibit intestinal carriage of this pathogen without any overt symptoms (Lorber, 1997).

Listeria monocytogenes can enter food processing facilities by several vehicles including raw agricultural products of plant or animal origin, clothing and shoes of workers, and possibly air-borne dust. Once this hardy pathogen enters the food processing environment it is easily spread to several areas by movement of raw materials, workers, tools, wheels of carts and actions that cause cross-contamination. *L. monocytogenes* is commonly found in moist areas such as floor drains, condensates on pipes, stagnant water, and residual food material on food processing equipment (Ryser and Marth, 2007).

Foodborne outbreaks

Listeria monocytogenes was first recognized as a human pathogen in 1929; however, it emerged as a serious food-borne pathogen in the 1980s and has been increasingly associated with food recalls and foodborne listeriosis outbreaks (Teratanavat and Hooker 2004). Five of the largest listeriosis outbreaks occurred in 1981 (Canada), 1983 (New England), 1985 (California), 1989 (multistate), and 2002 (multistate). In 1981, an outbreak related to consumption of contaminated coleslaw, resulted in 34 infected pregnant women, 9 of which experienced stillbirths, 23 had infected infants, and 2 had healthy births. There were 77 other adults who developed listeriosis with nearly 30% mortality. The outbreak in 1983 was caused by drinking a specific brand of pasteurized whole or 2% milk contaminated by *L.*

monocytogenes serotype 4b, resulted in 49 patients acquiring listeriosis; 7 fetuses or infants and 42 immunocompromised adults; of these 14 patients died (Fleming et al., 1985). In California (1985), 142 people developed listeriosis from consuming a certain brand of pasteurized soft cheese that had been cross contaminated with raw milk during the manufacturing process. Of those 93 cases were perinatal, 49 non pregnant, and 48 immunosuppressed individuals, of which 30 fetuses or newborn infants and 18 adults died (ICMSF, 1996). The 1989 multistate outbreak linked to meat frankfurters, resulted in 108 cases, with 14 associated deaths and 4 miscarriages or stillbirths (Mead et al., 2006). In 2002, a multistate outbreak of *L. monocytogenes* infection with 46 culture-confirmed cases, seven deaths, and three stillbirths or miscarriages in eight states was linked to eating sliced turkey deli meat. USDA established a zero tolerance policy for *L. monocytogenes* in ready-to-eat meat products (USDA, 1987, 1989). Many different foods have been associated with being contaminated with *L. monocytogenes*: minced meats, improperly cooked chicken, dairy products, fish and seafood, eggs, pâté, pork, rice salad, butter, and potato salad (Schlech et al., 1983; Cox 1989; Schuchat et al., 1992; Vasseur et al. 1999; Rocourt et al., 2000; Pawar et al., 2000; Ryser and Marth, 2007). *Listeria* is responsible for the foodborne illness listeriosis and is of significant concern to the food industry (Cox, 1989; Wong, 1998).

Characteristics of listeriosis

Listeriosis, an opportunistic disease, represents a small fraction of all illnesses related to bacterial foodborne pathogens but accounts for 3.8% of hospitalizations and 27.6% of deaths (Mead et al., 1999). Foodborne infection occurs after consuming contaminated food, enabling listeria to penetrate the epithelial lining and cross the intestinal barrier. The pathogen is believed to become attached to epithelial cells of the GI tract by means of D-

galactose residues on the bacterial surface which adhere to D-galactose receptors on the host cells (Dramsi et al., 1993). The organism becomes engulfed by macrophages and encased in a vacuole. Within the macrophage the pathogen eventually lyses the vacuole and invades the cytoplasm, where it begins to multiply. A virulence factor that helps the pathogen to escape from the vacuole is listeriolysin O (LLO), and hemolysin which produces a zone of beta-hemolysis around the listerial colony on blood agar (Lorber, 1997; Salyers and Whitt, 2002). The internalized pathogen is transported to lymph nodes then to the liver, which is a primary area of infection. Within the macrophage the pathogen can spread through the bloodstream to other body tissues also while being protected from the host's antibodies. Except for macrophages which are naturally phagocytic, other host cells such as enterocytes are invaded by the pathogen via special membrane proteins (InlA and InlB) called internalins (Gaillard et al., 1991) which facilitate adhesion to and invasion of the host cell (Lorber, 1997; Salyers and Whitt, 2002).

Spread of *L. monocytogenes* in the human body can cause infection at several sites leading to septicemia, meningitis, abortions and stillbirths, gastroenteritis and death. Listeriosis is regarded as a major problem especially in high risk groups including pregnant women, fetuses, neonates, the elderly and immunocompromised individuals (30 to 40% fatality) (Farber and Peterkin, 1991). *L. monocytogenes* is one of very few infectious microorganisms that are capable of traversing the placental barrier and infesting the fetus. An infected pregnant woman may experience mild flu-like symptoms; however, the fetus usually develops a systemic infection, which is highly lethal (Lorber, 1997). The tragic reality of listeriosis in pregnant women is that there is no early warning sign of the disease.

The only first symptom is an infected neonate or a stillborn infant. In immunocompromized individuals, the first sign of listeriosis infection is usually fever and the disease rapidly progresses to other complications, commonly meningitis (Lorber, 1997). Healthy individuals may not develop symptoms or show very mild symptoms. Common symptoms for healthier individuals are mainly enteric (abdominal cramps, diarrhea, nausea) along with a slight fever and headache. A major reason why listeriosis is an uncommon disease among immunocompetent persons is that the pathogen has a relatively high infectious dose (ID₅₀). The ID₅₀ is a measure of infectivity and refers to the number of viable cells of the pathogen required to cause infection in 50% of experimentally infected animals. Due to the high ID₅₀ of *L. monocytogenes*, healthy persons have to ingest large numbers of viable cells of the pathogen to contract a mild infection. Treatments such as antacids or H₂ blockers that compromise the acid protection in the stomach can increase a person's susceptibility to listeriosis. Pregnancy also increases a person's susceptibility to this foodborne infection (Lorber, 1997). Symptoms of listeriosis appear between 11 and 70 days (average 30 days) after ingestion of contaminated food. This protracted incubation period presents a major impediment to accurately tracing the source of an outbreak because people are unable to accurately recall food consumed 30 days ago (Broome, 1993).

Salmonella

Morphology and characteristics

Salmonella is a genus of the *Enterobacteriaceae* family. It is morphologically classified as a Gram-negative, non spore-forming, usually motile, medium rod (1 x 4 µm). The normal habitat, of this mesophilic, facultative anaerobe is the gastrointestinal tract of animals, birds, insects, and some reptiles (turtles and frogs). The *Salmonella* genus is

divided into two species, *Salmonella enterica* and *S. bongori*. *S. enterica* is further divided into six subspecies: (I) *S. enterica* subsp. *enterica*, (II) *S. enterica* subsp. *salame*, (IIIa) *S. enterica* subsp. *arizonae*, (IIIb) *S. enterica* subsp. *diarizonae*, (IV) *S. enterica* subsp. *indica*, and (IV) *S. enterica* subsp. *houstenae* (Porwollik et al., 2004; Ray 2004). Within subspecies I there are over 2500 serovars and all are considered human enteric pathogens.

The growth range for *Salmonella* is from 5-46°C and optimum growth temperature is between 35 and 37°C. *Salmonella* are generally sensitive to heat (pasteurization temperature and time) and high acidity (pH 4.5 or below), depending on food product and processing. Heat resistance can be developed in dry products (powdered milk) or low water activity products (peanut butter - $a_w < 0.35$) (Burnett et al., 2000; Shacher and Yaron, 2006). As the water activity decreases in a food product the pathogen becomes more heat resistant. As with other pathogens food composition (fat and protein content) must be considered when determining possible heat resistance of *Salmonella*. The cells also have the ability to survive (not multiplying) in suboptimal (frozen, dried, or nutrient starved) states for long periods (Foster and Spector 1995).

Reservoirs

The widespread occurrence of *Salmonella* in the natural environment is most likely attributed to several reservoirs of this pathogen. The continued prevalence of *Salmonella* in the global food chain is due to the pathogen's widespread occurrence along with intensive production practices employed by poultry, cattle, swine, fish and shellfish industries and the recycling of animal visceral organs and inedible raw materials into animal feeds (D'Aoust, 2000). For several decades, poultry and poultry products have been well known as important

reservoirs for salmonellae and have obscured the importance of meats including beef, pork, and mutton as potential sources of *Salmonella* infection in humans (Hedberg et al., 1999; Todd, 1994; World health Organization, 1988). In its attempt to address this problem, the USDA Food Safety and Inspection Service (FSIS) published the “Final Rule on Pathogen Reduction and Hazard Analysis and Critical Control Point (HACCP) Systems” in July 1996. According to the Final Rule the meat and poultry processors are required to implement HACCP plans and systematically sample final products for fecal indicator (*Escherichia coli* biotype 1). Also, FSIS performs testing of the finished meat products for *Salmonella* to ascertain that the HACCP system is adequate to control of this pathogen. Before implementation of the Final Rule, the *Salmonella* contamination rate in processed broiler chickens was 24%. By 1999 the contamination rate decreased to approximately 11%. In a more recent survey of 20 U.S. broiler processing plants, the contamination rate was 20% for post-chill carcasses (Berrang et al., 2009). Based on these findings, future improvement in reducing prevalence of *Salmonella* in meat and poultry products will require stringent control measures on animal production farms, and during processing, distribution and handling at retail sites.

Apart from poultry and other meats, eggs have been frequently identified as a reservoir of *Salmonella* and a vehicle for the spread of the pathogen in humans. The pandemic linked to *Salmonella enterica* serotype Enteritidis phage type 4 and phage type 8 in Europe and North America, respectively, has been associated with the consumption of eggs (raw or lightly-cooked) and egg-containing products. This pandemic further justifies the need for continued stringent pathogen control measures in poultry and egg production. More focus should also be placed on pathogen control in breeder flocks because it is known that *S.*

Enteritidis can infect the ovaries of laying hens and enter the egg before the shell is produced. This process is termed transovarian transmission in which the infective agent contaminates the egg prior to deposition of the shell. Obviously, no amount of surface sanitizing of whole eggs can destroy the pathogen which is inside of the egg and remains viable.

Intensive fish farming involving improper management practices have created a relatively new reservoir of *Salmonella*. Within the past three decades there has been an increase in aquaculture of various fish and shellfish species globally as alternative sources of these food products. This trend resulted from the noticeable depletion of feral stocks of fish and shellfish and led to intensive (high-density) inland farming of these fish to satisfy the growing consumer demand. This type of intensive aquaculture has contributed to the widespread contamination of fish in unprotected earth, concrete, or other types of ponds that are continuously exposed to environmental contaminants. In the U.S. a substantial amount of aquaculture products are imported from south American, Asian, and African countries where aquaculture farmers may be using questionable management practices that may increase the populations of salmonellae in pond-raised fish. In some countries it is common practice to feed pond-raised fish with raw pieces of meat, offal, night soil and/or animal feeds that may be contaminated with various serovars of *S. enterica*. The feeding of pond-raised fish with night soil is a serious public health concern because that type of feed could potentially carry typhoid and paratyphoid salmonellae. Such practices perpetuate the occurrence of high populations of salmonellae and other human enteric pathogens and create a constant source of contamination in the production environment (D'Aoust, 1994). In addition, they at times increase the populations of fish pathogens which cause sub-optimum growth or death of the

fish and severe economic loss to aquaculture farmers. To safeguard the health of fish and shell fish and reduce financial losses, aquaculture farmers are relying on the sub-therapeutic use of antibiotics such as chloramphenicol, sulfa drugs, ampicillin and fluoroquinolones. This management practice is short-sighted and irresponsible because such misuse of medically important antimicrobials in animal production selects for antibiotic-resistant *Salmonella* serovars (D'Aoust, 1994; D'Aoust et al., 1992). This in turn can have severe health consequences when consumers eat pond-raised fish in a sushi meal or in a lightly cooked form, or shellfish, contaminated with antibiotic-resistant salmonellae (D'Aoust, 1994; D'Aoust et al., 1992).

In recent years fresh fruits and vegetables have emerged as vehicles of human salmonellosis (Harris et al., 2003). Factors that contribute to that situation include increased imports of fresh and dehydrated vegetables from foreign countries that have a tropical climate year round. Also, in those countries, hygienic conditions during crop production, harvesting, and distribution may not meet minimum U.S. standards thus facilitating microbial contamination of fresh produce. More importantly, the fertilization of crops with untreated sewage or non-composted animal manure, irrigation of fields and washing of fresh produce with contaminated water, improper handling of produce by workers, and numerous other opportunities for environmental contamination of dried vegetables and spices during the drying process are factors that facilitate the accumulation of human enteric pathogens including *Salmonella* for which several reservoirs exist.

Foodborne outbreaks

The current increase in salmonellosis in the U.S. may be related to four factors: (1)

increased number of antimicrobial-resistant *Salmonella* isolates, (2) increased immunodeficient individuals, (3) increased egg-associated *Salmonella* Enteritidis contamination due to increased laying hens with infected ovaries, and (4) centralized food production that increase the risk of widespread distribution of contaminated food to cause large outbreaks (Tauxe, 1984). *Salmonella enterica* serotype Typhimurium and *Salmonella enterica* serotype Enteritidis have been the most frequently reported causes of foodborne illnesses since 1993. The Center for Disease Control and Prevention (CDC) 2011 Estimates of Foodborne Illness report linked to *Salmonella* are 1,027,561 illnesses, 19,336 hospitalizations, and 378 deaths. The twenty most common serotypes of *Salmonella* in 2006 represented 70% of all *Salmonella* isolates. The four most common serotypes were Typhimurium, Enteritidis, Newport, and Heidelberg (45% of all isolates; CDC, 2008). There were 121 outbreaks in 2006, and more than 3,300 illnesses (Enteritidis (26), Typhimurium (26), Newport (10), and Heidelberg (10); CDC, 2006). Raw or undercooked chicken and eggs are usually the foods thought to be most commonly associated with *Salmonella*; however, from 2006 to 2011, various serovars of *Salmonella* have been linked to contamination of the other foods including: tomatoes, banquet pot pies, dry pet food, peanut butter, cantaloupes, Malt-O-Meal Rice/wheat cereals, pistachios, alfalfa sprouts, red and black pepper, gourmet seasoning, soup/dip mixes, vegetable broth, granola bars, ceviche, potato chips, and eggs (<http://www.cdc.gov/salmonella/outbreaks.html>). During 2004, a multistate outbreak of serotype Javiana infections associated with tomatoes at a gas station deli chain affected more than 400 people in 5 states (CDC, 2008). *Salmonella* Tennessee was involved in a notable outbreak associated with peanut butter distributed worldwide, and caused over 700 cases of illness in 48 states (CDC, 2007a). In 2006, two *Salmonella*

(Newport and Typhimurium) outbreaks were associated with consumption of raw tomatoes in restaurants causing a total of 309 illnesses (CDC, 2007b). In 2009 there was a multistate outbreak of *Salmonella* Typhimurium associated with peanut butter and peanut butter containing products resulting in 529 people infected in 43 states, 116 people hospitalized and 8 deaths. In August 2010, there were two recalls related to eggs contaminated with *Salmonella* Enteritidis from two farms located in Iowa. Approximately 550 million eggs were recalled nationwide; more than 2000 people were sickened, between May 1, 2010 and Nov 30, 2010 (CDC, 2010). Based on these previously mentioned outbreaks, salmonellosis continues to be a major food safety problem in the United States. Therefore, much greater attention needs to be paid to control the pathogen at all points in the food chain from farm to consumer.

Characteristics of salmonellosis

Salmonellosis in humans can cause serious ailments including enteric (typhoid) fever, enterocolitis, and systemic infections. Typhoid and paratyphoid serovars of the pathogen are able to invade and survive in human intestinal cells and subsequently cause enteric fever, a serious human disease. Symptoms of enteric fever occur after an incubation period of 7 to 28 days and may include nausea, diarrhea, fever, abdominal pain, headache, and prostration (D'Aoust, 1991). Disease diagnosis depends on isolation of the pathogen from the patient's blood and urine in the initial stages of infection or from fecal material after the onset of symptoms (D'Aoust, 1989). Following the acute phase involving enteric fever, the patient usually enters an asymptomatic chronic carrier state. Antibiotics such as chloramphenicol, ampicillin, or trimethoprim-sulfamethoxazole are used to treat patients with enteric fever to

eliminate the systemic infection. Within the past two decades marked global increases in antibiotic-resistant *Salmonella* have challenged the efficacy of antibiotic therapies. This problem is further exacerbated in developing countries where outbreaks of enteric fever are frequently associated with salmonellae that exhibit multiple antibiotic resistance and cause unusually high mortality rates (Carmen Palomino et al., 1986; Rajajee et al., 1995).

Salmonellosis caused by non-typhoid *Salmonella* serovars usually results in enterocolitis and abdominal pain although systemic infections may occur in some instances. The pathogen invades the mucosa of the small intestine, proliferates in the epithelial cells, produces a toxin and causes inflammation. After invasion of the epithelial cells it multiplies and produces an enterotoxin. Symptoms may appear in 6 to 48 hours after ingestion of contaminated food or water. Acute symptoms include nausea, vomiting, abdominal cramps, non-bloody diarrhea, chills, fever, headache and prostration lasting 2 to 3 days or more. Generally, the disease is self-limiting and regression of diarrhea and abdominal pain occurs within 5 days after the onset of symptoms (D'Aoust, 1989). Although it was previously thought that relatively large amounts of the pathogen had to be ingested to cause disease, the infectious dose of *Salmonella* can be relatively small (15-20 cells) depending on the serotype. Chronic symptoms include enlargement of the liver and spleen, infection of tissues surrounding the brain and spinal cord, and reactive arthritis (Mayo Clinic, 2009, April 17). Salmonellosis can be fatal to elderly, infants and immune compromised individuals.

Staphylococcus aureus

Morphology and characteristics

The etymological root of the bacterium's name: *staphylo* (bunch of grapes) is derived

from Greek and *aureus* (golden) is derived from Latin. The organism's name pertains to the gold colonies that are formed on agar medium. *Staphylococcus aureus* is gram-positive, non-spore forming, non motile, spherical cells (0.5 to 1 μm) occurring singly, in pairs, or in grape-like clusters. The main habitat of this mesophilic, facultative anaerobe is on human skin, mouth or nose, and animal hides, feathers, and skin. The genus *Staphylococcus* belongs to the bacterial family *Staphylococcaceae*. More than 20 species of *Staphylococcus*, some pathogenic and some non-pathogenic, have been delineated by DNA hybridization (Kloos and Schleifer, 1986; Freney et al., 1988). Several *Staphylococcus* species have been implicated in human infections, notably *S. aureus*, *S. saprophyticus*, *S. epidermidis*, *S. lugdunensis*, and *S. schleiferi* (Tenover and Gayne 2000; von Eiff et al., 2002; Choi et al., 2006; Otto, 2009). Many strains are known to be enterotoxin producers; however, their involvement as foodborne pathogens is unknown (Becker et al., 2001). *S. aureus* is the most common strain associated with human foodborne illness.

Staphylococcus aureus strains can be classified into the following 6 biotypes according to their human or animal origin: a) human, b) non- β -hemolytic human, c) avian, d) bovine, e) ovine, and f) nonspecific (Devriese, 1984). Generally, *S. aureus* strains produce at least 34 different extracellular enzymes, toxins, and other chemical components; however, strain differences occur and no one strain is capable of producing all of these proteins (Landolo, 1989). Nearly all strains of *S. aureus* produce the enzymes coagulase, thermonuclease, and hemolysin and are also resistant to digestive proteolytic enzymes, such as trypsin and pepsin (Le Loir et al., 2003). There are two groups of heat stable enterotoxins produced by *S. aureus*, Group 1: A, B, C1, C2, C3, D, and E; Group 2: G, H, I, J, K, L, M, N,

O, P, Q, R, and U (Dinges et al., 2000; Jarraud et al., 2001; Baba et al., 2002; Omoe et al., 2002).

Several factors influence enterotoxin production in foods and the extent to which the toxin may cause foodborne illness. Factors include numbers of viable *S. aureus* in the food, type and composition of food, holding temperature, amount of food consumed, presence of inhibitors, health of the consumer, susceptibility to the toxin, and type of toxin produced (Doyle et al., 1997). Rate of toxin production by a strain is directly related to its rate of growth and cell concentrations. Viable cells of *S. aureus* at a level greater than 10^5 or 10^6 per gram of food have been associated with enterotoxin production at concentrations sufficient to cause disease even though lower viable numbers of the pathogen have at times been implicated in causing foodborne disease. Enterotoxin concentrations ranging from 1- 5 μg have been demonstrated in foods involved in many *S. aureus* food poisoning outbreaks; however, enterotoxin doses as little as 10 nanograms or less can cause food poisoning (Doyle et al., 1997).

Growth temperature of *S. aureus* ranges from 7 to 47.8 °C and optimal growth temperature range is 30 – 35 °C (Schmitt et al., 1990). Cells can be killed by heating at 66 °C for 12 minutes and at 72 °C for 15 seconds. The pH range for growth of the pathogen is 4.5 to 9.3 (Scott, 1953) whereas, the optimum pH for growth is between 7.0 and 7.5 (Bergdoll, 1989; Martin and Myers, 1994). *S. aureus* is highly tolerant to salt (up to 10%) and sugars and can grow within a water activity (a_w) range 0.83-0.99. Although the pathogen may grow at a_w as low as 0.83, a minimum a_w of 0.86 is required for enterotoxin production (Lund, 2000). The ability of *S. aureus* to grow at 6–7 °C, low pH, and low water activity levels depends on other intrinsic or extrinsic factors being optimal for the pathogen

(Jay, 1992). Ability to grow under several adverse conditions allows the pathogen to multiply without intense competition from normal microflora present in food. Eradication of staphylococci is not always successful due to their ability to form biofilms (Nostro 2007). *Staphylococcus aureus* strains are frequently involved in foodborne intoxications.

Reservoirs

Humans and animals are important reservoirs of *S. aureus*. This pathogen and other staphylococci can be found on skin and skin glands of humans, other mammals and birds. It has been isolated from nostrils, mouth, upper respiratory tract, mammary glands, intestinal tract and genitourinary tract of infested hosts (Murray et al., 1999). The anterior part of the nasal passages is a well-known site for colonization of *S. aureus*, although the pathogen can be found on the skin. It is estimated that about 50% of healthy humans naturally carry *S. aureus* as part of their normal microflora (Jay et al., 2005). Contamination of food with this pathogen can occur by direct contact of food with contaminated hands of workers. Also, food contamination may occur indirectly from shed skin cells or aerosols generated from coughing and or sneezing, which expel droplets from the respiratory tract. The majority of sources of staphylococcal food poisoning have been traced to humans who contaminated foods during preparation or improper handling following preparation. Generally, animals may harbor very high populations of *S. aureus* (Jay et al., 2005). Colonization of animals with this human enteric pathogen is of major public health concern because of the potential for contamination of foods of animal origin before or during processing. Other sources for transfer of the pathogen to food include equipment and materials used in food processing such as improperly cleaned and sanitized meat grinders, saws blades, knives, cutting boards,

and food storage containers. Apart from being on the human body or on food processing implements, *S. aureus* can persist for extended periods of time in a desiccated state. This pathogen is one of the most resistant non-spore-forming pathogens and has been isolated from air, dust, water and sewage (Doyle et al., 1997).

Foodborne outbreaks

The growth and proliferation of *S. aureus* in foods presents a potential hazard to consumer health since many strains of *S. aureus* produce enterotoxins. The association of staphylococci with foodborne illness dates back as early as 1884, when a large food poisoning outbreak in the U.S. was linked to contaminated cheese. In 1989 the consumption of canned mushrooms contaminated with *S. aureus* enterotoxin A, caused multiple staphylococcal foodborne outbreaks (Feb. 13, Feb. 28, and April 17; Hard-English et al., 1990). These outbreaks led to 82 sick individuals, and of those 12 were hospitalized. Between 1993 and 1997, 42 outbreaks occurred, involving 1,413 cases, and one death (CDC, 2002). Based on the Center for Disease Control and Prevention (CDC) 2011 Estimates of Foodborne Illness for 2011, approximately 241,148 illnesses will be associated with *S. aureus*. Foods commonly associated with staphylococcal food poisoning include meat and meat products, fish, eggs, fruits and vegetables (mashed potatoes), salads, cream-filled bakery products, dry pasta and dairy products (Bean and Griffin, 1987; Evenson et al., 1988; Bean et al., 1990; Ray, 2005). Many of these items are contaminated post processing, during food preparation in homes, food service establishments, or mishandling prior to consumption (temperature abuse, poor hygiene). Human intoxication is caused by ingesting enterotoxins

produced in food, usually because of temperature abused food (hot holding temperature below 60°C or cold holding temperature above 4 °C).

Characteristics of *Staphylococcus aureus* intoxication

Staphylococcal food poisoning (staphylococcal gastroenteritis) is a self-limiting illness due to the absorption of staphylococcal enterotoxins preformed in the food (Le Loir et al., 2003). Generally, a toxin level that results in human illness is reached when *S. aureus* populations exceed 10^6 colony forming units. The infectious dose varies and a healthy adult must consume 30 g or ml of food containing 100 to 200 ng toxins to experience illness whereas, lower amounts of the toxin have caused illness in immune-compromised individuals (Bergdoll, 1990; Ray, 2005). Severity of symptoms is directly related to an individual's susceptibility, general health, potency and amount of toxin ingested. The onset of symptoms can occur anywhere from 30 min to 8 hours (2 - 6 hours average) after consumption of contaminated food containing preformed toxin (Dack, 1956). Primary symptoms are abdominal cramps, nausea, vomiting, occasionally followed by diarrhea (never diarrhea alone) and prostration. Secondary symptoms include dehydration, headache, chills, fever, transient changes in blood pressure and pulse rate. Recovery typically takes 2 days. More serious symptoms include toxic shock syndrome (release of super antigens into the blood stream), pneumonia, mastitis, phlebitis, meningitis, urinary tract infections, osteomyelitis and endocarditis. Death related to staphylococcal food poisoning is very rare. For the general public the fatality rate is about 0.03%; however, this rate increases to about 4.4% for more susceptible groups such as young children and the elderly (Holmberg and Blake, 1984).

References

- Abe, Y., Itoh, T., and Adachi, S. 1982. Fractionation and characterization of hen's egg yolk phosvitin. *J. Food Sci.* 47: 1903-1907.
- Abee, T. and Delves-Broughton, J. 2003. Bacteriocins - nisin. In *Food Preservatives*, edited by N.J. Russell and G.W. Gould. Kluwer Academic, New York. Pp 146-178.
- Abee, T., Krockel, L., and Hill, C. 1995. Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. *Int. J. Food Microbiol.* 28:169-185.
- Achinewhu, S.C., Ogbonna, C.C., and Hart, A.D. 1995. Chemical composition of indigenous wild herbs, spices, nuts and leafy vegetables used as food. *Plant Foods Hum. Nutr.* 32: 31-36.
- Albright, K.J., Gordon, D.T., Cotterill, O.J. 1984. Release of iron from phosvitin by heat and food additives. *J. Food Sci.* 49:78-81.
- Arfa, B.A., Combes, S., Preziosi-Belloy, L., Gontard, N., and Chalier, p. 2006. Antimicrobial activity of carvacrol related to its chemical structure. *Lett. in Appl. Microbiol.* 43:149-154.
- Arrebola, M.L., Navarro, M.C., Jimenez, J., and Ocana, F.A. 1994. Yield and composition of the essential oil of *Thymus serpylloides* subsp. *serpylloides*. *Phytochemistry.* 36:67-72.
- Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K., Oguchi, A., Nagai, Y., Iwama, N., Asano, K., Naimi, T., Kuroda, H., Cui, L., Yamamoto, K., and Hiramatsu, K. 2002. Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet.* 359:1819-1827.
- Bacon, R.T. and Sofos, J.N. (2003). Characteristics of biological hazards in foods. In: *Food Safety Handbook* (edited by R.H.Scchmidt & G.E. Rodrick). Pp. 157–195. Hoboken, NJ: JohnWiley & Sons.
- Bagamboula, C.F., Uyttendaele, M., and Debevere. 2004. Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and p-cymene towards *Shigella sonnei* and *S. flexneri*. *Food Microbiol.* 21:33-42.
- Bailey, F.J. and Hurst, A. 1971. Preparation of a highly active form of nisin from *Streptococcus lactis*. *Can. J. Microbiol.* 17:61-67.
- Bauer, R., and Dicks, L. M. (2005). Mode of action of lipid II-targeting lantibiotics. *Int. J. Food Microbiol.* 101:201-216.

- Bean, N.H. and Griffin, P.M., 1990. Foodborne disease outbreaks in the United States, 1973-1987. *J. Food Prot.* 53:804.
- Bean, N.H., Griffin, P.M., Goulding, J.S., and Ivey, C.B., 1990. Foodborne disease outbreaks, 5- year summary, 1983-1987. *Morbidity and Mortality Weekly Report*. Vol. 39 No. SS-1 pp. 15-57.
- Becker, K., Keller, B., Von Eiff, C., Brück, M., Lubritz, G., Etienne, J. and Peters, G. (2001). Enterotoxigenic potential of *Staphylococcus intermedius*. *Appl. Environ. Microbiol.* 67: 5551-5557.
- Bell, B.P., Goldoft, M., Griffin, P.M., Davis, M.A., Gordon, D.C., Tarr, P.I., Bartleson, C.A., Lewis, J.H., Barrett, T.J., Wells, J.G., et al., 1994. A multistate outbreak of *Escherichia coli* O157:H7-associated bloody diarrhea and hemolytic uremic syndrome from hamburgers: the Washington experience. *JAMA*. 272:1349-1353.
- Bergdoll, M.S. 1989. *Staphylococcus aureus*. In: *Foodborne Bacterial Pathogens* (Doyle, M.P., ed.). Marcel Dekker, Inc., New York, NY, USA, pp. 463-523.
- Bergdoll, M.S. 1990. Staphylococcal food poisoning. In: DO Cliver, ed. *Foodborne Diseases*. San Diego: Academic Press, Inc., pp. 85–106.
- Besser, R. E., Griffin, P.M., and Slutsker, L. 1999. *Escherichia coli* O157:H7 gastroenteritis and the hemolytic uremic syndrome: an emerging infectious disease. *Annu. Rev. Med.* 50: 355-367.
- Beuchat, L.R. 1994. Antimicrobial properties of spices and their essential oils. In *Natural Antimicrobial Systems and Food Preservation*, eds. V.M. Dillon., and R.G. Board, pp.167-179. Wallingford, UK: CAB Intl.
- Beutin, L., Geier, D., Steinruck, H., Zimmerman, S., and Schueutz, F. 1993. Prevalence and some properties of verotoxin (Shiga-like toxin)-producing *Escherichia coli* in seven different species of healthy domestic animals. *J. Clin. Microbiol.* 31:2483-2488.
- Boyce, T.G., Swerdlow, D.L., and Griffin, P.M. 1995. *Escherichia coli* O157:H7 and the hemolytic-uremic syndrome. *N. Engl. J. Med.* 333:364-368.
- Boziaris, I. S., and Adams, M. R. 1999. Effect of chelators and nisin produced in situ on inhibition and inactivation of gram negatives. *Int. J. Food Microbiol.* 53:105-113.
- Branen, J.K., and Davidson, P.M. 2004. Enhancement of nisin, lysozyme, and monolaurin antimicrobial activities by ethylenediaminetetraacetic acid and lactoferrin. *Int. J. Food Microbiol.* 90:63-74.
- Breuer, T., Benkel D.H., Shapiro, R.L., Hall, W.N., Winnett, M.M., Linn, M.J., Neimann, J.,

- Barrett, T.J., Dietrich, S., Downes, F.P., Toney, D.M., Pearson, J.L., Rolka, H., Slutsker, L., and Griffin, P.M. 2001. A multistate outbreak of *Escherichia coli* O157:H7 infections linked to alfalfa sprouts grown from contaminated seeds. *Emerg. Infect. Dis.* 7:977-982.
- Broome, C.V. 1993. Listeriosis: can we prevent it? *ASM News* 59:444-446.
- Bruno, M.E. and Montville, T.J. 1993. Common mechanistic action of bacteriocins from lactic Acid bacteria. *Appl. Environ. Microbiol.* 59:3003-10.
- Buchana, R.L. and Doyle, M.P. 1997. Foodborne disease significance of *Escherichia coli* O157:H7 and enterohemorrhagic *E. coli*. *Food Technol.* 51:69-75.
- Bunduki, M.M.C., Flanders, K.J., and Donnelly, C.W. 1995. Metabolic and structural sites of damage in heat- and sanitizer-injured populations of *Listeria monocytogenes*. *J. Food Prot.* 58:410-415.
- Burley, R.W., and Vadhera, D.V. 1989. The avian egg chemistry and biology. John Wiley, New York, New York, USA.
- Burnett S, Gehm E, Weissinger, W.R, and Beuchat, L.R. 2000. Survival of *Salmonella* in peanut butter and peanut butter spread. *J. Appl. Microbiol.* 89(3):472-477.
- Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods –a review. *Int. J. Food Microbiol.* 94:223-253.
- Byrne, B.,M., van het Schip, A.D., van de Klundert, J.A.M., Arnberg, A.C., Gruber, M., and Ab, G. 1984 Amino acid sequence of phosvitin derived from the nucleotide sequence of part of the chicken vitellogenin gene. *Biochemistry.* 23:4275-4279.
- Cabo, M.L., Torres, B., Herrera, J.J., Bernardez, M., and Pastoriza, L. 2009. Application of nisin and pediocin against resistance and germination of *Bacillus* spores in sous vide products. *J. Food Prot.* 72:515-523.
- Calderwood, S., Acheson, D., Keusch, G., Barrett, T., Griffin, P., Strockbine, N., Swaminathan, B., Kaper, J., Levine, M., Kaplan, B., Karch, H., O'Brien, A., Obrig, T., Takeda, Y., Tarr, P., and Wachsmuth, I. 1996. Proposed new nomenclature for SLT (VT) family. *ASM News* 62:118-119.
- Carmen Palomino, W., Lucia Aguad, R., Manuel Rodriguea, L., Graciela Cofre, G., and Villanueva, J. 1986. Clinical course of infections by *Salmonella typhi*, paratyphus A and paratyphus B in releas to the sensitivity of the etiological agent to chloamphenicol. *Rev. Méd. Chile.* 114:919-927.
- Castellani, O., Guerin-Dubiard, C., David-Driand, E., and Anton, M. 2004. Influence of

physiochemical conditions and technological treatments on the iron binding capacity of egg yolk phosvitin. *Food Chem.* 85:569-577.

Castellani, O., Martinet, V., Davied-Briand, E., Dubiard, G., and Anton, M. 2003. Egg yolk phosvitin: preparation of metal-free purified protein by fast protein liquid chromatography using aqueous solvents. *Journal of Chromatography B.* 791:273-284.

CDC. 2006. Annual Listing of Foodborne Disease Outbreaks, United States.

CDC. 2007a. Multistate Outbreak of Salmonella Serotype Tennessee Infections Associated with Peanut Butter --- United States, 2006-2007. *MMWR* 56:521-524. <http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5621a1.htm>

CDC. 2007b. Multistate Outbreaks of Salmonella Infections Associated with Raw Tomatoes Eaten in Restaurants --- United States, 2005-2006. *MMWR* 56:909-911. <http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5635a3.htm>

CDC. 2009. Multistate Outbreak of Salmonella Infections Associated with Peanut Butter and Peanut Butter--containing products --- United States, 2008-2009. *MMWR* 58: 1-6. <http://www.cdc.gov/mmwr/preview/mmwrhtml/mm58e0129a1.htm>

CDC, 2011. "CDC - Outbreaks Involving Salmonella." *Centers for Disease Control and Prevention*. Web. 24 Mar. 2011. <<http://www.cdc.gov/salmonella/outbreaks.html>>

Centers for Disease Control Surveillance Summaries 1993–1997, *MMWR*, U.S. DHHS, Atlanta, GA. Centers for Disease Control and Prevention, 2000, 49, #SS-1.

Chami, F., Chami, N., Bennis, S., Bouchikhi, T., and Remmal, A. 2005. Oregano and clove essential oils induce surface alteration of *Saccharomyces cerevisiae*. *Phytother. Res.* 19:405-408.

Cheeseman, G. C. and Berridge, N. J. 1957. An improved method of preparing nisin. *Biochem. J.* 65:603-608.

Choi, I., Jung, C., Choi, H., Kim, C., and Ha, H. 2005. Effectiveness of phosvitin peptides on enhancing bioavailability of calcium and its accumulation in bones. *Food Chem.* 93:577-583.

Chun, W. and Hancock, R.E. 2000. Action of lysozyme and nisin mixtures against lactic acid bacteria. *Int. J. Food Microbiol.* 60:25-32.

Cizke, A., Alexa, P., Literak, I., Hamrik, J., Novaak, P., and Smola, J. 1999. Shiga toxin-producing *Escherichia coli* O157:H7 in feedlot cattle and Norwegian rats from a large-scale farm. *Lett. Appl. Microbiol.* 28:435-439.

- Clark, R.C. 1985. The primary structure of avian phosvitins (contributions through the edman degradation of methylmercaptovitins prepared from the constituent phosphoproteins). *Int. J. Biochem.* 17:983-988.
- Cody, S.H., Glynn, M.K., Farrar, J.A., Cairns, K.L., Griffin, P.M., Kobayashi, J., Fyfe, M., Hoffman, R., King, A.S., Lewis, J.H., Swaminathan, B., Bryant, R.G., and Vugia, D.J. 1999. An outbreak of *Escherichia coli* O157:H7 infection from unpasteurized commercial apple juice. *Ann-Intern-Med.* 130(3):202-209.
- Cohen, M.B. 1996. *Escherichia coli* infections: a frequent cause of bloody diarrhea and the hemolytic-uremic syndrome. *Adv. Pediatr.* 43:171-207.
- Connelly, C., and Taborsky, G. 1961. Chromatographic fractionation of phosvitin. *J. Biol. Chem.* 236:1364-1368.
- Cosentino, S., Tuberoso, C.I.G., Pisano, B., Satta, M., Mascia, V., Arzedi, E., Palmas, F., 1999. In vitro antimicrobial activity and chemical composition of Sardinian Thymus essential oils. *Lett. Appl. Microbiol.* 29:130-135.
- Cowan, M.M. 1999. Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* 12:564-582.
- Cray, W.C., Jr., and Moon, H.W. 1995. Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 61:1586-1590.
- Cummings, K., Barrett, E., Mohle-Boetani, J.C., Brooks, J.T., Farrar, J., Hunt, T., Fiore, A., Komatsu, S., Werner, B., and Slutsker, L. 2001. A multistate outbreak of *Salmonella enterica* serotype Baildon associated with domestic raw tomatoes. *Emerg. Infect. Dis.* 7: 1046-1048.
- Cutter C.N. and Siragusa G.R. 1996. Reduction of *Brochothrix thermosphacta* on beef surfaces following immobilization of nisin in calcium alginate gels. *Lett. App. Microbiol.* 23:9-12.
- Dack, G.M. 1956. The role of enterotoxin of *Micrococcus pyogenes* var. *aureus* in the etiology of pseudomembranous enterocolitis. *Am. J. Surg.* 92:765-769.
- D'Aoust, J.-Y. 1989. *Salmonella*. In M.P. Doyle (ed.), *Foodborne Bacterial Pathogens*. Marcel Dekker, Inc., New York, N.Y. p. 327-445.
- D'Aoust, J.-Y. 1991. Pathogenicity of foodborne *Salmonella*. *Int. J. Food Microbiol.* 12:17-40.
- D'Aoust, J.-Y. 1994. *Salmonella* and the international food trade. *Int. J. Food Microbiol.* 24: 11-31.

- D'Aoust, J.-Y., Sewell, A.M., Daley, E. and Greco, P. 1992. Antibiotic resistance of agricultural and foodborne *Salmonella* isolates in Canada: 1986-1989. *J. Food Prot.* 55: 428-434.
- De Vlaming, V.L. Wiley, H.S. Delajunty, G. and Wallace, R.A. 1980. Goldfish (*Carassius auratus*) vitellogenin: induction, isolation, properties and relationship to yolk proteins. *Comp. Biochem. Physiol.* 67B:613-623.
- De Vos, W.M., Mulders, J.W., Hugenholtz, J., and Kuipers O.P. 1993. Properties of nisin Z and distribution of its genes, *nisZ*, in *Lactococcus lactis*. *Appl. Environ. Microbiol.* 59:213-218.
- Dean-Nystrom, E.A., Bosworth, B.T., Cray, W.C., Jr., and Moon, H.W. 1997. Pathogenicity of *Escherichia coli* O157:H7 in the intestines of neonatal calves. *Infect. Immun.* 65: 1842-1848.
- Delves- Broughton, J., Blackburn, P., Evans, R. E., and Hugenholtz, J. 1996. Applications of the bacteriocin, nisin. *Anton. von Leeuwen.* 69:193-202.
- Delves-Broughton, J. and Gasson, M. J. 1994. Nisin. In *Natural Antimicrobial Systems and Food Preservation.* eds. V. M. Dillon and R. G. Board, pp. 99-131. UK: CAB International.
- Devriese, L.A. 1984. A simplified system for biotyping *Staphylococcus aureus* strains isolated from different animal species. *J. Appl. Bacteriol.* 56:215-220.
- Di Pasqua, R., Betts, G., Hoskins, N., Edwards, M., Ercolini, D., and Mauriello, G. 2007. Membrane toxicity of antimicrobial compounds from essential oils. *J. Agric. Food Chem.* 55:4863-4870.
- Dinges, M.M., Orwin, P.M. and Schlievert, P.M. 2000. Enterotoxins of *Staphylococcus aureus*. *Clin. Microbiol. Rev.* 13:16-34.
- Dorman, H. J. D., and S. G. Deans. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J. Appl. Microbiol.* 88:308-316.
- Doyle, M. P., Beuchat, L. R., and T. J. Montville (eds.). 1997. *Food Microbiology: Fundamentals and Frontiers.* ASM Press, Washington, DC.
- Drams, S., Dehoux, P., and Cossart, P. 1993. Common features of Gram-positive bacterial proteins involved in cell recognition. *Mol. Microbiol.* 9:1119-1122.
- Dutreux, N., Notermans, S., Góngora-Nieto, M.M., Barbosa-Cánovas, G.V., and Swanson, B.G. 2000. Effects of combined exposure of *Micrococcus luteus* to nisin and pulsed electric fields. *Int. J. Food Microbiol.* 60:147-152.

- Elder, R.O., Keen, J.E., Siragusa, G.R., Barkocy-Gallagher, G.A., Koohmaraie, M., and Laegreid, W.W. 2000. From the cover: correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Proc. Natl. Acad. Sci. USA* 97: 2999-3003.
- Evenson, M.L., Hinds, M.W., Bernstein, R.S., Bergdoll, M.S. 1988. Estimation of human dose of staphylococcal enterotoxin A from a large outbreak in staphylococcal food poisoning involving chocolate milk. *Int. J. Food Microbiol.* 7:311–316.
- FAO/WHO Expert Committee on Food Additives. 1969. Specifications for identity and purity of some antibiotics. 12th Report. WHO Technical Report Series, No. 430.
- Farber, J. M., Coates, F., and Daley, E. 1992. Minimum water activity requirements for the growth of *Listeria monocytogenes*. *Lett. Appl. Microbiol.* 15:103-105.
- Farber, J.M. and Peterkin, P.I. 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Mol. Biol. Rev.* 55:476-511.
- FDA (Food and Drug Administration). 1988. Nisin preparation: Affirmation of GRAS status as a direct human food ingredient. *Fed. Reg.* 53: 11247.
- Fenlon, D. R., J. Wilson, and W. Donachie. 1996. The incidence and level of *Listeria monocytogenes* contamination of food sources at primary production and initial processing. *J. Appl. Bacteriol.* 81:641–650.
- Firstenberg-Eden, R. and Sullivan, N.M. 1997. EZ coli rapid detection system: a rapid method for the detection of *Escherichia coli* O157 in meat and other foods. *J. of Food Prot.* 60:219-225.
- Fitzgerald, D.J., Stratfore, M., Gasson, M.J., Ueckert, J., Bos, A., and Narbad, A. 2004. Mode of antimicrobial action of vanillin against *Escherichia coli*, *Lactobacillus plantarum* and *Listeria innocua*. *J. Appl. Microbiol.* 97:104-113.
- Fleming, D.W., Cochi, S.L., MacDonald, K.L., Brondum, J., Hayes, P.S., Plikaytis, B.D., Holmes, M.B., Audurier, A., Broome, C.V., and Reingold, A.L. 1985. Pasterurized milk as a vehicle of infection in an outbreak of listeriosis. *New Engl. J. Med.* 404-407.
- Foster, J.W. and Spector, M.P. 1995. How *Salmonella* survive against the odds. *Annu. Rev. Microbiol.* 49:145-174.
- Freney, J., Brun, Y., Bes, M., Meugnier, H., Grimont, F., Grimont, P.A.D., Nervi, C., and Fleurette, J. 1988. *Staphylococcus lugdunensis* sp. nov. and *Staphylococcus schlegeleri* sp. nov., two species from human clinical specimens. *Int. J. Syst. Bacteriol.* 38:168-172.

- Friedman, M., Henika, P.R., and Mandrell, R.E. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. J. Food Prot. 65:1545-1560.
- Friedman, M., Henika, P.R., Levin, C. E., and Mandrell, R.E. 2004. Antibacterial activities of plant essential oils and their components against *Escherichia coli* O157:H7 and *Salmonella enterica* in apple juice. J. Agric. Food Chem. 52:6042-6048.
- Friedman, M., L. Zhu, Y., Feinstein, and Ravishankar, S. 2009. Carvacrol facilitates heat-induced inactivation of *Escherichia coli* O157:H7 and inhibits formation of heterocyclic amines in grilled ground beef patties. J. Agric. Food Chem. 57:1848-1853.
- Fukase, K. Kitazawa, M., Sano, A., Shimbo, K., Fujita, H., Horimoto, S., Wakamiya, T., and Shiba, T. 1988. Total synthesis of peptide antibiotic nisin. Tetr. Lett: 29:795-798.
- Gaillard, J.L. Berche, P., Frehel, C., Gouin, E., and Cossart, P. 1991. Entry of *Listeria monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens form Gram-positive cocci. Cell. 65:1127-1141.
- Galvez, A., Abriouel, H., Lopez, R.L., and Omar, N.B. Bacteriocin-based strategies for food biopreservation. Int. J. Food Microbiol. 120:51-70.
- Gao, F.H., Abee, T., and Konings, W.N. 1991. Mechanism of action of the peptide antibiotic nisin in liposomes and cytochrome c oxidase-containing proteoliposomes. Appl. Environ. Microbiol. 57:2164-70.
- Gaysinsky, S., Davidson, P.M., Bruce, B.D., and Weiss, J. 2005. Growth inhibition of *Escherichia coli* O157:H7 and *Listeria monocytogenes* by carvacrol and eugenol encapsulated in surfactant micelles. J. Food Prot. 68: 2259-2566.
- Gellin, B.G and Broome, C.V. 1989. Listeriosis. JAMA. 261: 1313-1320.
- Gill, A.O., and Holley, R.A. 2006. Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant oil aromatics. Int. J. Food Microbiol. 108:1-9.
- Goulas, A. Triplett, E.L., and Taborsky, G. 1996. Oligophosphopeptides of varied structural complexity derived from the egg phosphoprotein, phosvitin. J. Protein Chem. 15:1-9.
- Greengard, O., Sentenac, A., and Mendelsohn, N.1964. Phosvitin, the iron carrier of egg yolk. Biochim. Biophys. Acta. 90:406-407.
- Griffin, P.M. 1995. *Escherichia coli* O157:H7 and other enterhemorrhagic *Escherichia coli*,

- p. 739-761. In M.J. Blaser, P.D. Smith, J.I. Ravdin, H.B. Grennberg and R.L. Guerrant (ed.), *Infections of Gastrointestinal Tract*. Raven Press, New York, N.Y.
- Griffin, P.M., Bell, B.P., Cieslak, P.R., Tuttle, J., Barrett, T.J., Doyle, M.P., McNamara, A.M., Shefer, A.M., and Wells, J.G. 1994. Large outbreak of *Escherichia coli* O157:H7 infections in the western United States: the big picture. In: Recent Advances in Verocytotoxin-producing *Escherichia coli* Infections (Karmali, M.A. and Goglio, A.G., Eds.), pp. 7–12. Elsevier, Amsterdam.
- Grizzuti, K. and Permann, G.E. 1973. Binding of magnesium and calcium ions to the phosphoglycoprotein phosvitin. *Biochemistry*. 12:4399-4403.
- Grogan, J., and Taborsky, G. 1987. Iron binding by phosvitin: variable mechanisms of release by phosvitin of diverse species characterized by different degrees of phosphorylation. *J. Inorg. Biochem.* 29:33-47.
- Gross, E. and Morell, J.L. 1971. The structure of nisin. *J. Am. Soc.* 93:4634-4635.
- Guerin-Dubiard, C. Anton, M., Dhene-Garcia, A., Martinet, V., and Brule, G. 2002. Hen egg and fish egg phosvitins: composition and iron binding properties. *Eur. Food Res. Technol.* 214:460-464.
- Hammer, K. A., C. F. Carson, and T. V. Riley. 1999. Antimicrobial activity of essential oils and other plant extracts. *J. Appl. Microbiol.* 86:985–990.
- Hanlin, M.B., Kalchayanad, N., Ray, P. and Ray, B.1993. Bacteriocins of lactic acid bacteria in combination have greater antibacterial activity. *J. Food Protect.* 56:252–255.
- Hard-English, P., York, G. Stier, R., and Cocotas, P.1990. Staphylococcal food poisoning outbreaks caused by canned mushrooms from China. *Food Technol.* 44(12): 74.
- Hasper, H. E., Kramer, N. E., Smith, J. L., Hillman, J. D., Zachariah, C., Kuipers, O. P., de Kruijff, B. and Breukink, E. 2006. An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. *Science*. 313:1636-1637.
- Heald, P.J. and McLachlan, P.M. 1963. Isolation of phosvitin from the plasma of the laying hen. *J. Biochem.* 87:571-576.
- Hedberg, C.W., Angulo, F.J., White, K.E., Langkop, C.W., Schell, W.L., Stobierski, M.G., Schuchat, A., Besser, J.M., Dietrich, S., Helsel, L., Griffin, P.M., McFarland, J.W., and Osterholm, M.T. 1999. Outbreaks of salmonellosis associated with eating uncooked tomatoes: implications for public health. The Investigation Team. *Epidemiol. Infect.* 122: 385-393.
- Hegenauer, J., Sltman, P., and Nace, G. 1979. Iron (III)-phosphoprotein chelates:

stoichiometric equilibrium constant for interaction of iron (III) and phosphorylserine residues of phosvitin and casein. *Biochemistry*. 18:3865-3879.

Helander, I.M., Alakomi, H., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E.J., Gorris, L.G.M. and von Wright, A. 1998. Characterization of the action of selected essential oil components on Gram-negative bacteria. *J. Agr. Food Chem.* 46:3590–3595.

Hirsch, A., Grinsted, E., Chapman, H. R., and Mattick, A. 1951. A note on the inhibition of an anaerobic sporeformer in Swiss-type cheese by a nisin-producing *Streptococcus*. *J. Dairy Res.* 18: 205.

Holmberg, S. D., and Blake, P. A. 1984. Staphylococcal food poisoning in the United States. *J. Amer. Med. Assoc.* 251:487-489.

Holmberg, S.D. and Blake, P.A. Staphylococcal food poisoning in the United States. New facts and old misconceptions. *J. Am. Med. Ass.*, 251, 487, 1984.
http://www.cdc.gov/foodborneoutbreaks/documents/2006_line_list/2006_line_list.pdf

Hurst, A. 1981. Nisin. *Adv. Appl. Microbiol.* 27: 85-123.

Hurst, A., and Hoover, D. G. 1993. Nisin. In *Antimicrobials in Foods*. eds. P. M. Davidson, and A. L. Branen, pp. 369-394. 2nd Edition. New York: Marcel Dekker.

ICMSF. 1996. Characteristics of microbial pathogens –*Listeria monocytogenes*. In: *Microorganisms in Foods 5*. Pp 141–182. London: Blackie Academic and Professional.

Ishikawa, S., Yano, Y., Arihara, K., and Itoh, M. 2004. Egg yolk phosvitin inhibits hydroxyl radical formation from the fenton reaction. *Biosci. Biotechnol. Biochem.* 68:1324-1331.

Ishikawa, S.I., Asano, T., Takenoshita, S., Nozawa, Y., Arihara, K. and Itoh, M. 2009. Egg yolk proteins suppress azoxymethane-induced aberrant crypt foci formation and cell proliferation in the colon of rats. *Nutr. Res.* 29:64-69.

Ishikawa, S.I., Ohtsuki, S., Tomita, K., Arihara, K. and Itoh, M. 2005. Protective effect of egg yolk phosvitin against ultraviolet-light-induced lipid peroxidation in the presence of iron ions. *BioBTER*. 105:249-256.

Ishikawa, S.I., Tamaki, S., Arihara, K., and Itoh, M. 2007. Egg yolk protein and egg yolk phosvitin inhibit calcium, magnesium, and iron absorptions in rats. *J. Food Sci.* 72:S412-S419.

Itoh, T., Abe, Y., and Adachi, S. 1983. Comparative studies on the α and β -phosvitin from hen's egg yolk. *J. Food Sci.* 48:1755-1757.

Jaquette C.B. and Beuchat L.R. 1998. Combined effects of pH, nisin, and temperature on

growth and survival of psychrotrophic *Bacillus cereus*. J. Food Prot. 61:563-570.

Jarraud, S., Peyrat, M.A., Lim, A., Tristan, A., Bes, M., Mougél, C., Etienne, J., Vandenesch, F., Bonneville, M., and Lina, G. 2001. *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. J. Immunol. 166:669-677.

Jay, J.M., 2000. Modern Food Microbiology, sixth ed. Aspen Publishers, Inc., Gaithersburg, MD, p. 679.

Jay, J.M. 1992. Staphylococcal gastroenteritis In: JM Jay, ed. Modern Food Microbiology. New York: Van Nostrand Reinhold. pp. 455–478.

Jay, J. M., Loessner, M. J., and Golden, D. A. 2005. Modern Food Microbiology, 7th edition, Springer Science + Business Media, LLC, Pubs., New York, NY.

Jiang, B. and Mine, Y. 2000. Preparation of novel functional oligophosphopeptides from hen egg yolk phosvitin. J. Agric. Food Chem. 48:990-994.

Jiang, B. and Mine, Y. 2001. Phosphopeptides derived from hen egg yolk phosvitin: effect of molecular size on the calcium-binding properties. Biosci Biotechnol Biochem. 65:1187-1190.

Johnson, W.M., Lior, H., and Bezansons. 1983. Cytotoxic *Escherichia coli* O157:H7 associated with haemorrhagic colitis in Canada. Lancet i:76.

Joubert, F. J. and Cook, W. H. 1958. Preparation and characterization of phosvitin from hen egg yolk. Can. J. Physiol. Pharmacol. 36:399-408.

Juneja, V.K. and Friedman, M. 2007. Carvacrol, Cinnamaldehyde, Oregano oil, and thymol inhibit *Clostridium perfringens* spore germination and outgrowth in ground turkey during chilling. J. Food Prot. 70:218-222.

Juneja, V.K. and Friedman, M. 2008. Carvacrol and Cinnamaldehyde facilitate thermal destruction of *Escherichia coli* O157:H7 in raw ground beef. J. Food Prot. 71:1604-1611.

Juntilla, J.R., Niemela, S.I., and Hirn, J. 1988. Minimum growth temperatures of *Listeria monocytogenes* and nonhemolytic *Listeria*. J. Appl. Bacteriol. 65:321-327.

Karatzas, A.K., Kets, E.P.W., Smid, E.J., and Bennik, M.H.J. 2001. The combined action of carvacrol and high hydrostatic pressure on *Listeria monocytogenes* Scott A. J. Appl Microbiol. 90: 463-469.

Karch, H., Russman, H., Schmidt, H., Schwarzkopf, A., and Heesemann, J. 1995. Long-term

- shedding and clonal turnover of enterhemorrhagic *Escherichia coli* O157 in diarrheal diseases. *J. Clin. Microbiol.* 33:1602-1605.
- Karmali, M.A., Petric, M., Lim, C., Fleming, P.C., Arbus, G.S., and Lior, H. 1985. The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing *Escherichia coli*. *J. Infect. Dis.* 151:775-782.
- Katayama, S. Xu, X., Fan, M.Z. and Mine, Y. 2006. Antioxidative stress activity of oligophosphopeptides derived from hen egg yolk phosvitin in Caco-2 cells. *J. Agric. Food Chem.* 54:773-778.
- Kim, J., Marshall, M.R., Wei, C.I. 1995a. Antibacterial activity of some essential oil components against five foodborne pathogens. *J. Agric. Food Chem.* 43:2839-2845.
- Kim, J.M., Marshall, M.R., Cornell, J.A., Preston, J.F.III, Wei, C.I. 1995. Antibacterial activity of carvacrol, citral, and geraniol against *Salmonella typhimurium* in culture medium and on fish cubes. *J. Food. Sci.* 60:1364-1368.
- Kisko, G. and Roller, S. 2005. Carvacrol and p-cymene inactivate *Escherichia coli* O157:H7 in apple juice. *BioMed Central Microbiol.* 1-9.
- Klaenhammer, T.R. 1993. Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol. Rev.* 12:39-85.
- Kloos, W.E., and Schleifekr, H. 1986. Genus IV. *Staphylococcus* Rosenbach 1884, 1 gAL, (Nom. Cons. Opin. 17 Jud. Comm. 1958. 153). In: *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 101 3-1035. Edited by P. H. A. Sneath. Baltimore: Williams & Wilkins.
- Knight K.P., Bartlett F.M., McKellar R.C., and Harris L.J. 1999. Nisin reduces the thermal resistance of *Listeria monocytogenes* Scott A in liquid whole egg. *J. Food Prot.* 62:999-1003.
- Knowles, J.R., Roller, S., Murray, D.B., and Naidu, S. 2005. Antimicrobial action of carvacrol at different stages of dual-species biofilm development by *Staphylococcus aureus* and *Salmonella enterica* serovar Typhimurium. *Appl Environ. Microb.* 71:797-803.
- Komitopoulou, E., Boziaris, I. S., Davies, E. A., Delves-Broughton, J. and Adams, M. R. 1999. *Alicyclobacillus acidoterrestris* in fruit juices and its control by nisin. *Int.J. Food Sci. Techn.* 34:81-85.
- Kordel, M., Schuller, F., and Sahl, H. G. 1989. Interaction of the pore forming-peptide antibiotics Pep 5, nisin and subtilin with non-energized liposomes. *FEBS Letts.* 244:99-102.

- Kotula, K.L. and Kotula, A.W. 2000. Microbial ecology of different types of food-fresh red meats. In: B.M. Lund, T.C. Baird-Parker and G.W. Gould, Editors, *The microbiological safety and quality of food*, Aspen Publishers, Inc, Gaithersburg, MD, pp. 359-388.
- Koutosomanis, K., Lambropoulou, K., Nychas, G.J. 1999. A predictive model for the nonthermal inactivation of *Salmonella enteritidis* in a food model system supplemented with a natural antimicrobial. *Int. J. Food Microbiol.* 49:63-74.
- Kramer, N.E., Smid, E.J., Kok, J., de Kruijff, B., Kuipers, O.P., and Breukink, E. 2004. Resistance of Gram-positive bacteria to nisin is not determined by lipid II levels. *FEMS Microbiol Lett.* 239:157-161.
- Kuwano, K., Tanaka, N., Shimizu, T., Nagatoshi, K., Nou, S., and Sonomoto, K. 2005. Dual antibacterial mechanisms of nisin Z against Gram-positive and Gram-negative bacteria. *Int. J. Antimicrob. Agents.* 26:396-402.
- Kwon, J.A., Yu, C.B., and Park, H.D. 2003. Bactericidal effects and inhibition of cell separation of cinnamic aldehyde on *Bacillus cereus*. *Lett. Appl. Microbiol.* 37:61-65.
- La Storia, A., Ercolini, D., Marinello, F., Di Pasqua, R., Villani, F., Mauriello, G. 2011. Atomic force microscopy analysis shows surface structure changes in carvacrol-treated bacterial cells. *Res. Microbiol.* 162:164-172.
- Lacroix, M., Borsa, J., Chiasson, F., and Ouattara, B. 2004. The influence of atmosphere conditions on *Escherichia coli* and *Salmonella typhi* radiosensitization in irradiated ground beef containing carvacrol and tetrasodium pyrophosphate. *Radiat. Phys. Chem.* 71:59-62.
- Lagouri, V.G., Blekas, M., Tsimidou, S., Kokkini, S., and Boskou, D. 1993. Composition and antioxidant activity of essential oils from oregano plants grown wild in Greece. *Z. Lebensm Unters. For-schung.* 197:20-23.
- Lambert, R.J.W., Skandamis, P.N., Coote, P.J., and Nychas, G.-J.E. 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J. Appl. Microbiol.* 91:453-462.
- Landolo, J.J. 1989. Genetic analysis of extracellular toxins of *Staphylococcus aureus*. *Annu. Rev. Microbiol.* 43:375-402.
- Laridi, R., Kheadr, E.E., Benech, R.O., Vuilleumard, J.C., Lacroix, C., and Fliss, I. 2003. Liposome encapsulated nisin Z: optimization, stability and release during milk fermentation. *Int. Dairy J.* 13:325-336.
- Lauková A. 1995. Inhibition of ruminal staphylococci and enterococci by nisin in vitro. *Lett. Appl. Microbiol.* 20:34-36.

- Le Loir, Y., Baron, F. and Gautier, M. 2003. *Staphylococcus aureus* and food poisoning. *Genet. Mol. Res.* 2:63-76.
- Lee, J., and Kaletunç, G. 2010. Inactivation of *Salmonella Enteritidis* strains by combination of high hydrostatic pressure and nisin. *Int. J. Food Microbiol.* 140:49-56.
- Lee, S. H., and Kim, H. U. 1985. Studies on the antibiotic nisin produced by *Streptococcus lactis* IFO12007. *Korean J. Anim. Sci.* 27:480-483.
- Lee, S.K., Han, J.H., Decker, E.A. 2002. Antioxidant activity of phosvitin in phosphatidylcholine liposomes and meat model systems. *J. Food Sci.* 67:37-41.
- Lorber, B. 1997. Listeriosis. *Clin. Infect. Dis.* 24:1-11.
- Losso, J.N., Bogumil, R., and Nakai, S. 1993. Comparative studies of phosvitin from chicken and salmon egg yolk. *Comp. Biochem. Phys. B.* 106:919-923.
- Lu, C. and Baker, R.C. 1986. Characteristics of egg yolk as antioxidant for inhibiting metal-catalyzed phospholipid oxidations. *Poultry Sci.* 65:2065-2070.
- Lund, B.M., Baird-Parker, T.C., and Gould, G.W. (eds.). 2000. *The Microbial Safety and Quality of Food.* Aspen Publishers, Gaithersburg, MD.
- Mabuchi, N., Yamamura, J.-I., Adachi, T., Aoki, N., Nakamura, R., and Nakamura, T. 1996. *EMBL/GenBank/DDBJ databases* (P87498).
- MacDonald, C. L., O'Leary, L. M., Cohen, M. L., Norris, P., Wells, G., Noll, E., Kobayashi, J. M., and Blake, P. A. 1998. *Escherichia coli* O157:H7, an emerging gastrointestinal pathogen. *J. Am. Med. Assoc.* 259:3567-3570.
- Mangalassary, S., Han, I., Rieck, J., Acton, J., Jiang, X., Sheldon, B., and Dawson, P. 2007. Effect of combining nisin and/or lysozyme with in-package pasteurization on thermal inactivation of *Listeria monocytogenes* in ready-to-eat turkey bologna. *J. Food Prot.* 70:2503-2511.
- Mantovani, H.C., and Russell, J.B. 2001. Nisin resistance of *Streptococcus bovis*. *Appl. Environ. Microbiol.* 67:808–813.
- Martin, D. L., MacDonald, K.L, White, K. E. Soler, J. T., and Osterholm, M. T. 1990. The epidemiology and clinical aspects of the hemolytic uremic syndrome in Minnesota. *J. Am. Med. Assoc.* 323:1161-1166.
- Martin, S.E. and Myers, E.R. 1994. *Staphylococcus aureus*. In: YH Hiu, JR Gorham, KD

- Murrell, D.O. Cliver, eds. Foodborne Disease Handbook, Diseases Caused by Bacteria. New York: Marcel Dekker, 1994, pp. 345–394.
- Matsudomi, N., Ito, Kazunari, and Yoshika, Y. 2006. Preventive effect of egg yolk phosphovitin on heat-insolubilization of egg white protein and its application to heat-induced egg white gel. *Biosci. Biotechnol. Biochem.* 70:836-842.
- Mayo Clinic. (2007, April 12). *Salmonella*. Retrieved March 23, 2011 from *Mayo Clinic* Web site, <http://www.mayoclinic.com/health/salmonella/DS00926>.
- McAuliffe, O., Ross, R.P., and Hill, C. 2001. Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiol. Rev.* 25:285-308.
- Mead, P.S., Dunne, E.F., Graves, L., Wiedmann, G., Patrick, M., Hunter, S., Salehi, E., Mostashari, F., Craig, A., Mshar, P., Bannerman, T., Sauders, B.D., Hayes, P., Dewitt, W., Sparling, P., Griffin, P., Morse, D., Slutsker, L., Swaminathan, B., and for the Listeria Outbreak Working Group. 2006. Nationwide outbreak of listeriosis due to contaminated meat. *Epidemiol. Infect.* 134:744-751.
- Mead, P.S., Slutsker, Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., and Tauxe, R.V. 1999. Food-related illnesses and death in the United States. *Emerg. Infect. Dis.* 5:607-625.
- Mecham, D.K., and Olcott, H.S. 1949. Phosvitin, the principal phosphoprotein of egg yolk. *J. Am. Chem. Soc.* 74:3670-3676.
- Meljlholm, O. and Dalgaard, P. 2002. Antimicrobial effect of essential oils on the seafood spoilage microorganism *Photobacterium phosphoreum* in liquid media and fish products. *Lett. Appl. Microbiol.* 34:27-31.
- Mena, C., Almeida, G., Carneiro, L., Teixeira, P., Hogg, T. and Gibbs, P.A. 2004. Incidence of *Listeria monocytogenes* in different food products commercialized in Portugal. *Food Microbiol.* 21:213–216.
- Millette, M., Smoragiewicz, W., and Lacroix, M. 2004. Antimicrobial potential of immobilized *Lactococcus lactis subsp. lactis* ATCC 11454 against selected bacteria. *J. Food Prot.* 67:1184-1189.
- Moll, G.N., Clark, J., Chan, W.C., Bycroft, B.W., Roberts, G.C., Konings, W.N., and Driessen, A.J. 1997. Role of transmembrane pH gradient and membrane binding in nisin pore formation. *J. Bacteriol.* 179:135-40.
- Moll, G.N., Roberts, G.C., Konings, W.N., and Driessen, A.J. 1996. Mechanism of lantibiotic-induced pore-formation. *Antonie Van Leeuwenhoek.* 69:185-91.

- Morris, E.R. and Greene, F.E. 1972. Utilization of the iron of egg yolk for hemoglobin formation by the growing rat. *J. Nutr.* 102:901-908.
- Mulders, J. W. M., Boerrigter, I. J., Rollema, H. S., Siezen, R. J., and de Vos, W. M. 1991. Identification and characterization of the lantibiotic nisin Z, a natural nisin variant. *Eur. J. Biochem.* 201:581-584.
- Murray, P.R., Baron, E.J., Pfaller, M.A., Tenover, F.C., and Tenover, R.H. (eds). 1999. *Manual of Clinical Microbiology*, 7th ed. ASM Press, Washington DC.
- Natvig, E.E., Ingham, S.C., Ingham, B.H., Cooperband, L.R., and Roper, T. R. 2002. *Salmonella enterica* serovar Typhimurium and *Escherichia coli* contamination of root and leaf vegetables grown in soils with incorporated bovine manure. *Appl. Environ. Microb.* 68: 2737-2744.
- Nazer, A.I., Kobilinsky, A., Tholozan, J.-L., and Dubois-Brissonnet, F. 2005. Combinations of food antimicrobials at low levels to inhibit the growth of *Salmonella* sv. Typhimurium: a synergistic effect?. *Food Microbiol.* 22:391-398.
- Obaidat, M.M. and Frank, J.F. 2009. Inactivation of *Escherichia coli* O157:H7 on the intact and damaged portions of lettuce and spinach leaves by using allyl isothiocyanate, carvacrol, and cinnamaldehyde in vapor phase. *J. Food Prot.* 72:2046-2055.
- Okereke, A., and Montville, T.J. 1992. Nisin dissipates the proton motive force of the obligate anaerobe *Clostridium sporogenes* PA 3679. *Appl. Environ. Microb.* 58:2463-2467.
- Olasupo, N.A., Fitzgerald, D.J., Narbad, A., and Gasson, M.J. 2004. Inhibition of *Bacillus subtilis* and *Listeria innocua* by nisin in combination with some naturally occurring organic compounds. *J. Food Prot.* 67:596-600.
- Omoe, K. et al. 2002. Detection of *seg*, *seh*, and *sei* genes in *Staphylococcus aureus* isolates and determination of the enterotoxin productivities of *S. aureus* isolates harboring *seg*, *seh*, or *sei* genes. *J. Clin. Microbiol.* 40: 857.
- Orskov, F., Orskov, I., and Villar, J.A. 1987. Cattle as reservoir of verotoxin-producing *Escherichia coli* O157:H7. *Lancet* ii:276.
- Osaki, S., Sexton, R.C., Pascual, E., and Frieden, E. 1975. Iron oxidation and transferrin formation by phosvitin. *J. Biochem.* 151:519-525.
- Oussalah, M., Caillet, S., Saucier, L., and Lacroix, M. 2007. Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli* O157:H7, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes*. *Food Control.* 18:414-420.

- Ousslah, M., Caillet, S., and Lacroix, M. 2006. Mechanism of action of Spanish oregano, Chinese cinnamon, and savory essential oils against cell membranes and walls of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. J. Food Prot. 69: 1046-1055.
- Parente, E., Giglio, M.A., Ricciardi, A., and Clemente, F. 1998. The combined effect of nisin, leucocin F10, pH, NaCl and EDTA on the survival of *Listeria monocytogenes* in broth. Int J. Food Microbiol. 40:65-75.
- Pawar, D.D., Malik, S.V.S., Bhilegaonkar, K.N., Barbuddhe, S.B., 2000. Effect of nisin and its combination with sodium chloride on the survival of *Listeria monocytogenes* added to raw buffalo meat mince. Meat Sci. 56:215-219.
- Pei, R-S., Zhou, F., J., B-P., and Xu, J. 2009. Evaluation of combined antibacterial effects of eugenol, cinnamaldehyde, thymol, and carvacrol against *E. coli* with an improved method. J. Food Sci. 74: M379-M383.
- Perez-Conessa, D., Cao, J., Chen, L., Mclandsborough, L., and Weiss, J. 2011. Inactivation of *Listeria monocytogenes* and *Escherichia coli* O157:H7 biofilms by micelle-encapsulated eugenol and carvacrol. J. Food Prot. 74:55-62.
- Periago, P.M., Delgado, B., Fernandez, P.S. and Palop, A. 2004. Use of carvacrol and cymene to control growth and viability of *Listeria monocytogenes* cells and predictions of survivors using frequency distribution functions. J. Food Prot. 67:1405-1416.
- Pol, I.E. and Smid, E.J. 1999. Combined action of nisin and carvacrol on *Bacillus cereus* and *Listeria monocytogenes*. Lett Appl. Microbiol. 29:166-170.
- Porwollik, S., Boyd, E.F., Choy, C., Cheng, P., Florea, L., Proctor, E., and McClelland, M. 2004. Characterization of *Salmonella enterica* Subspecies I Genovars by use of microarrays. J. Bacteriol. 186:5883-5898.
- Poulose AJ, Croteau R. 1978. Biosynthesis of aromatic monoterpenes: conversion of g-terpinene to p-cymene and thymol in *Thymus vulgaris* L. Arch. Biochem. Biophys. 187: 307-314.
- Rajajee, S., Anandi, T.B., Subha, S., Vatsala, B.R. 1995. Patterns of resistant *Salmonella typhi* infection in infants. J. Trop. Pediatr. 41:52-54.
- Ramseier, H.R. 1960. The mode of action of nisin on *Clostridium butyricum*. Arch. Mikrobiol. 37:57-94.
- Ravishankar, S., Zhu, L., Reyna-Granados, J., Law, B., Joens, L., and Friedman, M. 2010. Carvacrol and cinnamaldehyde inactivate antibiotic-resistant *Salmonella enterica* in buffer and on celery and oysters. J. Food Prot. 73:234-240.

- Ray, B. 1992. Nisin of *Lactococcus lactis* subsp. *lactis* as a food preservative. In *Food Biopreservatives of Microbial Origin*. eds. B. Ray and M. A. Daeschel, pp. 207-264. USA: CRC Press.
- Ray, Bibek. "Foodborne Infections." *Fundamental Food Microbiology*. 3rd ed. Boca Raton: CRC, 2004. 371-74. Print.
- Ray, Bibek. "Foodborne Infections." *Fundamental Food Microbiology*. 3rd ed. Boca Raton: CRC, 2004. 362-366. Print.
- Remmal, A., Bouchikhi, T., Rhayour, K., Ettayebi, M., 1993. Improved method for the determination of antimicrobial activity of essential oils in agar medium. *J. Essent Oil Res* 5:179-184.
- Renugopalakrishnan, V., Horowitz, P.M., and Glimcher, M.J. 1985. Structural studies of phosvitin in solution and in the solid state. *J. Biol. Chem.* 260:11406-11413.
- Rocourt J, Jacquet C, Reilly A. 2000. Epidemiology of human listeriosis and seafoods. *Int. J. Food Microbiol.* 62:197–209.
- Rogers, L. A., and Whittier, E. O. 1928. Limiting factors in lactic fermentation. *J. Bacteriol.* 16: 211-229.
- Roller, S., and Seedhar, P. 2002. Carvacrol and cinnamic acid inhibit microbial growth in fresh-cut melon and kiwifruit at 4° and 8°C. *Lett. Appl. Microbiol*, 35:390-394.
- Rosenow, E. M., and Marth, E. H. 1986. Growth patterns of *Listeria monocytogenes* in skim, whole and chocolate milk and in whipping cream at 4, 13, 21, and 35o C. *J. Food Prot.* 49: 847-848.
- Ryser, E.T. and Marth E. H. 2007. *Listeria*, listeriosis, and food safety. 23rd edn. New York: Marcel Dekker Inc.; Foodborne listeriosis; pp. 305–356.
- Sahl, H.-G. 1991. Pore formation in bacterial membranes by cationic lantibiotics. In *Nisin and Novel Lantibiotics*, Jung, G. and Sahl, H.G., Eds., Escom, Leiden, The Netherlands. 347-358.
- Sahl, H.-G., Jack, R.W., and Bierbaum, G. 1995. Biosynthesis and biological activities of lantibiotics with unique post-translational modifications. *Eur. J. Biochem.* 230:827-853.
- Salyers, A. A., and Smith, D. D. 2002. *Bacterial Pathogenesis: A Molecular Approach*, 2nd edition. ASM Press, Washington, DC.
- Sattar Khan, M.A., Nakamura, S., Ogawa, M., Akita, E., Azakami, H., and Kato, A. 2000.

- Bactericidal action of egg yolk phosvitin against *Escherichia coli* under thermal stress. J. Agric. Food Chem. 48:1503-1506.
- Scallan, E., Hoekstra, R.M., Angulo, F.J, Tauxe, R.V., Widdowson, M-A., Roy, S.L., et al. 2011. Foodborne illness acquired in the United States-major pathogens. Emerg. Infect. Dis. [Epub ahead of print]
- Schlech, W.F., III, Lavigne, P.M., Bortolussi, R.A., Allen, A.C., Haldane, E.V., Wort, A.J., Hightower, A.W., Johnson, S.E., King, S.H., Nicholls, E.S., and Broome, C.V. 1983. Epidemic listeriosis – evidence for transmission by food. N. Engl. J. Med. 308:203-206.
- Schmidt, J.J. and Spero, L. 1983. The complete amino acid sequence of staphylococcal enterotoxin C1. J. Biol. Chem. 258:6300-6306.
- Schuchat, A., Deaver, K.A., Wenger, J.D., et al. 1992. Role of foods in sporadic listeriosis. I. Case-control study of dietary risk factors. J. Am. Med. Assoc. 267:2041–2045.
- Scott, W.J. Water relations of *Staphylococcus aureus* at 30°C. Aust. J. Biol. Sci., 6:549-1953.
- Shachar D, and Yaron S. 2006. Heat Tolerance of *Salmonella enterica* Serovars Agona, Enteritidis, and Typhimurium in Peanut Butter. J Food Prot. 11:2687-2691.
- Sikkema, J., de Bont, J.A.M. and Poolman, B. 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiol. Rev. 59:201-222.
- Sivropoulou, A., Papanikolaou, E., Nikolaou, C., Kokkini, S., Lanaras, T., and Arsenakis, M. 1996. Antimicrobial and cytotoxic activities of origanum essential oils. Journal of Agric Food Chem. 44:1201-1205.
- Smith-Palmer, A., Stewart, J., and Fye, L. 2001. The potential application of plant essential oils as natural food preservatives in soft cheeses. Food Microbiol. 18:463-470.
- Sofos, J.N., Beuchat, L., R., Davidson, M.P., and Johnson, E.A. 1998. Naturally occurring antimicrobials in food. Regul Toxicol Pharm. 28:71-72.
- Solomakos, N., Govaris, A., Koidis, P., and Botsoglou, N. 2008. The antimicrobial effect of thyme essential oil, nisin, and their combination against *Listeria monocytogenes* in minced beef during refrigerated storage. Food Microbiol. 25:120-127.
- Sundararajan, T. A., Sampath Kumar, K. S. V. and Sarma, P. S. 1960. A simplified procedure for the preparation of phosvitin and vitellin. Biochim. Biophys. Acta. 38:360-362.
- Taborsky, G. 1963. Interaction between phosvitin and iron and its effect on a rearrangement

- of phosvitin structure. *Biochemistry*. 2:266-271.
- Taborsky, G. 1974. Phosphoproteins. *Adv. Protein Chem.* 28:1-210.
- Taborsky, G. 1983. Phosvitin. *Adv. Inorg. Biochem.* 5:235-279.
- Taborsky, G. and Allende C.C. 1962. A rearrangement in the structure of phosvitin. *Biochemistry*. 1: 406-411.
- Taborsky, G. and Mok, C. 1967. Phosvitin homogeneity and molecular weight. *J. Biol Chem.* 242: 1495-1501.
- Teixeira, P., Lima, J., Azeredo, J., and Oliveira, R. 2008. Adhesion of *Listeria monocytogenes* to materials commonly found in domestic kitchens. *Int. J. Food Sci. Technol.* 43:1239-1244.
- Tereatanavat, R., and Hooker, N.H. 2004. Understanding the characteristics of United States meat and poultry recalls: 1994-2002. *Food Control*.15:359-367.
- Thomas L.V. and Wimpenny J.W. 1996. Investigation of the effect of combined variations in temperature, pH, and NaCl concentration on nisin inhibition of *Listeria monocytogenes* and *Staphylococcus aureus*. *Appl. Environ. Microbiol.* 62:2006-2012.
- Tsai, Y., and Ingham, S. C. 1997. Survival of *Escherichia coli* O157:H7 and *Salmonella* spp. in acidic condiments. *J. Food Prot.* 60:751-755.
- Ukuku, D.O., Zhang, H, and Huang, L. 2009. Growth parameters of *Escherichia coli* O157:H7, *Salmonella* spp., *Listeria monocytogenes*, and aerobic mesophilic bacteria of apple cider amended with nisin-EDTA. *Foodborne Pathog. Dis.* 6:487-94.
- Ultee, A. and Smid, E.J. 2001. Influence of carvacrol on growth and toxin production by *Bacillus cereus*. *Int. J. Food Microbiol.* 64:373-378.
- Ultee, A., Bennik, M.H.J., and Moezelaar, R. 2002. The phenolic hydroxyl group of carvacrol is essential for action against food-borne pathogen *Bacillus cereus*. *Appl. Environ. Microbiol.* 68:1561-1568.
- Ultee, A., Gorris, L.G.M., and Smid, E.J. 1998. Bactericidal activity of carvacrol towards the food-borne pathogen *Bacillus cereus*. *J. Appl. Microbiol.* 85:211-218.
- Ultee, A., Kets, E.P.W. and Smid, E.J. 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. *Appl. Environ. Microbiol.* 65:4606-4610.
- Ultee, A., Kets, E.P.W., Alberda, M., Hoekstra, F.A., and Smid, E.J. 2000a. Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol. *Arch. Microbiol.* 174:233-238.

- Ultee, A., Slump, R.A., Steging, G., and Smid, E.J. 2000b. Antimicrobial activity of carvacrol toward *Bacillus cereus* on rice. *J. Food Prot.* 63:620-624.
- US Department of Agriculture. 1987. *Testing for Listeria monocytogenes*. Federal Register. 52:7464.
- US Department of Agriculture. 1989. *Revised policy for controlling Listeria monocytogenes*. Federal Register 54:22345-6.
- Valero, M., and Salmeron, M.C. 2003. Antibacterial activity of 11 essential oils against *Bacillus cereus* in tyndallized carrot broth. *Int. J. Food Microbiol.* 85:73-81.
- Veldhuizen, E.J.A., Tjeerdsma-Van Bokhoven, J.L.M., Zweijtzter, C., Burt, S.A., and Haagsman, H.P. 2006. Structural requirements for the antimicrobial activity of carvacrol. *J. Agric. Food Chem.* 54:1874-1879.
- Veldhuizen, E.J.A., Creutzberg, T.O., Burt, S.A., and Haagsman, H.P. 2007. Low temperature and binding to food components inhibit the antibacterial activity of carvacrol against *Listeria monocytogenes* in steak tartare. *J. Food Prot.* 70:2127-2132.
- Vogel, H.J. 1983. Structure of hen phosvitin: A ^{31}P NMR, ^1H NMR, and laser photochemically induced dynamic nuclear polarization ^1H NMR study. *Biochemistry.* 22:668-674.
- Wachsmuth, I.K., Sparling, P.H., Barrett, T.J., and Potter, M.E. 1997. Enterohemorrhagic *Escherichia coli* in the United States. *FEMS Immunol. Med. Microbiol.* 18:233-239.
- Wallace, J., Cheasty, S., and Jones, K. 1997. Isolation of Vero cytotoxin-producing *Escherichia coli* O157 from wild birds. *J. Appl. Microbiol.* 82:299-404.
- Wallace, R.A. and Morgan, J.P. 1986. Chromatographic resolution of chicken phosvitin. *Biochem. J.* 240:871-878.
- Watkins, B.A. 1995. The nutritive value of the egg. In: Stadelman WI, Cottertill OI, editor. *Egg science and technology*. 4th ed. Binghamton, N.Y.: Food Products Press. P177-194.
- Webb, J., Multani, J.S., Saltman, P., Beach, N.A., Gray, H.B. 1973. Spectroscopic and magnetic studies of iron (III) phosvitins. *Biochemistry.* 12:1797-1802.
- Wells, J.G., Davis, B.B., Wachsmuth, I.K., Rile, L.W., Remis, R.S., Sokolow, R., and Morris, G.K. 1983. Laboratory investigation of hemorrhagic colitis outbreaks associated with a rare *Escherichia coli* serotype. *J. Clin. Microbiol.* 18:512-520.
- Wessels, S., Jelle, B., and Nes, I. F. 1998. *Bacteriocins of the Lactic Acid Bacteria: An*

Overlooked Benefit for Food. Danish Toxicology Centre, Denmark.

- Wiedemann, I., Benz, R., and Sahl, H-G. 2004. Lipid II-mediated pore formation by the peptide antibiotic nisin: a black lipid membrane study. *J. Bacteriol.* 186:3259-3261.
- Williams, N. C., and Ingham, S. C. 1997. Change in heat resistance of *Escherichia coli* O157:H7 following heat shock. *J. Food Prot.* 60:1128-1131.
- Wong, A.C.L. 1998. Biofilms in food processing environments. *J. Dairy Sci.* 81:2765-2770.
- World Health Organization. 1988. *Salmonellosis Control: the Role of Animal and Product Hygiene.*, Technical Report Series 774. World Health Organization, Geneva, Switzerland.
- Xu, J., Zhou, F., Ji, B.-P., Pei, R.-S., and Xu, N., 2008. The antibacterial mechanism of carvacrol and thymol against *Escherichia coli*. *Lett. Appl Microbiol.* 47:174-179.
- Xu, X., Katayama, S., and Mine, Y. 2007. Antioxidant activity of tryptic digests of hen egg yolk phosphovitin. *J. Sci. Food Agric.* 87:2604 -2608.
- Yamashoji, S., Yoshida, H. and Kajimoto, G. 1979. Effects of amino acids on the photosensitized oxidation of linolenic acid in an aqueous ethanol solution. *Agric. Biol. Chem.* 43:1505-1511.
- Yasui, S.C., Pancoska, P., Dukor, R.K., Keiderling, T.A., Renugopalakrishnan, V., Glimcher, M.J. and Clark, R.C. 1989. Conformational transitions in phosphovitin with pH variation. *J. Biol. Chem.* 265:3780-3788.
- Yuste, J., Mor-Mur, M., Capellas, M., Guamis, B. and Pla, R., 1998. Microbiological quality of mechanically recovered poultry meat treated with high hydrostatic pressure and nisin. *Food Microbiol.* 15:407-414.
- Zhao, T., Doyle, M.P., Shere, J., and Garber, L. 1995. Prevalence of enterohemorrhagic *Escherichia coli* O157:H7 in a survey of dairy herds. *Appl. Environ. Microbiol.* 61: 1290-1293.
- Zhou, F., Ji, B., Zhang, H., Jiang, H., Li, J.J., Li, J., Ren., Y. and Yan, W. 2007a. Synergistic effect of thymol and carvacrol combined with chelators and organic acids against *Salmonella Typhimurium*. *J. Food Prot.* 70:1704-1709.
- Zhou, F., Ji, B., Zhang, H., Jiang, H., Yang, Z., Li, J.J., Li, J., and Yan, W. 2007b. The antibacterial effect of cinnaldehyde, thymol, carvacrol and their combinations against the foodborne pathogen *Salmonella typhimurium*. *J. Food Safety.* 27:124-133.

CHAPTER 3

INFLUENCE OF PHOSVITIN, CARVACROL AND THEIR COMBINATION ON THE VIABILITY OF *SALMONELLA ENTERICA* AND *LISTERIA MONOCYTOGENES* IN ONION MUSHROOM SOUP HELD AT 12 °C OR 35 °C

Shecoya White¹, Aubrey Mendonca^{1*}, Aura Daraba³, and Dong Ahn²

¹Department of Food Science and Human Nutrition, and ²Department of Animal Science, Iowa State University, Ames IA, USA 50011; ³Faculty of Food Science and Engineering, University “Dunarea de Jos” Galati, Romania.

*Corresponding author: Department of Food Science and Human Nutrition, 2312 Food Sciences Bldg., Iowa State University, Ames, IA, USA 50011.

Phone: (515) 294-9078. Fax: (515) 294-8181. Email: amendon@iastate.edu.

Key words: *Listeria*, *Salmonella*, phosvitin, carvacrol, soup

Abstract

The antibacterial effect of phosvitin or carvacrol alone or combined, against *Salmonella enterica* and *Listeria monocytogenes* in brain heart infusion (BHI) broth and onion mushroom soup was investigated. Broth with added phosvitin (10, 20, 40, 60, 80 or 100 mg/ml), or carvacrol (0.09, 0.12, 0.14, 0.19, 0.38, or 0.75 mg/ml) was inoculated with *S. enterica* or *L. monocytogenes* to a final concentration of 5.0 log CFU/ml per pathogen. Broth or soup without antimicrobials served as control. Growth of *S. enterica* and *L. monocytogenes* in BHI (35 °C, 24 hours) was monitored using a Bioscreen C turbidometer (OD 600nm). Growth of the pathogens in soup (12 °C, 8 days) and (35 °C, 24 hours) was monitored by surface plating samples. In BHI broth the minimum inhibitory concentration (MIC) of phosvitin and carvacrol was 80 mg/ml and 0.14 mg/ml, respectively, for both *S. enterica* and *L. monocytogenes*. In soup (12 °C) all antimicrobial treatments tested decreased viable counts of both pathogens after 2 days of storage. In control soup viable numbers increased to 6.95 and 8.72 log CFU/ml at day 8 for *S. enterica* and *L. monocytogenes*, respectively. In soup (35 °C) viable counts of the pathogens were decreased by the higher concentration of carvacrol (0.40 mg/ml) and phosvitin (60 mg/ml) plus carvacrol concentrations (0.20 or 0.40 mg/ml). The combination of phosvitin (60 mg/ml) and carvacrol (0.40 mg/ml) exerted the greatest cidal effect throughout storage against *S. enterica* and *L. monocytogenes*.

Introduction

The growing number of foodborne illness-related outbreaks due to human enteric pathogens

is an increasing concern to regulatory agencies, the food industry, and consumers. While effective control of foodborne pathogens is crucial to ensuring microbial food safety, methods of control involving synthetic chemical preservatives are becoming less desirable to consumers. This consumer attitude is part of a growing trend towards “Green Consumerism” emphasizing the reduction or elimination of preservative agents such as nitrite and sodium chloride from foods (5) and encouraging the use of natural antimicrobials from animal, plant or microbial sources to preserve foods, promote healthier foods and improve food safety.

Phosvitin is a 35 kDa glycoprotein found exclusively in the granule fraction of chicken egg yolk, making up 7% of the yolk protein and 80% of the protein bound phosphorus (39, 49). This 217 amino acid peptide is composed of over 50% serine residues, of which 90% are phosphorylated (6, 7, 41). Phosvitin contains between 3 to 6 iron atoms per molecule depending on the method of isolation from hens’ eggs; however, it has a binding capacity of about 60 iron molecules (2, 26, 40, 51). Phosvitin is a strong metal chelator and could be used as an antimicrobial agent. To date there is only one published report demonstrating the antibacterial activity of phosvitin (37). Researchers found that the combination of phosvitin and thermal stress (50 °C) was bactericidal against *Escherichia coli*. It is likely that phosvitin sensitized this organism to the lethal effects of heat in a laboratory broth medium.

Herbs and spices have been used for centuries specifically for medicinal purposes, flavor, fragrances, and as natural preservative methods for a variety of foods (1, 8). The majority of essential oils are classified as Generally Recognized As Safe (GRAS). The phenolic compounds in the essential oil fraction of the plant possess antimicrobial properties

(4). Carvacrol is an essential oil found in the leaves and flowering plant of both thyme (*Thymus vulgaris*) and oregano (*Origanum vulgare*) (5, 31). The percent composition of carvacrol in oregano ranges from trace amounts to 82% and 2 to 45% in thyme (3, 5, 23). The hydrophobic phenolic compound in its structure is thought to be the main contributor to its antimicrobial properties (47). Several studies have demonstrated that carvacrol has both bacteriostatic and bactericidal activity against microorganisms including: *Listeria monocytogenes* (10, 12, 17, 31, 33, 34, 35, 38, 48), *Bacillus cereus* (35, 42-47), *Escherichia coli* O157:H7 (10-12, 14, 16, 29, 31-33, 52), *Staphylococcus aureus* (20, 24, 31), and *Salmonella enterica* (11, 14, 20, 22, 28, 36, 53). Carvacrol has been shown to possess stronger antimicrobial effect with increased temperature and storage time, and at a lower pH (5, 11).

The antimicrobial properties of both of these natural antimicrobials may be used to improve microbial safety of foods, with combined use possibly increasing the effectiveness of the antimicrobial treatments against foodborne pathogens. Also, synergy between two of these antimicrobials may permit the use of relatively lower amounts of each antimicrobial and thereby reducing cost of the antimicrobial treatment. While there is a growing body of knowledge on the antimicrobial effects of carvacrol against various foodborne pathogens, such published research involving phosvitin is scarce. To our knowledge there is no published research involving the combined use of phosvitin and carvacrol against *Salmonella enterica* and *Listeria monocytogenes*. Accordingly the objective of the present study was to evaluate the antimicrobial efficacy of phosvitin, carvacrol and their combinations in onion mushroom soup held at 12 °C or 35 °C.

Materials and Methods

Bacterial cultures and culture conditions. Five strains of *Listeria monocytogenes* (H7962-4b, H7762-4b, H7596-non4b, H7969-4b, and NADC-2045-4b) and five serotypes of *Salmonella enterica* (Enteritidis-ATCC13076, Heidelberg, Typhimurium-ATCC 14802, Gaminara-8324, Oranienburg-9329) were obtained from the culture collection of the Microbial Food Safety Laboratory, Iowa State University, Ames, IA. Stock cultures were kept frozen (-70 °C) in Brain Heart Infusion (BHI) broth (Difco, Becton, Dickinson, Sparks, MD) supplemented with 10% (vol/vol) glycerol. Stock cultures were activated in BHI broth (pH 7.2) and incubated at 35 °C. At least two consecutive 18 to 22-h transfers of each stock culture were carried out before using the cells as inocula in each experiment.

Preparation of Inoculum. An equal volume of the individual working cultures of *L. monocytogenes* or *S. enterica* 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 (American Laboratory Trading, Inc., East Lyme, CT) and washed once in 0.1% (wt/vol) peptone. The pelleted cells were suspended in fresh 0.1% (wt/vol) peptone to obtain a final viable cell concentration of approximately 10^9 CFU ml⁻¹. Viable counts of the washed cell suspensions were evaluated by surface plating serially diluted (10-fold) samples on tryptic soy agar supplemented with 0.6% yeast extract. The individual cell suspensions were used to inoculate BHI broth or commercial onion mushroom soup.

Antimicrobials. Phosvitin was supplied by Dr. Dong Ahn from the Department of Animal Science at Iowa State University, Ames, IA. A commercial preparation of carvacrol

was purchased from Sigma-Aldrich (Aldrich W224502).

Total phenolic content of carvacrol. The concentration of total phenolics of carvacrol was determined using the method described by Waterman and Mole (50). The assay involves reduction of ferric iron to the ferrous state by phenolic compounds that results in the formation of the Prussian blue complex $\text{Fe}_4[\text{Fe}(\text{CN})_6]_3$ with a potassium ferricyanide reagent. The Prussian blue complex was determined colorimetrically (18). Two separate 1% (vol/vol) solutions of carvacrol in 10% (vol/vol) ethanol and deionized water were prepared. The absorbance of three sub-samples from each solution was measured at 720 nm and the total phenolic content expressed as the number of catechin equivalents $\times 100$.

Preparation of treatment solutions for Bioscreen C Assay. BHI broth with added phosvitin (10, 20, 40, 60, 80, 100 mg/ml) or carvacrol (0.09, 0.12, 0.14, 0.19, 0.38, or 0.75 mg/ml) was filter sterilized using 0.22 μm pore size Millipore filters (Fisher Scientific, Pittsburgh, PA). Samples (2.5-ml) of the treatment solutions and control (BHI with no added antimicrobial) were each inoculated with 25 μl of diluted (1:100) *L. monocytogenes* or *S. enterica* cell suspension to obtain a final concentration of approximately 10^5 CFU/ml of sample.

Bioscreen C assay. Aliquots (200- μl) of inoculated samples were added in triplicate to the wells of microtiter plate for the Bioscreen C Turbidometer (Growth Curves, Piscataway, NJ), an automated microbial growth analyzer and incubator. Plates were incubated in the Bioscreen C at 35 °C for 24 h and the instrument was programmed to record optical density (OD) measurements at 600 nm every 30 min, with shaking of samples before

each OD reading. Minimum inhibitory concentration (MIC) was defined as the lowest treatment concentration that completely inhibited microbial growth for 24 hr (< 0.05 OD unit increase).

Preparation and inoculation of soup. Dehydrated onion mushroom soup/dip mix was purchased from a local grocery store. The soup mix was hydrated with sterile distilled water and prepared according to the directions stated on the package. Soup ingredients are shown in Table 1. After boiling, the soup was tempered to ~ 35 °C and 10-ml portions of the soup were aseptically transferred into sterile 30 ml screw cap Pyrex tubes. Phosvitin, carvacrol or combinations were added to each tube to yield the following concentrations: phosvitin (60 mg/ml), carvacrol (0.20 mg/ml or 0.40 mg/ml), phosvitin (60 mg/ml) + carvacrol (0.20 mg/ml) and phosvitin (60 mg/ml) + carvacrol (0.40 mg/ml). Phosvitin was added to the tube prior to the addition of the soup to prevent aggregation and facilitate its dissolving in the soup. Tubes of soup without added antimicrobial served as control. The treatments were then warmed or cooled to the target temperature. Tubes of soup with added antimicrobials were mixed by vortexing and inoculated with a suspension of washed cells of *S. enterica* or *L. monocytogenes* to generate a final cell concentration of approximately 10^5 CFU/ml for each pathogen. After inoculation, each tube of soup was gently mixed by vortexing and held at either 12 °C or 35 °C.

Microbiological analysis. Inoculated tubes, of soup held at 12 °C, were tested for survivors of the pathogens every 2 days for a total of 8 days whereas, soup, held at 35 °C, were tested at 4, 8, 12 and 24 h. Ten-fold serial dilutions of the soup were prepared using 0.1% (wt vol/vol) peptone with added Tween 80. Aliquots (1.0 ml or 0.1ml) of the soup or

diluted samples of soup were surface plated (in duplicate) on xylose lysine desoxycolate (XLD) agar (for *S. enterica*) and modified oxford medium (for *L. monocytogenes*). All inoculated plates were incubated at 35 °C for 48h before counting bacterial colonies.

Measurement of pH and water activity. 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 made using the Aqualab CX2 water activity meter (Decagon Services, Pullman, Washington). The initial pH and water activity of each sample of soup, including controls, was measured at day 0 (prior to inoculation). Measurements of pH were also taken periodically during storage of the soup at 12 °C or 35 °C.

Statistical analysis. Three independent replications of each experiment were performed. Mean numbers of viable *L. monocytogenes* or *S. enterica* were statistically analyzed using SAS statistical software version 8.1 (SAS Institute Inc., Cary, N.C.). Treatment means were evaluated for statistically significant differences using Tukey's test. Significant differences were defined at $P < 0.05$ for all the experimental data.

Results

Inhibitory effect of phosvitin in BHI broth. Figures 1 and 2 show the effect of various levels of phosvitin on the growth of *Salmonella enterica* and *Listeria monocytogenes*, respectively, in Brain Heart Infusion (BHI) broth at 35 °C. For *S. enterica* the optical density (OD) of control and BHI broth containing 10 mg/ml phosvitin increased rapidly and reached OD greater than 1.2 within 18 hours. Growth inhibition of *S. enterica* markedly increased

with higher phosvitin concentrations. Compared to control and 10 mg/ml, all concentrations of phosvitin extended the lag phase of the pathogen. Phosvitin concentrations of 80 and 100 mg/ml were bacteriostatic. The minimum inhibitory concentration (MIC) of phosvitin for *S. enterica* was 80 mg/ml.

Growth of *L. monocytogenes* increased rapidly in control broth; the OD of the culture increased from 0.1 (at 7h) to greater than 0.6 (at 18h). As observed with *Salmonella* all phosvitin concentrations inhibited growth of *L. monocytogenes* with higher concentrations causing greater growth inhibition. Greater extensions of the lag phase were observed with increased phosvitin concentrations. Phosvitin at 80 and 100 mg/ml was bacteriostatic to *L. monocytogenes* and the MIC of phosvitin for this pathogen was also 80 mg/ml.

Inhibitory effect of carvacrol in BHI broth. Growth of *S. enterica* and *L. monocytogenes* in BHI supplemented with various concentrations of carvacrol is shown in Figures 3 and 4, respectively. For *S. enterica* the OD of control increased from 0.1 to greater than 1.0 within 16 hours. Of all carvacrol concentrations tested, increases in OD were only observed in cultures with the two lowest concentrations (0.09 and 0.12 mg/ml). At 24 hours the OD values of control, and BHI broth with 0.09 mg/ml and 0.12 mg/ml were 1.03, 0.68 and 0.39, respectively. All other concentrations (0.14 to 0.75 mg/ml) of carvacrol completely inhibited growth of *S. enterica*. The MIC of carvacrol for *S. enterica* was 0.14 mg/ml. Except for 0.09 and 0.12 mg/ml, all other carvacrol concentrations tested completely prevented an increase in OD of *L. monocytogenes* cultures. Broth with 0.09 mg/ml had little or no effect on the growth of this pathogen; increases in OD were very similar to that of the control. The MIC of carvacrol for *L. monocytogenes* was 0.14 mg/ml.

Antibacterial effect of phosvitin and carvacrol in soup at 12 °C. The total phenolic content of the 1% solution of carvacrol was 0.98 catechin equivalents. This result coincides with the manufacturer's claim of "up to 98% total polyphenols". The initial pH of the soup was 6.1 and water activity 0.994. Tables 2 and 3 show viable counts of *S. enterica* and *L. monocytogenes*, respectively, in onion mushroom soup (12 °C) with phosvitin, carvacrol, or combinations of those antimicrobials. The initial viable count of *S. enterica* or *L. monocytogenes* in soup was $\sim 10^5$ CFU/ml. For both pathogens the initial viable counts in control soup declined slightly after two days of storage; 0.38- and 0.40- log decreases were observed for *S. enterica* and *L. monocytogenes*, respectively. All antimicrobial treatments tested decreased viable counts of both pathogens after two days of storage.

In control soup viable counts of *S. enterica* increased from 4.60 log CFU/ml (day 2) to 6.95 log CFU/ml at day 8 (Table 2). Phosvitin (60 mg/ml) or carvacrol (0.20 or 0.40 mg/ml) inhibited growth of this pathogen; viable counts (log CFU/ml) at day 8 of storage were 5.28, 4.99, and 2.27 respectively, in soup containing phosvitin (60 mg/ml), carvacrol (0.20 mg/ml) and carvacrol (0.40 mg/ml). The combination of phosvitin (60 mg/ml) and carvacrol (0.40 mg/ml) exerted the greatest cidal effect against *S. enterica* at day 2; viable counts were decreased by 3.91 log. From day 4 through day 8 viable counts of the pathogen were undetected (< 10 CFU/ml); however, results of enrichment tests performed on the soup samples were positive for the pathogen (data not shown).

Viable numbers of *L. monocytogenes* increased from 4.62 log CFU/ml (day 2) to 8.72 log CFU/ml at day 8 in control soup. In soup that contained phosvitin (60 mg/ml) initial numbers of the pathogen were decreased by 1.05 log at day 2 and survivors increased slowly

to reach 5.37 log CFU/ml at day 8. Carvacrol at 0.20 mg/ml reduced initial counts of *L. monocytogenes* by 1.1 log CFU/ml; however, survivors grew relatively fast and reached counts of 8.81 log CFU/ml at day 8. Viable counts (log CFU/ml) at day 8 for the remaining 4 treatments were 4.77 (0.40 mg/ml carvacrol), 4.38 (60 mg/ml phosvitin + 0.20 mg/ml carvacrol), and 3.17 (60 mg/ml phosvitin + 0.40 mg/ml carvacrol).

Antibacterial effect of phosvitin and carvacrol in soup at 35 °C. Log reduction in the initial viable count of *S. enterica* following 4 hours of exposure to antimicrobials in onion mushroom soup at 35 °C is shown in Figure 5. The pathogen grew in control soup and in soup with added phosvitin (60 mg/ml) or the lower concentration of carvacrol (0.20 mg/ml). Soup with carvacrol (0.40 mg/ml), 60 mg/ml phosvitin + 0.20 mg/ml carvacrol, or 60 mg/ml phosvitin + 0.40 mg/ml carvacrol exhibited a cidal effect on the pathogen; initial viable counts (control 7.63 log CFU/ml) were reduced by 5.48, 4.48, and 7.33, respectively ($P < 0.05$).

Tables 4 and 5 show the viability of *S. enterica* and *L. monocytogenes* respectively, in onion mushroom soup (35 °C) with added phosvitin (60 mg/ml), carvacrol (0.20 or 0.40 mg/ml) or their combinations. In control soup viable numbers of *S. enterica* increased from 7.63 log CFU/ml at 4 hours to 11.31 log CFU/ml at 24 hours. Compared to control, phosvitin significantly inhibited growth of the pathogen up to 12 hours at 35 °C ($P < 0.05$). Of the two concentrations of carvacrol tested the lower one (0.20 mg/ml) did not significantly inhibit growth of the pathogen in the soup for 24 hours ($P > 0.05$). In fact, viable counts (log CFU/ml) of the pathogen in soup with 0.20 mg/ml carvacrol increased from 6.39 at 4 hours to 10.14 in 24 hours (Table 4). However, when 0.20 mg/ml carvacrol was used in

combination with phosvitin, a much stronger inhibitory effect against the pathogen was observed compared to that produced by phosvitin or carvacrol (0.20 mg/ml) used singly ($P<0.05$). The extent of growth inhibition produced by that combination of phosvitin and carvacrol was similar to that observed with the higher concentration of carvacrol (0.40 mg/ml). Phosvitin combined with 0.40 mg/ml carvacrol exhibited the greatest cidal effect against *S. enterica* ($P<0.05$); no viable counts of the pathogen could be detected in the soup from 8 to 24 hours (Table 4). In all instances when no viable counts were detected by plating, enrichment tests gave positive results for presence of the pathogen (data not shown).

Viable numbers of *L. monocytogenes* in control soup increased from 4.60 log CFU/ml at 4 hours to 8.01 log CFU/ml at 24 hours (Table 5). Phosvitin suppressed the growth of *L. monocytogenes* throughout the duration of the study and its inhibitory effect was significantly greater from that of the control from 8 to 24 hours ($P<0.05$). Compared to control, carvacrol (0.20 mg/ml) alone did not significantly inhibit growth of *L. monocytogenes* ($P>0.05$). The higher concentration of carvacrol (0.40 mg/ml) and both phosvitin/carcacrol combinations tested significantly inhibited growth of the pathogen from 8 to 24 hours ($P<0.05$).

Phosvitin plus carvacrol (0.20 mg/ml) was able to suppress growth of the pathogen until 24 hours of incubation. The combination of phosvitin and carvacrol (0.40 mg/ml) was significantly different from the control after 4 hours. This combination caused a decline in viability of the pathogen in the soup at 35 °C which is highly conducive to prolific growth of the pathogen throughout storage.

Discussion

The application of naturally derived antimicrobials in foods is gaining increasing

attention from the food industry as consumers become concerned over possible negative health effects from long-term consumption of synthetic food preservatives. Certain naturally-derived organic compounds have good potential for use in inhibiting microbial growth or destroying foodborne organisms to extend the microbial shelf-life of foods (27). Based on the results of the present study of phosvitin used alone or combined with carvacrol to control foodborne pathogens in a food product offers further evidence on the antibacterial potential of these compounds.

While there is a growing body of knowledge on the antimicrobial effects of carvacrol, there is a scarcity of published reports on the antimicrobial effects of phosvitin. Also, there are no published reports on the combined antibacterial effectiveness of phosvitin and carvacrol against foodborne human enteric pathogens. In the present study phosvitin strongly inhibited growth of both *S. enterica* and *L. monocytogenes* in BHI broth at 35 °C (Figures 1 and 2). Plate counts to determine numbers of viable pathogens in BHI with phosvitin at the minimum inhibitory concentration (MIC) for each organism (80 mg/ml) revealed that phosvitin exerted a bacteriostatic effect on both pathogens (data not shown). This observed inhibition is likely due to phosvitin binding divalent metal ions such as iron (30) which is an essential mineral for microbial growth (15). Approximately ninety-five percent of iron in hen's egg yolk is strongly bound by phosvitin in a stable conformation to avoid being used by microbes (13). Such iron ligation can result in suppression of microbial growth in the hen's egg to protect the developing chick embryo from microbial attack.

Compared to phosvitin, carvacrol completely inhibited growth of *S. enterica* and *L. monocytogenes* at a far lower concentration (MIC = 0.014 mg/ml) (Figs 3 and 4). Plate

counts on cultures of the pathogens in BHI broth with MIC of carvacrol revealed that this antimicrobial was bactericidal; viable numbers of both pathogens decreased over time (data not shown). The antibacterial effectiveness of carvacrol against *S. enterica* and *L. monocytogenes* has been previously reported (17, 35, 36, 53). The MIC of carvacrol observed for *S. enterica* in the present study is within the same range reported in previous studies (28) although higher MICs have been reported (5). Variations in MICs may be attributed to variables such as strain of the organisms tested, type of growth medium and temperature, and potency of the antimicrobial.

The main focus of this study was to determine the antimicrobial activity of phosvitin combined with carvacrol against *S. enterica* and *L. monocytogenes* in an actual food system namely, onion mushroom soup. Considering the fact that improper temperature control can compromise the microbial safety of foods we intentionally held the soup at two abusive temperatures (12 °C and 35 °C). At those temperatures the antimicrobial efficacy of phosvitin alone or combined with carvacrol was evaluated. Holding soup at 12 °C simulates possible temperature conditions in a defective refrigerator that fails to maintain safe temperatures (≤ 4.4 °C). Holding the soup at 35 °C simulates improper temperature control during hot holding especially if there is a failure of hot holding equipment to maintain temperatures at 60 °C or higher. Also, 35 °C is within the optimum growth range of both pathogens. Both *Salmonella* and *Listeria* grew very well in soup at 12 °C (Tables 2 and 3) and at 35 °C (Tables 4 and 5) indicating the potential of this food product to cause foodborne illness although it contains onion powder and garlic powder on the label (Table 1). Both onion and garlic are known to contain antimicrobials including allicin (27); however, it is

likely that the amount of allicin in the soup is at miniscule levels because both onion powder and garlic powder are minor ingredients as shown on the label (Table 1).

Based on the FDA Food Code potentially hazardous foods should not be held for more than four hours between 4.4 °C and 60 °C (9). In that temperature range called the “temperature danger zone”, foodborne pathogens can multiply rapidly. For example, in the present study *S. enterica* reached 7.63 log CFU/ml of soup within 4 hours in soup held at 35 °C (Table 4). This relatively rapid growth in onion mushroom soup represented approximately 2.63 log increase from the initial population (log 5.0 CFU/ml) of this pathogen. A recent recall of onion soup/dip mix because of contamination with *Salmonella* (17) reinforces the need for antimicrobial interventions to ensure the microbial safety of these potentially hazardous products.

The observed variability in antimicrobial effectiveness of phosvitin or carvacrol used singly in soup reinforces the fact that combined use of antimicrobials offers a more effective approach for controlling pathogens in foods (25). In this regard the best and most reliable antimicrobial treatment was a combination of phosvitin (60 mg/ml) and carvacrol (0.40 mg/ml) which maintained consistent control of both pathogens irrespective of temperature. The results of this study further emphasize the potential of using phosvitin in combination with other naturally derived antimicrobials to enhance its efficacy in controlling foodborne pathogens. The antimicrobial effectiveness observed from the combined use of phosvitin and carvacrol could allow the use of lower concentrations of each antimicrobial for improved food preservation. However, it is well accepted that the practical application of such

preservative systems would require further research on their impact on the sensory properties of foods.

References

1. Achinewhu, S. C., C. C. Ogbonna, and A. D. Hart, 1995. Chemical composition of indigenous wild herbs, spices, nuts and leafy vegetables used as food. *Plant Foods Hum. Nutr.* 32:31-36.
2. Albright, K. J., D. T. Gordon, O. J. Cotterill. 1984. Release of iron from phosvitin by heat and food additives *J. Food Sci.* 49:78-81.
3. Arrebola, M. L., M. C. Navarro, J. Jimenez, and F. A. Ocana. 1994. Yield and composition of the essential oil of *Thymus serpylloides* subsp. *serpylloides*. *Phytochemistry*. 36:67-72.
4. Beuchat, L. R. 1994. Antimicrobial properties of spices and their essential oils, p. 167-179. *In* Natural Antimicrobial Systems and Food Preservation, eds. V.M. Dillon., and R.G. Board, Wallingford, UK: CAB Intl.
5. Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods –a review. *Int. J. Food Microbiol.* 94:223-253.
6. Byrne, B. M., A. D. van het Schip, J. A. M. van de Klundert, A. C. Arnberg, M. Gruber, and G. Ab. 1984. Amino acid sequence of phosvitin derived from the nucleotide sequence of part of the chicken vitellogenin gene. *Biochemistry*. 23:4275-4279.
7. Clark, R.C. 1985. The primary structure of avian phosvitins (contributions through the edman degradation of methylmercaptovitins prepared from the constituent phosphoprotiens). *Int. J. Biochem.* 17:983-988.

8. Cowan, M. M. 1999. Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* 12:564-582.
9. FDA. 2009. Food Code 2009. <http://www.fda.gov/Food/FoodSafety/RetailFoodProtection/FoodCode/FoodCode2009/>
10. Friedman, M., P. R. Henika, and R. E. Mandrell. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J. Food Prot.* 65:1545-1560.
11. Friedman, M., P. R. Henika, C. E. Levin, and R. E. Mandrell. 2004. Antibacterial activities of plant essential oils and their components against *Escherichia coli* O157:H7 and *Salmonella enterica* in apple juice. *J. Agri. Food Chem.* 52:6042-6048.
12. Gaysinsky, S., P. M. Davidson, B. D. Bruce, and J. Weiss. 2005. Growth inhibition of *Escherichia coli* O157:H7 and *Listeria monocytogenes* by carvacrol and eugenol encapsulated in surfactant micelles. *J. Food Prot.* 68:2259-2566.
13. Greengard, O., Sentenac, A., and Mendelsohn, N. 1964. Phosvitin, the iron carrier of egg yolk. *Biochim. Biophys. Acta.* 90:406-407.
14. Helander, I. M., H. Alakomi, K. Latva-Kala, T. Mattila-Sandholm, I. Pol, E. J. Smid, L. G. M. Gorris, and A. von Wright. 1998. Characterization of the action of selected essential oil components on Gram-negative bacteria. *J. Agri. Food Chem.* 46:3590–3595.
15. Jay, J. M., Loessner, M. J., and Golden, D. A. 2005. Modern Food Microbiology, 7th edition, Springer Science + Business Media, LLC, Pubs., New York, NY.

16. Juneja, V. K., and M. Friedman. 2008. Carvacrol and Cinnamaldehyde facilitate thermal destruction of *Escherichia coli* O157:H7 in raw ground beef. *J. Food Prot.* 71:1604-1611.
17. Karatzas, A. K., E. P. W. Kets, E. J. Smid, and M. H. J. Bennik. 2001. The combined action of carvacrol and high hydrostatic pressure on *Listeria monocytogenes* Scott A. *J. Appl. Microbiol.* 90:463-469.
18. Khiyami, M. A., A. L. Pometto III, and R. C. Brown. 2005. Detoxification of corn stover and corn starch pyrolysis liquors by ligninolytic enzymes of *Phanerochaete chrysosporium*. *J. Agric. Food Chem.* 53:2969–2977.
19. Kisko, G., and S. Roller. 2005. Carvacrol and p-cymene inactivate *Escherichia coli* O157:H7 in apple juice. *BMC Microbiol.* 1-9.
20. Knowles, J.R., S. Roller, D. B. Murray, and S. Naidu. 2005. Antimicrobial action of carvacrol at different stages of dual-species biofilm development by *Staphylococcus aureus* and *Salmonella enterica* serovar Typhimurium. *Appl. and Environ. Microbiol.* 71:797-803.
21. Kroger. 2011: accessed at http://www.kroger.com/services/Pages/recall_information.aspx
22. Lacroix, M., J. Borsa, F. Chiasson, and B. Ouattara. 2004. The influence of atmosphere conditions on *Escherichia coli* and *Salmonella typhi* radiosensitization in irradiated ground beef containing carvacrol and tetrasodium pyrophosphate. *Radiat. Phys. and Chem.* 71:59-62.
23. Lagouri, V. G., M. Blekas, S. Tsimidou, S. Kokkini, and D. Boskou. 1993. Composition and antioxidant activity of essential oils from oregano plants grown wild in Greece. *Z. Lebensm Unters. For-schung.* 197:20-23.

24. Lambert, R. J. W., P. N. Skandamis, P. J. Coote, and G. -J. E. Nychas, 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J. Appl. Microbiol.* 91:453-462.
25. Leistner, L. 2000. Basic aspects of food preservation by hurdle technology. *Int. J. Food Microbiol.* 55:181-186.
26. Mecham, D. K., and H. S. Olcott. 1949. Phosvitin, the principal phosphoprotein of egg yolk. *J. Am. Chem. Soc.* 74:3670-3676.
27. Naidu, A. S. 2000. Natural food antimicrobial systems. CRC Press, Boca Raton, FL.
28. Nazer, A.I., A. Kobilinsky, J.-L. Tholozan, and F. Dubois-Brissonnet. 2005. Combinations of food antimicrobials at low levels to inhibit the growth of *Salmonella* sv. Typhimurium: a synergistic effect?. *Food Microbiol.* 22:391-398.
29. Obaidat, M. M. and J. F. Frank. 2009. Inactivation of *Escherichia coli* O157:H7 on the intact and damaged portions of lettuce and spinach leaves by using allyl isothiocyanate carvacrol, and cinnamaldehyde in vapor phase. *J. Food Prot.* 72:2046-2055.
30. Osaki, S., Sexton, R.C., Pascual, E., and Frieden, E. 1975. Iron oxidation and transferrin formation by phosvitin. *J. Biochem.* 151:519-525.
31. Oussalah, M., S. Caillet, L. Saucier, M. and Lacroix. 2007. Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli* O157:H7, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes*. *Food Control.* 18:414-420.
32. Pei, R-S., F. Zhou, B-P. Ji, and J. Xu. 2009. Evaluation of combined antibacterial effects of eugenol, cinnamaldehyde, thymol, and carvacrol against *E. coli* with an improved method. *J. Food Sci.* 74:M379-M383.

33. Perez-Conessa, D., J. Cao, L. Chen, L. Mclandsborough, and J. Weiss. 2011. Inactivation of *Listeria monocytogenes* and *Escherichia coli* O157:H7 biofilms by micelle-encapsulated eugenol and carvacrol. *J. Food Prot.* 74:55-62.
34. Periago, P. M., B. Delgado, P. S. Fernandez, and A. Palop. 2004. Use of carvacrol and cymene to control growth and viability of *Listeria monocytogenes* cells and predictions of survivors using frequency distribution functions. *J. Food Prot.* 67:1405-1416.
35. Pol, I. E., and E. J. Smid. 1999. Combined action of nisin and carvacrol on *Bacillus cereus* and *Listeria monocytogenes*. *Lett. Appl. Microbiol.* 29:166-170.
36. Ravishankar, S., L. Zhu, J. Reyna-Granados, B. Law, L. Joens, and M. Friedman. 2010. Carvacrol and cinnamaldehyde inactivate antibiotic-resistant *Salmonella enterica* in buffer and on celery and oysters. *J. Food Prot.* 73:234-240.
37. Sattar Khan, M. A., S. Nakamura, M. Ogawa, E. Akita, H. Azakami, and A. Kato, 2000. Bactericidal action of egg yolk phosvitin against *Escherichia coli* under thermal stress. *J. Agric. Food Chem.* 48:1503-1506
38. Solomakos, N., A. Govaris, P. Koidis, and N. Botsoglou. 2008. The antimicrobial effect of thyme essential oil, nisin, and their combination against *Listeria monocytogenes* in minced beef during refrigerated storage. *Food Microbiol.* 25:120-127.
39. Stadelman WJ, Cotterill OJ. 1977. Egg Science and Technology. p. 323. Westport, CT: AVI publishing.
40. Taborsky, G. 1963. Interaction between phosvitin and iron and its effect on a rearrangement of phosvitin structure. *Biochemistry.* 2:266-271.
41. Taborsky, G. 1983. Phosvitin. *Adv. Inorg. Biochem.* 5:235-279.

42. Ultee, A. and E. J. Smid. 2001. Influence of carvacrol on growth and toxin production by *Bacillus cereus*. *Int. J. Food Microbiol.* 64:373-378.
43. Ultee, A., E. P. W. Kets, and E. J. Smid. 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. *Appl. and Environ. Microbiol.* 65:4606-4610.
44. Ultee, A., E. P. W. Kets, M. Alberda, F. A. Hoekstra, and E. J. Smid. 2000a. Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol. *Arch. Microbiol.* 174:233-238.
45. Ultee, A., Gorris, L.G.M., and Smid, E.J. 1998. Bactericidal activity of carvacrol towards the food-borne pathogen *Bacillus cereus*. *J. Appl. Microbiol.* 85:211-218.
46. Ultee, A., R. A. Slump, G. Steging, and E. J. Smid. 2000b. Antimicrobial activity of carvacrol toward *Bacillus cereus* on rice. *J. Food Prot.* 63:620-624.
47. Veldhuizen, E. J. A., J. L. M. Tjeerdsma-Van Bokhoven, C. Zweijtzter, S. A. Burt, and H. P. Haagsman. 2006. Structural requirements for the antimicrobial activity of carvacrol. *J. Agric. Food Chem.* 54:1874-1879.
48. Veldhuizen, E. J. A., T. O. Creutzberg, S. A. Burt, H. P. and Haagsman. 2007. Low temperature and binding to food components inhibit the antibacterial activity of carvacrol against *Listeria monocytogenes* in steak tartare. *J. Food Prot.* 70:2127-2132.
49. Wallace, R. A. and J. P. Morgan. 1986. Chromatographic resolution of chicken phosvitin. *Biochemisry.* 240:871-878.
50. Waterman, P. G., and S. Mole. 1994. Analysis of phenolic plant metabolites, p. 85–88. In J. H. Lawton and G. E. Likens (ed.), *Methods in ecology*. Blackwell Scientific, Oxford.
51. Webb, J., J. S. Multani, P. Saltman, N. A. Beach, and H. B. Gray. 1973. Spectroscopic and magnetic studies of iron (III) phosvitins. *Biochemistry.* 12:1797-1802.

52. Xu, J., F. Zhou, B. -P. Ji, R.-S. Pei, and N. Xu. 2008. The antibacterial mechanism of carvacrol and thymol against *Escherichia coli*. *Lett. Appl. Microbiol.* 47:174-179.
53. Zhou, F., B. Ji, H. Zhang, H. Jiang, J. J. Li, J. Li, Y. Ren, and W. Yan. 2007. Synergistic effect of thymol and carvacrol combined with chelators and organic acids against *Salmonella Typhimurium*. *J. Food Prot.* 70:1704-1709.

TABLE 1. ^a*Ingredients in dehydrated onion and mushroom soup/dip mix.*

Cornstarch
Dehydrated onions
Salt
Monosodium glutamate
Maltodextrin
Wheat flour
Dehydrated mushrooms
Partially hydrogenated soybean oil
Hydrolyzed protein (corn, soy, wheat)
Autolyzed yeast extract
Caramel color
Onion powder
Guar gum
Garlic powder
Natural flavors
Disodium guanylate
Disodium inosinate

*Sodium 700 mg- 29%

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

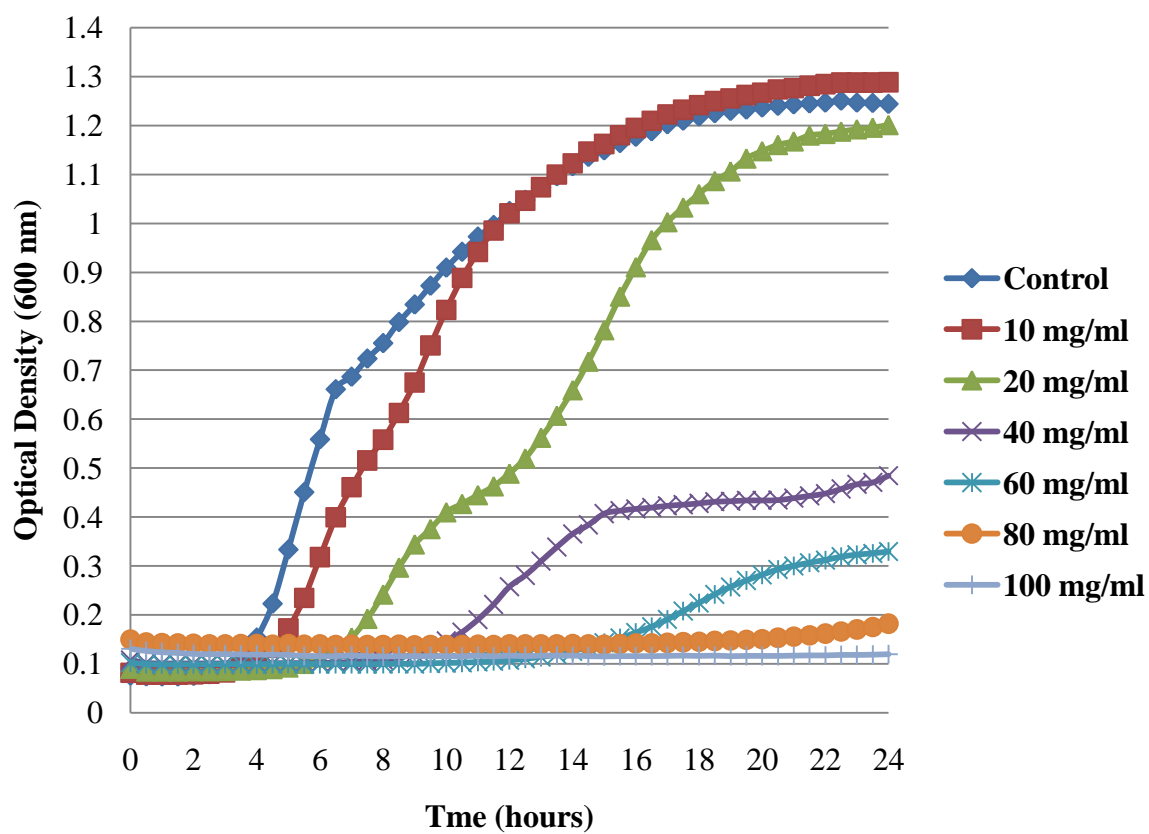


FIGURE 1. *Growth of Salmonella enterica in BHI broth (35 °C) supplemented with various concentrations of phosvitin.*

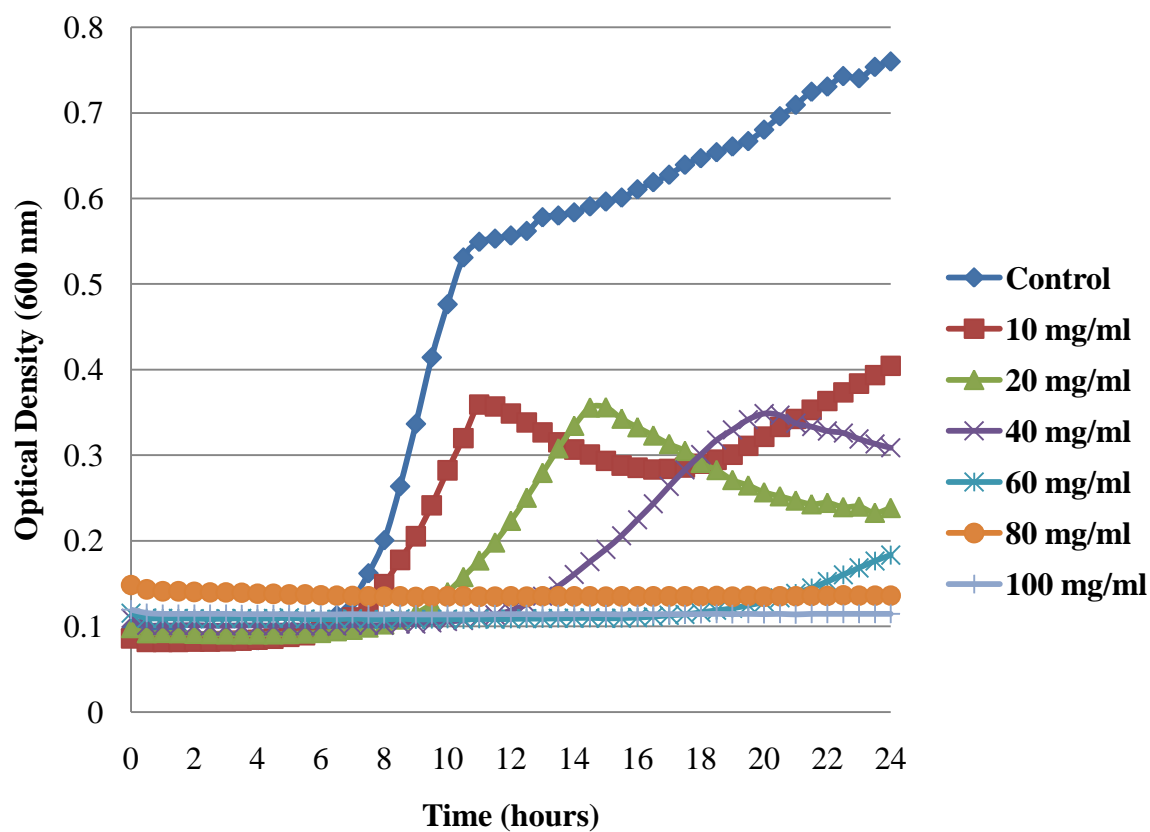


FIGURE 2. *Growth of Listeria monocytogenes in BHI broth (35 °C) supplemented with various concentrations of phosvitin.*

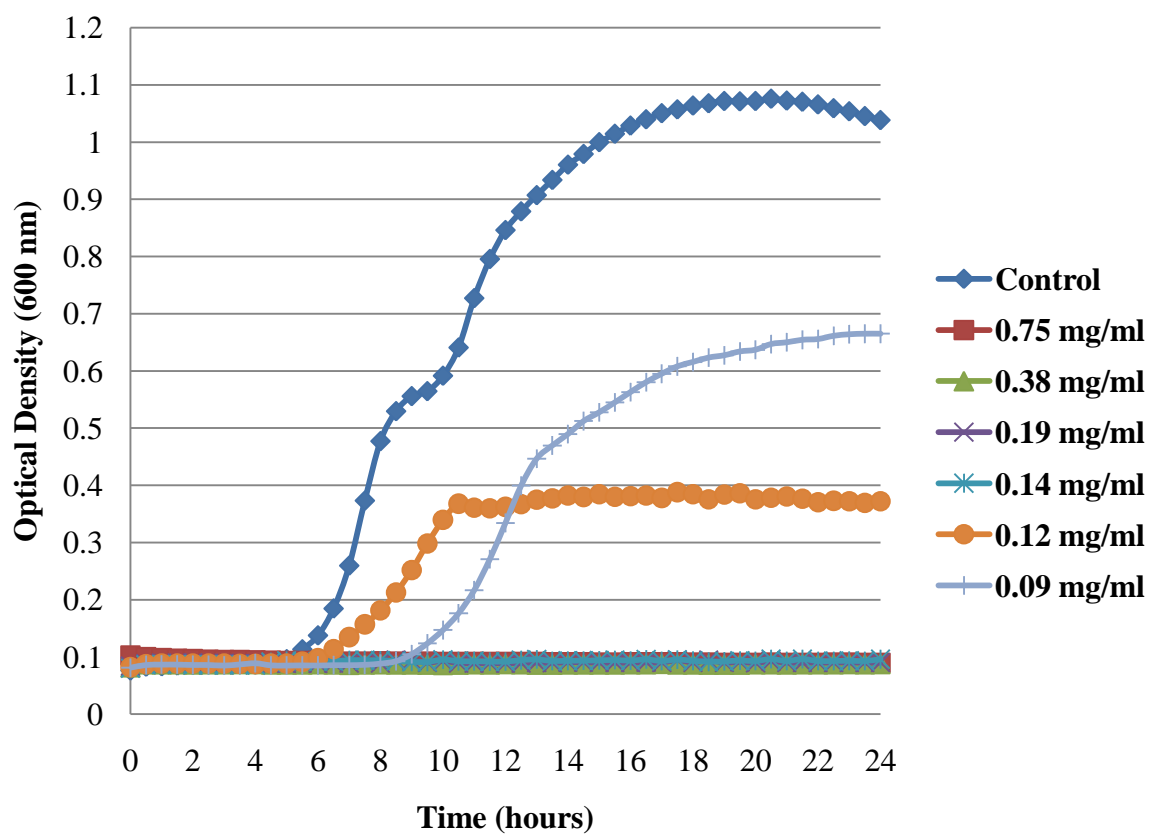


FIGURE 3. *Growth of Salmonella enterica in BHI broth (35 °C) supplemented with various concentrations of carvacrol.*

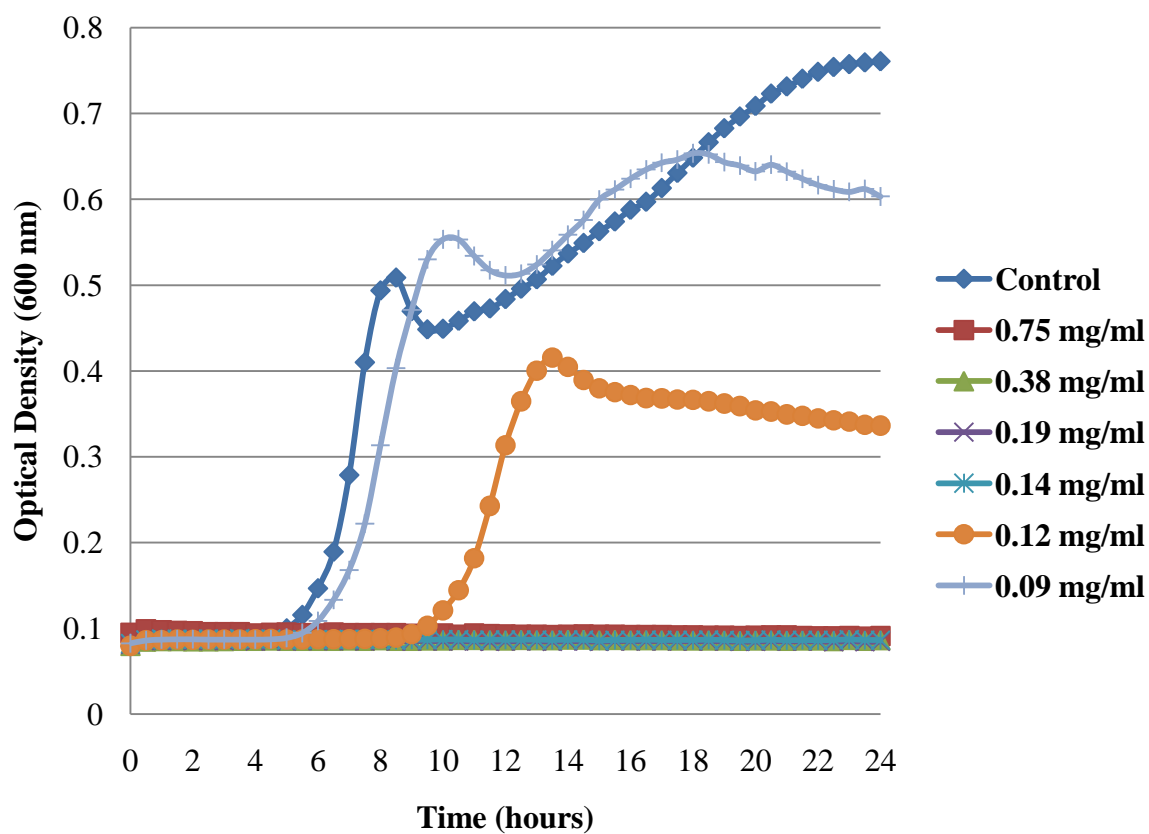


FIGURE 4. *Growth of Listeria monocytogenes in BHI broth (35 °C) supplemented with various concentrations of carvacrol.*

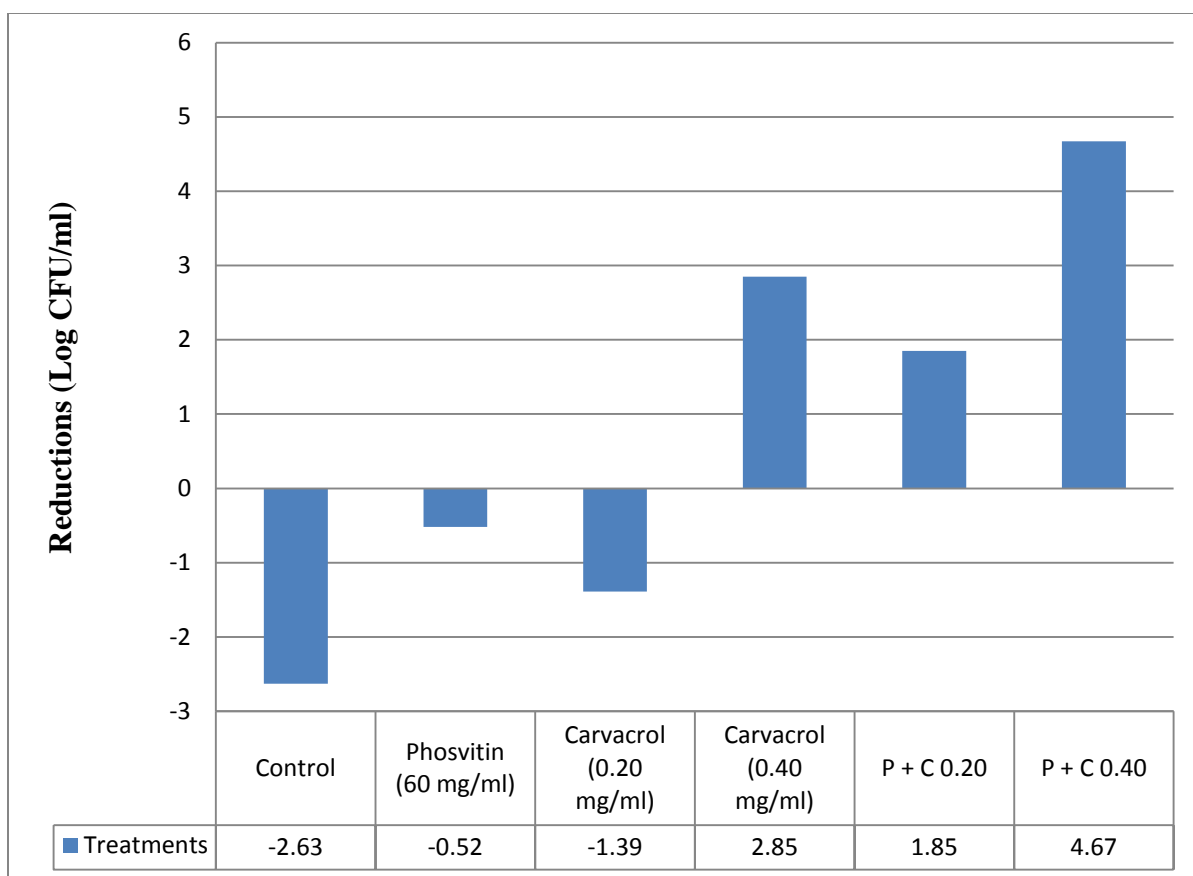


FIGURE 5. *Log reduction in initial count of Salmonella enterica following 4 hr of exposure to antimicrobials in onion mushroom soup at 35 °C.*

TABLE 2. Antibacterial effectiveness of phosvitin (60 mg/ml) alone or combined with carvacrol against *Salmonella enterica* in onion mushroom soup held at 12 °C for 8 days.

Treatment (mg/ml)	Viable count (Log ₁₀ CFU/ml) ^x			
	2d	4d	6d	8d
Control	4.60 ± 0.05ax	6.26 ± 2.51ax	6.02 ± 3.44ax	6.95 ± 3.62ax
Phosvitin (60)	4.42 ± 0.34ax	4.53 ± 0.14abx	4.49 ± 0.42ax	5.28 ± 1.59ax
Carvacrol (0.20)	3.81 ± 0.20abx	5.24 ± 2.52abx	4.36 ± 3.86ax	4.99 ± 3.39ax
Carvacrol (0.40)	1.38 ± 2.40bcx	1.75 ± 3.03abx	2.21 ± 3.83ax	2.27 ± 3.93ax
Phos + Car (0.20)	2.75 ± 0.75abcx	2.36 ± 0.32abx	2.49 ± 0.28ax	1.90 ± 0.44ax
Phos + Car (0.40)	0.51 ± 0.89cx	0.00 ± 0.00bx	0.00 ± 0.00ax	0.00 ± 0.00ax

^xEach reported value for viable count represents the mean (standard deviation) of three independent replications of the experiment

^{a,b,c}Means with a different letter within a column differ significantly (P<0.05)

^{x,y,z}Means with a different letter within a row differ significantly (P<0.05)

TABLE 3. Antibacterial effectiveness of phosvitin (60 mg/ml) alone or combined with carvacrol against *Listeria monocytogenes* in onion mushroom soup held at 12 °C for 8 days.

Treatment (mg/ml)	Viable count (Log ₁₀ CFU/ml) ^x			
	2d	4d	6d	8d
Control	4.62 ± 1.14az	5.86 ± 1.12ayz	7.22 ± 0.07axy	8.72 ± 0.69ax
Phosvitin (60)	3.95 ± 0.69ax	4.02 ± 0.63ax	4.80 ± 0.46bcx	5.37 ± 1.33abx
Carvacrol (0.20)	3.90 ± 0.08az	5.60 ± 2.13ayz	7.16 ± 0.75abxy	8.81 ± 0.18ax
Carvacrol (0.40)	4.13 ± 0.40ax	4.31 ± 1.09ax	4.49 ± 1.57cx	4.77 ± 2.11bx
Phos + Car (0.20)	3.94 ± 0.75ax	3.96 ± 0.78ax	4.25 ± 0.56cx	4.38 ± 0.47bx
Phos + Car (0.40)	3.78 ± 0.70ax	4.00 ± 1.44ax	3.54 ± 0.96cx	3.17 ± 1.61bx

^xEach reported value for viable count represents the mean (standard deviation) of three independent replications of the experiment

^{a,b,c}Means with a different letter within a column differ significantly (P<0.05)

^{x,y,z}Means with a different letter within a row differ significantly (P<0.05)

TABLE 4. Antibacterial effectiveness of phosvitin (60 mg/ml) alone or combined with carvacrol against *Salmonella enterica* in onion mushroom soup held at 35 °C for 24 hours.

Treatment (mg/ml)	Viable count (Log ₁₀ CFU/ml) ^x			
	4h	8h	12h	24h
Control	7.63 ± 0.31 ^{ay}	9.46 ± 1.29 ^{ax}	10.28 ± 0.26 ^{awx}	11.31 ± 0.02 ^{aw}
Phosvitin (60)	5.52 ± 0.95 ^{by}	6.95 ± 0.51 ^{bxy}	8.12 ± 0.50 ^{bx}	10.99 ± 0.03 ^{aw}
Carvacrol (0.20)	6.39 ± 0.39 ^{abz}	8.13 ± 0.01 ^{aby}	8.85 ± 0.15 ^{abx}	10.14 ± 0.17 ^w
Carvacrol (0.40)	2.15 ± 0.60 ^{cx}	2.42 ± 1.02 ^{cx}	3.54 ± 1.12 ^{cw}	6.65 ± 0.96 ^{bw}
Phos + Car (0.20)	3.15 ± 0.56 ^{cx}	3.12 ± 0.22 ^{cx}	4.07 ± 1.15 ^{cx}	6.46 ± 1.26 ^{bw}
Phos + Car (0.40)	0.33 ± 0.58 ^{dw}	0.00 ± 00.0 ^{dw}	0.00 ± 0.00 ^{dw}	0.00 ± 0.00 ^{cw}

^xEach reported value for viable count represents the mean (standard deviation) of three independent replications of the experiment

^{a,b,c}Means with a different letter within a column differ significantly (P<0.05)

^{w,x,y,z}Means with a different letter within a row differ significantly (P<0.05)

TABLE 5. Antibacterial effectiveness of phosvitin (60 mg/ml) alone or combined with carvacrol against *Listeria monocytogenes* in onion mushroom soup held at 35 °C for 24 hours.

Treatment (mg/ml)	Viable count (Log ₁₀ CFU/ml) ^x			
	4h	8h	12h	24h
Control	4.60 ± 0.03az	6.04 ± 0.74ay	6.51 ± 0.24ay	8.01 ± 0.52ax
Phosvitin (60)	4.22 ± 0.25ax	4.42 ± 0.11bcx	4.68 ± 0.19bx	5.15 ± 0.85bcx
Carvacrol (0.20)	4.71 ± 0.63az	5.60 ± 0.55abyz	6.40 ± 0.45axy	7.57 ± 0.06abx
Carvacrol (0.40)	4.11 ± 0.71ax	4.19 ± 0.63bcx	4.27 ± 0.53bx	4.47 ± 0.18cdx
Phos + Car (0.20)	3.99 ± 0.70ax	4.17 ± 0.44cx	4.33 ± 0.32bx	4.29 ± 0.62cdx
Phos + Car (0.40)	3.42 ± 0.15ax	3.35 ± 0.36cx	3.23 ± 0.30cx	2.13 ± 1.84dx

^xEach reported value for viable count represents the mean (standard deviation) of three independent replications of the experiment

^{a,b,c}Means with a different letter within a column differ significantly (P<0.05)

^{x,y,z}Means with a different letter within a row differ significantly (P<0.05)

CHAPTER 4

GROWTH INHIBITION OF *STAPHYLOCOCCUS AUREUS*, *ESCHERICHIA COLI* O157:H7, AND BACKGROUND MICROFLORA BY PHOSVITIN AND CARVACROL ADDED TO ONION MUSHROOM SOUP

Shecoya White¹, Aubrey Mendonca^{1*}, Aura Daraba³, and Dong Ahn²

¹Department of Food Science and Human Nutrition, and ²Department of Animal Science, Iowa State University, Ames IA, USA 50011; ³Faculty of Food Science and Engineering, University “Dunarea de Jos” Galati, Romania.

*Corresponding author: Department of Food Science and Human Nutrition, 2312 Food Sciences Bldg., Iowa State University, Ames, IA, USA 50011.

Phone: (515) 294-9078. Fax: (515) 294-8181. Email: amendon@iastate.edu.

Key words: *Staphylococcus*, *Escherichia coli*, phosvitin, carvacrol, soup

Abstract

The antibacterial efficacy of phosvitin, or carvacrol alone, or combined against *Staphylococcus aureus* and *Escherichia coli* O157:H7 in brain heart infusion (BHI) broth and onion mushroom soup was investigated. Broth with added phosvitin (10 - 100 mg/ml), carvacrol (0.09 - 0.75 mg/ml) or combinations was inoculated with *S. aureus* or *E. coli* O157:H7. Broth or soup without antimicrobials served as control. Growth curves and minimum inhibitory concentrations (MICs) 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, 8 days) and (35 °C, 24 hours) was monitored by surface plating the samples on appropriate selective agar. The MIC of phosvitin and carvacrol was greater than 100 mg/ml and 0.12 mg/ml, respectively, for *S. aureus*; and 80 mg/ml and 0.12 mg/ml, respectively, for *E. coli*. No growth of *S. aureus* occurred in control or treated soup at 12 °C. Phosvitin (60 mg/ml) plus carvacrol (0.40 mg/ml) exhibited the greatest cidal effect against both pathogens, after day 2. With this combination, pathogens were undetected (<10 CFU/ml) from day 4 through day 8. In soup (35 °C, 24hr), phosvitin (60 mg/ml) and carvacrol (0.40 mg/ml) combination reduced viable cells of *S. aureus* and *E. coli* by 6.78 and 9.89 log CFU/ml, respectively. Phosvitin concentration alone, carvacrol (0.40 mg/ml) alone, and in combination were also effective towards growth inhibition of soup background microflora. The combination of phosvitin (60 mg/ml) and carvacrol (0.40 mg/ml) is highly effective for controlling *S. aureus* and *E. coli* in temperature abused onion mushroom soup and has good potential for improving the microbial safety of this potentially hazardous food product during temperature abuse.

Introduction

Control of human enteric foodborne pathogens to prevent of foodborne related illnesses is a goal of the government, food manufacturers, processors and retailers. The continued use of synthetic chemical preservatives to enhance microbial safety of control food has increased consumer health concerns, even though the use of these preservatives may be regarded as safe by regulatory authorities (Beuchat 2008). In this regard, food manufacturers are searching for new naturally derived alternatives to address the concern of consumers. To reduce chemical preservatives that are currently used in foods the following must be addressed when considering substitution with natural antimicrobials: assurance of microbiological food safety, efficacy and functionality in foods; toxicology and safety in food formulations; interactions with food components and other preservatives. Other important considerations include: mechanism of action against microorganisms; influence on food quality and sensory; extraction and isolation methods, and economical production on an adequate scale (Sofos and others 1998; Beuchat 2008). Naturally derived antimicrobials are obtained from either animal, plant, or microbial sources.

Phosvitin is a naturally derived phosphoprotein from egg yolk, containing approximately 120 phosphoserine residues (Castellanie and others 2004). The high phosphorus content allows phosvitin to bind up to 60 iron molecules, dependent on the isolation method (Mecham and Olcott 1949; Taborsky 1963; Webb and others 1973; Albright and others 1984). The ability of phosvitin to remove essential nutrients such as iron from the environment is believed to inhibit growth of foodborne pathogens (Sattar Khan and others 2000). To date, antimicrobial efficacy of phosvitin has been reported in one published paper

(Sattar Khan and others 2000). In that study *Escherichia coli* was inactivated when subjected to phosvitin (0.1 mg/ml) combined with heat (50 °C, 20 min) (Sattar Khan and others 2000).

Carvacrol, (2-methyl-5-(1-methylethyl)-phenol), is an essential oil from the leaves and flowering plant of both oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) (Burt, 2004; Oussalah 2007). The percent composition of carvacrol in oregano ranges from trace amounts to 82% and 2 to 45% in thyme (Lagouri and others 1993; Arrebola and others 1994; Burt 2004). Antimicrobial activity of carvacrol has been studied extensively in broth model systems as well as several foods including: cheese (Smith-Palmer and others 2001), apple cider (Friedman and others 2004, Kisko and Roller 2005), fruit (kiwi and melon) (Roller and Seedhar 2002), carrot broth (Valero and Salmeron 2003), salad (Koutsoumanis and others 1999), lettuce and spinach leaves (Obaidat and Frank 2009), rice (Ultee and others 2000b), turkey (Juneja and Friedman 2007), meat (Veldhuizen and others 2007; Juneja and Friedman 2008; Solomakos and others 2008; Friedman and others 2009), fish/seafood (Kim and others 1995; Meljholm and Dalgaard, 2002; Ravishankar and others 2010). It has been shown to have stronger antimicrobial effect with increased temperature and storage time, and at a lower pH (Burt 2004; Friedman and others 2004). This phenolic compound is thought to exert antibacterial action by causing structural and functional damage to cellular membranes (Sikkema and others 1995; Sivropoulou and others 1996; Ultee and others 1999). While there is a growing body of knowledge on the antimicrobial effects of carvacrol, against various foodborne pathogens, such published research involving phosvitin is scarce. To our knowledge there are no published research involving the combined use of phosvitin and carvacrol against *Staphylococcus aureus* and *Escherichia coli* O157:H7. The objectives of the present study were: 1) to establish the minimum inhibitory concentration of both

phosvitin and carvacrol in brain heart infusion broth and 2) to evaluate the antimicrobial efficacy of phosvitin, low concentrations of carvacrol both individually and their combinations against foodborne human enteric pathogens and background microflora in soup held at 12 °C and 35 °C.

Materials and Methods

Bacterial cultures and culture conditions

Three strains of *Staphylococcus aureus* (ATCC 6538, ATCC 25923, and BAA-44) and five strains of *Escherichia coli* O157:H7 (FRIK125, ATCC 35150, ATCC 43894, ATCC 43895, and 93-062) were used in the present study. All cultures were obtained from the culture collection of the Microbial Food Safety Laboratory, Iowa State University, Ames, IA. Stock cultures were kept frozen (-70 °C) in Brain Heart Infusion (BHI) broth (Difco, Becton, Dickinson, Sparks, MD) supplemented with 10% (vol/vol) glycerol. Stock cultures were activated in BHI broth (pH 7.2) and incubated at 35 °C. At least two consecutive 18 to 22-h transfers of each stock culture were carried out before using the cells as inocula in each experiment.

Preparation of inoculum

An equal volume of each of the working cultures of *S. aureus* or *E. coli* O157:H7 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 (American Laboratory Trading, Inc., East Lyme, CT) and washed once in 0.1% (wt/vol) peptone. The pelleted cells were suspended in fresh 0.1% (wt/vol) peptone to obtain a final viable cell concentration of

approximately 10^9 CFU ml⁻¹. Viable counts of the washed cell suspensions were evaluated by surface plating serially diluted (10-fold) samples on tryptic soy agar supplemented with 0.6% yeast extract. The individual cell suspensions were used to inoculate BHI broth or commercial onion mushroom soup.

Antimicrobials

Phosvitin was supplied by Dr. Dong Ahn from the Animal Science Department at Iowa State University, Ames, IA. A commercial preparation of carvacrol was purchased from Sigma-Aldrich (Aldrich W224502).

Total phenolic content of carvacrol

The concentration of total phenolics of carvacrol was determined using the method described by Waterman and Mole (1994). The assay involves reduction of ferric iron to the ferrous state by phenolic compounds that results in the formation of the Prussian blue complex $\text{Fe}_4[\text{Fe}(\text{CN})_6]_3$ with a potassium ferricyanide reagent. The Prussian blue complex is determined colorimetrically (Khiyami and others 2005). Two separate 1% (vol/vol) solutions of carvacrol in 10% (vol/vol) ethanol and deionized water were prepared. The absorbance of three sub- samples from each solution was measured at 720 nm and the total phenolic content expressed as the number of catechin equivalents x 100.

Preparation of treatment solutions for Bioscreen C assay

BHI broth with added phosvitin (10, 20, 40, 60, 80, 100 mg/ml) or carvacrol (0.09, 0.12, 0.14, 0.19, 0.38, or 0.75 mg/ml) were filter sterilized using 0.22 μm pore size Millipore filters (Fisher Scientific, Pittsburgh, PA). Samples (2.5-ml) of the treatment solutions and

control (BHI with no added antimicrobial) were each inoculated with 25 μ l of diluted (1:100) *S. aureus* or *E. coli* O157:H7 cell suspension to obtain a final concentration of approximately 10^5 CFU/ml of sample.

Bioscreen C assay

Aliquots (200 μ l) of inoculated samples were added in triplicate to the wells of microtiter plate for the Bioscreen C Turbidometer (Growth Curves, Piscataway, NJ), an automated microbial growth analyzer and incubator. Plates were incubated in the Bioscreen C at 35 °C for 24 h and the instrument was programmed to take optical density (OD) measurements at 600 nm every 30 min, with shaking of samples before each OD reading. Minimum inhibitory concentration (MIC) was defined as the lowest treatment concentration that completely inhibited microbial growth for 24 hr (< 0.05 OD unit increase).

Preparation and inoculation of soup

Dehydrated onion mushroom soup/dip mix was purchased from a local grocery store. The soup mix was hydrated with sterile distilled water and prepared according to the directions stated on the package. Soup ingredients are shown in Table 1. After boiling, the soup was tempered to ~ 35 °C and 10-ml portions of the soup were aseptically transferred into sterile 30 ml screw cap Pyrex tubes. Phosvitin, carvacrol or combinations were added to each tube to give the following concentrations: phosvitin (60 mg/ml), carvacrol (0.20 mg/ml or 0.40 mg/ml), phosvitin (60 mg/ml) + carvacrol (0.20 mg/ml) and phosvitin (60 mg/ml) + carvacrol (0.40 mg/ml). Phosvitin was added to the tubes prior to the addition of the soup to prevent aggregation and facilitate its dissolving in the soup. Tubes of soup without added

antimicrobial served as control. The tubes of soup were then warmed or cooled to the desired temperature (12 °C or 35 °C). Tubes of soup with added antimicrobials were mixed by vortexing and inoculated with a suspension of washed cells of the foodborne pathogens to give a final cell concentration of approximately 10^5 CFU/ml for each pathogen. After inoculation, each tube of soup was gently mixed by vortexing and held at either 12 °C or 35 °C.

Microbiological analysis

Inoculated tubes of soup held at 12 °C were tested for survivors of the pathogens every 2 days for a total of 8 days whereas, soup held at 35 °C were tested at 4, 8, 12 and 24 h. Ten-fold serial dilutions of the soup were prepared using 0.1% (vol/vol) peptone with added Tween 80 (0.5% v/v). Aliquots (1.0 ml or 0.1ml) of the soup or diluted samples of soup were surface plated (in duplicate) on mannitol salt agar (for *S. aureus*) and sorbitol MacConkey agar (for *E.coli*). All inoculated plates were incubated at 35 °C for 48h before counting bacterial colonies.

Measurement of pH and water activity

The initial pH and water activity of each sample of soup including controls were measured at day 0 (prior to inoculation). Measurements of pH were also taken periodically during storage of the soup at 12 °C or 35 °C. 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).

Statistical analysis

Three independent replications of each experiment were performed. Mean numbers of survivors *S. aureus* or *E.coli* O157:H7 were statistically analyzed using SAS statistical software version 8.1 (SAS Institute Inc., Cary, N.C.). Treatment means were evaluated for statistically significant differences using Tukey's test. Significant differences were defined at $P < 0.05$ for all the experimental data.

Results

Growth inhibition by phosvitin in BHI broth

The effect of phosvitin on the growth of *Staphylococcus aureus* and *Escherichia coli* O157:H7 in Brain Heart Infusion (BHI) broth is shown in Figures 1 and 2. For *S. aureus*, the optical density (OD) of control and broth containing 10 and 20 mg/ml phosvitin increased rapidly and slightly exceeded an OD of 0.8 after 17 hours. Growth inhibition and extension of the lag phase of *S. aureus* increased with higher phosvitin concentrations. The MIC of phosvitin for *S. aureus* was greater than 100 mg/ml. For *E. coli* O157:H7, the OD of the culture increased from 0.1 (at 3h) to greater than 1.2 (at 17h). All phosvitin concentrations, except 10 mg/ml, inhibited growth of *E. coli* O157:H7 with higher concentrations causing more pronounced growth inhibition. Phosvitin at 80 and 100 mg/ml were bacteriostatic. Greater extensions of the lag phase and lower maximum OD values were observed with increased phosvitin concentrations. The MIC of phosvitin for *E. coli* O157:H7 was 80 mg/ml.

Growth inhibition by carvacrol in BHI broth

Growth of *S. aureus* and *E. coli* O157:H7 in BHI supplemented with various concentrations of carvacrol is shown in Figures 3 and 4. The OD of control increased from 0.1 to greater than 0.6 within 18 hours. Increase in OD occurred only in BHI containing 0.09 mg/ml carvacrol. At 24 hours the control and carvacrol (0.09 mg/ml)-containing cultures achieved an OD > 0.7 and 0.45, respectively. All other concentrations of carvacrol (0.12 - 0.75 mg/ml) completely inhibited growth of the organism. The MIC of carvacrol for *S. aureus* was 0.12 mg/ml. Except for 0.09 mg/ml, all other carvacrol concentrations tested against *E. coli* O157:H7 completely inhibited growth of the pathogen. Brain heart infusion broth with 0.09 mg/ml decreased the growth of the pathogen compared to control. At 18 hours the OD of control and carvacrol (0.09 mg/ml) was ~ 1.0 and 0.65, respectively. The MIC of carvacrol for *E. coli* O157:H7 was 0.12 mg/ml.

Growth inhibition by phosvitin and carvacrol in onion mushroom soup at 12 °C

The total phenolic content of the 1% solution of carvacrol was 0.98 catechin equivalents. This result coincides with the manufacturer's claim of "up to 98% total polyphenols". The initial pH and water activity of the soup was 6.1 and 0.994, respectively. The initial viable count of *S. aureus* or *E. coli* O157:H7 in onion mushroom soup was Log 5.0 CFU/ml. Viable counts of *S. aureus* or *E. coli* O157:H7 in soup (12 °C) with phosvitin alone or combined with carvacrol are shown in Tables 2 and 3. No growth of *S. aureus* occurred in control or treated soup at 12 °C. In control soup viable numbers of *S. aureus* decreased by 0.86 Log CFU/ml at 4 days and numbers of surviving bacteria remained constant throughout the storage period. Numbers of survivors in soup with phosvitin (60

mg/ml) or carvacrol (0.20 mg/ml) alone were not significantly ($P>0.05$) different from those in control soup. Carvacrol (0.40 mg/ml) and phosvitin (60 mg/ml) plus carvacrol (0.20 or 0.40mg/ml) decreased cell survival during the storage period.. The combination of phosvitin and carvacrol (0.40 mg/ml) was cidal to *S. aureus*; initial counts decreased by 4.32 log CFU/ml at 2 days of storage. Viable counts of the pathogen decreased further and were undetectable (<10 CFU/ml) from day 4 to day 8.

In control soup at 12 °C viable numbers of *E. coli* O157:H7 increased to 6.90 log CFU/ml at day 8. Phosvitin (60mg/ml) exerted a bacteriostatic action on the pathogen; no growth occurred throughout the duration of the study. At day 2, carvacrol (0.20 mg/ml) caused about a 1 log reduction in viable counts compared to the control, and number of survivors remained constant up to 8 days. By day 2, carvacrol (0.40 mg/ml) alone and the combination of phosvitin and carvacrol (0.40mg/ml) caused a log reduction of 2.39 and 4.05, respectively. After day 2 viable cells were undetected (<10 CFU/ml) in soup that contained either of the two treatments.

Growth inhibition by phosvitin and carvacrol in onion mushroom soup at 35 °C

Tables 4 and 5 show the effect of phosvitin, carvacrol and combinations against *S. aureus* and *E. coli* O157:H7 in onion mushroom soup (35 °C). In control soup viable numbers of *S. aureus* increased from 5.60 log at 4 hours to 8.83 log CFU/ml at 24 hours of incubation (Table 4). Compared to control, phosvitin alone strongly inhibited growth of the pathogen for 12 hours ($P<0.05$). Carvacrol (0.20 mg/ml) alone failed to suppress the growth of the pathogen; however, when it was combined with phosvitin the growth inhibitory effect of that combination was significantl ($P<0.05$). Viable counts in soup that contained carvacrol

(0.4 mg/ml) alone and the combination of phosvitin and carvacrol (0.40 mg/ml) were significantly ($P < 0.05$) lower than from the control after 4 hours; however, there was no significant difference between these two treatments ($P > 0.05$). At 24 hours carvacrol (0.40 mg/ml) and phosvitin plus carvacrol (0.40 mg/ml) reduced cell survival by 6.33 and 6.78 log CFU/ml, respectively.

Viable numbers of *E. coli* O157:H7 in control soup increased from 7.59 log at 4 hours to 10.69 log CFU/ml at 24 hours of incubation (Table 5). Viable counts of the pathogen in soup with added phosvitin (60 mg/ml), carvacrol (0.2 or 0.4 mg/ml) and their combinations were significantly ($P < 0.05$) lower than those of the control for the first four hours of incubation at 35°C. Phosvitin strongly inhibited the growth of the microorganisms for the first 8 hours. Carvacrol (0.20 mg/ml) lost effectiveness to suppress the growth of *E. coli* O157:H7 at 8 hours whereas, carvacrol (0.40mg/ml) alone or phosvitin combined with carvacrol (0.20 mg/ml) was effective in suppressing growth of the pathogen within that 8-hour period. Viable counts of soup with the combination of phosvitin and 0.4mg/ml of carvacrol were significantly ($P < 0.05$) lower than those of control throughout the entire storage period. Phosvitin combined with the highest level of carvacrol (0.40 mg/ml) produced a 9.89 log reduction in viable counts at 24 hours, compared to the control.

In addition to the antimicrobial activity of phosvitin and carvacrol against *S. aureus* and *E. coli*, growth inhibition of background microflora present in soup at 35° C for 12 hours (Figure 5) was evaluated. Background microflora in control soup increased from 1.4 log CFU/ml at time 0 to 4.28 log at 12 hours. At 12 hour, phosvitin (60 mg/ml), carvacrol (0.40 mg/ml), and their combination were significantly ($P < 0.05$) different from the control.

Phosvitin alone, carvacrol alone, and the phosvitin carvacrol combination reduced microflora by 1.39, 2.78, and 3.19 log CFU/ml, respectively.

Discussion

In recent years consumer awareness of food safety has increased substantially. Also consumers are demanding more natural food preservatives instead of synthetic ones. Such changes in consumer attitude are rapidly affecting the actions of the food industry. Food processors are now seeking naturally derived antimicrobials to substitute for currently used synthetic additives. Some naturally-derived antimicrobials include plant essential oils (EO) and their components (Burt, 2004). One component of EO that has very good antimicrobial activity is carvacrol. In the present study we used carvacrol and phosvitin (an antimicrobial protein from egg yolk) to control *S. aureus* and *E. coli* O157:H7 in an onion mushroom soup held at abusive temperatures. While several studies have been published to demonstrate the antibacterial efficacy of carvacrol, to date there is only one published report on the antibacterial effect of phosvitin (Sattar Khan and others 2000). In the present study phosvitin inhibited the growth of *S. aureus* and *E. coli* O157:H7 in BHI broth in a dose dependent manner (Figures 1 and 2). *E. coli* O157:H7 seemed to be more sensitive to phosvitin compared to *S. aureus*. This observed sensitivity might be due to the relatively strong chelation ability of phosvitin (Sattar Khan and others 2000). Carvacrol proved to be a more potent antimicrobial compared to phosvitin due to the very low concentrations required to inhibit the pathogens tested in BHI broth the present study.

It is generally well recognized that concentrations of antimicrobials that are effective in laboratory media require higher concentrations to be effective in actual food products.

Certain antimicrobials, such as essential oils, or their components, may effectively control microorganisms at concentrations that alter sensory quality. To circumvent this issue of high concentration, it might be best to use essential oils in system of antimicrobials consistent with hurdle technology concept (Adams and others 2003; Nychas and Skandamis 2003; Roller and Board 2003). In the present study we were able to use relatively low concentrations (0.20 and 0.40 mg/ml) of carvacrol were combined with phosvitin to control pathogens in onion mushroom soup held at abusive temperatures. Veldhuizen et al. (2007) reported reduced antimicrobial activity of similar concentrations of carvacrol when combined with egg yolk. Our data show that phosvitin alone extracted from the egg yolk actually increased the efficacy of carvacrol. In all instances the combination proved to strongly inhibitory and at times cidal to the pathogens (Tables 2 and 3). We intentionally held the soup at 12 °C and 35 °C to simulate temperature abuse situations. Both of these temperatures fall within the temperature danger zone in which pathogenic bacteria can grow rapidly and reach high populations within 4 hours. At both temperatures the combination of carvacrol and phosvitin was very effective in controlling *S. aureus* and *E. coli* O157:H7 in the soup. That combination also inhibited growth of background microflora in onion mushroom soup. Based on these findings the combination of phosvitin and carvacrol has good potential for enhancing the microbial safety of onion mushroom soup and preventing microbial spoilage of this product. Further research is needed to determine the influence of combined antimicrobials on the sensory characteristics and quality of food.

Acknowledgments

The authors thank Dr. Bill Collona on his instruction on the use of the bioscreen

and use of the anaerobic chamber. The authors also thank Michael Mendonca and David Manu for laboratory assistance.

References

Adams, M., and Smid, E. 2003. Nisin in multifactorial food preservation, p. 11-33. *In* S. Roller (ed.), Natural antimicrobials for the minimal processing of foods. Woodhead Publishing Ltd., Cambridge, United Kingdom.

Albright KJ, Gordon DT, Cotterill OJ. 1984. Release of iron from phosvitin by heat and food additives J Food Sci. 49:78-1.

Arrebola ML, Navarro MC, Jimenez J, Ocana FA. 1994. Yield and composition of the essential oil of *Thymus serpylloides* subsp. *serpylloides*. Phytochemistry. 36:67-2.

Beuchat LR. "Control of Foodborne Pathogens and Spoilage Microorganisms by Naturally Occurring Antimicrobials." *Microbial Food Contamination*. Boca Raton: CRC, 2008. Print

Burt S. 2004. Essential oils: their antibacterial properties and potential applications in foods – a review. Int J Food Microbiol. 94:223-53.

Castellani O, Guerin-Dubiard C, David-Driand E, Anton M. 2004. Influence of physiochemical conditions and technological treatments on the iron binding capacity of egg yolk phosvitin. Food Chem. 85:569-77.

Friedman M, Henika PR, Levin, CE, and Mandrell RE. 2004. Antibacterial activities of plant essential oils and their components against *Escherichia coli* O157:H7 and *Salmonella enterica* in apple juice. J Agric Food Chem. 52:6042-48.

- Friedman M, Zhu L, Feinstein Y, Ravishankar S. 2009. Carvacrol facilitates heat-induced inactivation of *Escherichia coli* O157:H7 and inhibits formation of heterocyclic amines in grilled ground beef patties. *J Agric Food Chem.* 57:1848-53.
- Juneja VK, Friedman M. 2007. Carvacrol, Cinnamaldehyde, Oregano oil, and thymol inhibit *Clostridium perfringens* spore germination and outgrowth in ground turkey during chilling. *J Food Prot.* 70:218-22.
- Juneja VK, Friedman M. 2008. Carvacrol and Cinnamaldehyde facilitate thermal destruction of *Escherichia coli* O157:H7 in raw ground beef. *J Food Prot.* 71:1604-11.
- Kim JM, Marshall MR, Cornell JA, Preston JF III, Wei CI. 1995. Antibacterial activity of carvacrol, citral, and geraniol against *Salmonella typhimurium* in culture medium and on fish cubes. *J Food Sci.* 60:1364-68.
- Kisko G, Roller S. 2005. Carvacrol and p-cymene inactivate *Escherichia coli* O157:H7 in apple juice. *Bio Med Central Microbiology.* 1-9.
- Koutsomanis K, Lambropoulo K, Nychas GJ. 1999. A predictive model for the nonthermal inactivation of *Salmonella enteritidis* in a food model system supplemented with a natural antimicrobial. *Int J Food Microbiol.* 49:63-74.
- Lagouri VG, Blekas M, Tsimidou S, Kokkini S, and Boskou D. 1993. Composition and antioxidant activity of essential oils from oregano plants grown wild in Greece. *Z. Lebensm Unters. For-schung.* 197:20-23.

Lamber, R.J.W., Skandamis, P.N., Coote, P., and nychas. 2001. A sudy of the minimum inhibitory concentration and mode if action of oregano essential oil, thymol and ccarv. J Appl Microbiol. 91:453-62.

Mecham DK, Olcott HS. 1949. Phosvitin, the principal phosphoprotein of egg yolk. J Am Chem Soc. 74:3670-76.

Meljlholm O, Dalgaard P. 2002. Antimicrobial effect of essential oils on the seafood spoilage microorganism *Photobacterium phosphoreum* in liquid media and fish products. Lett Appl Microbiol. 34:27-1.

Nychas, G.-J. E., and P.N. Skandamis. 2003. Antimicrobials form herbs and spices, p. 176-200. In. S. Roller (ed.), Natural antimicrobials for the minimal processing of foods. Woodhead Publishing LTd., Cambridge, United Kingdom.

Obaidat MM, Frank JF. 2009. Inactivation of *Escherichia coli* O157:H7 on the intact and damaged portions of lettuce and spinach leaves by using allyl isothiocyanate carvacrol, and cinnamaldehyde in vapor phase. J Food Prot. 72:2046-55.

Oussalah M, Caillet S, Saucier L, Lacroix M. 2007. Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli* O157:H7, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes*. Food Control. 18:414-20.

Ravishankar S, Zhu L, Reyna-Granados J, Law B, Joens L, Friedman M. 2010. Carvacrol and cinnamaldehyde inactivate antibiotic-resistant *Salmonella enterica* in buffer and on celery and oysters. J Food Prot. 73:234-40.

Roller S, Seedhar P. 2002. Carvacrol and cinnamic acid inhibit microbial growth in fresh-cut melon and kiwifruit at 4° and 8 °C. *Lett Appl Microbiol.* 35:390-4.

Roller, S., and R. G. Board. 2003. Naturally occurring antimicrobial systems, p. 262-290. *in* N. J. Russell and G.W. Gould (ed.), *Food Preservatives*, 2nd ed. Kluwer Academic, New York, N.Y.

Sattar Khan MA, Nakamura S, Ogawa M, Akita E, Azakami H, Kato A. 2000. Bactericidal action of egg yolk phosphovitin against *Escherichia coli* under thermal stress. *J Agric Food Chem.* 48:1503-6.

Sikkema J, de Bont JAM, Poolman B. 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol Rev.* 59:201-22.

Sivropoulou A, Papanikolaou E, Nikolaou C, Kokkini S, Lanaras T, Arsenakis M. 1996. Antimicrobial and cytotoxic activities of origanum essential oils. *J Agric Food Chem.* 44:1201-05.

Smith-Palmer A, Stewart J, and Fye L. 2001. The potential application of plant essential oils as natural food preservatives in soft cheeses. *Food Microbiol.* 18:463-70.

Sofos JN, Beuchat LR, Davidson MP, and Johnson EA. 1998. Naturally occurring antimicrobials in food. *Regul Toxicol Pharm.* 28:71-2.

Solomakos N, Govaris A, Koidis P, Botsoglou N. 2008. The antimicrobial effect of thyme essential oil, nisin, and their combination against *Listeria monocytogenes* in minced beef during refrigerated storage. *Food Microbiol.* 25:120-27.

- Taborsky G. 1963. Interaction between phosvitin and iron and its effect on a rearrangement of phosvitin structure. *Biochemistry*. 2:266-71.
- Ultee A, Slump RA, Steging G, Smid EJ. 2000b. Antimicrobial activity of carvacrol toward *Bacillus cereus* on rice. *J Food Prot*. 63:620-24.
- Valero, M, Frances, E. 2006. Synergistic bactericidal effect of carv, cinnamaldehyde or thymol and refrigeration to inhibit *Bacillus cereus* in carrot broth. *Food Microbiol*. 26:68-73.
- Valero M, Salmeron MC. 2003. Antibacterial activity of 11 essential oils against *Bacillus cereus* in tyndallized carrot broth. *Int J Food Microbiol*. 85:73-1.
- Veldhuizen EJA, Tjeerdsma-Van Bokhoven JLM, Zweijter C, Burt SA, Haagsman HP. 2006. Structural requirements for the antimicrobial activity of carvacrol. *J Agric Food Chem*. 54:1874-9.
- Veldhuizen, EJA, Creutzberg TO, Burt SA, and Haagsman HP. 2007. Low temperature and binding to food components inhibit the antibacterial activity of carvacrol against *Listeria monocytogenes* in steak tartare. *J Food Prot*. 70:2127-32.
- Webb J, Multani JS, Saltman P, Beach NA, Gray HB. 1973. Spectroscopic and magnetic studies of iron (III) phosvitins. *Biochemistry*. 12:1797-02.

TABLE 1. ^aIngredients in dehydrated onion and mushroom soup/dip mix.

Cornstarch
Dehydrated onions
Salt
Monosodium glutamate
Maltodextrin
Wheat flour
Dehydrated mushrooms
Partially hydrogenated soybean oil
Hydrolyzed protein (corn, soy, wheat)
Autolyzed yeast extract
Caramel color
Onion powder
Guar gum
Garlic powder
Natural flavors
Disodium guanylate
Disodium inosinate

*Sodium 700 mg- 29%

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

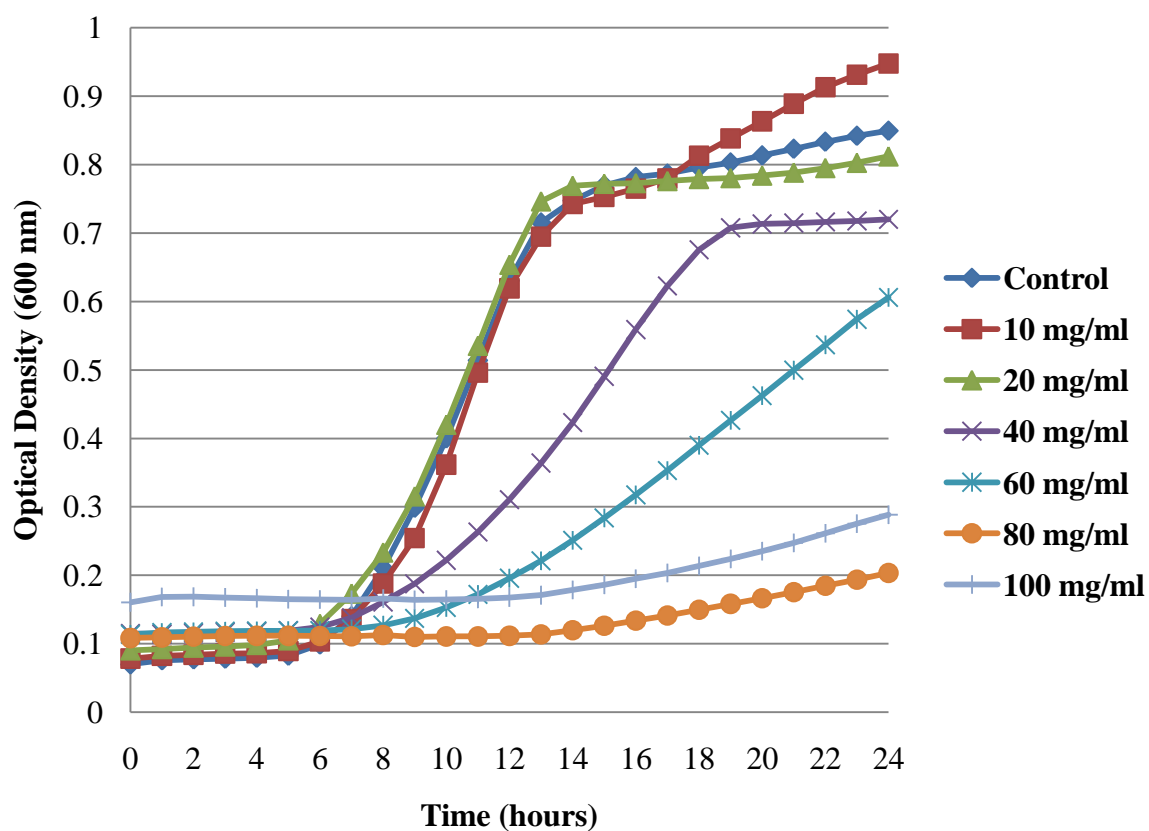


Figure 1. Growth of *Staphylococcus aureus* in BHI broth (35 °C) supplemented with various concentrations of phosvitin.

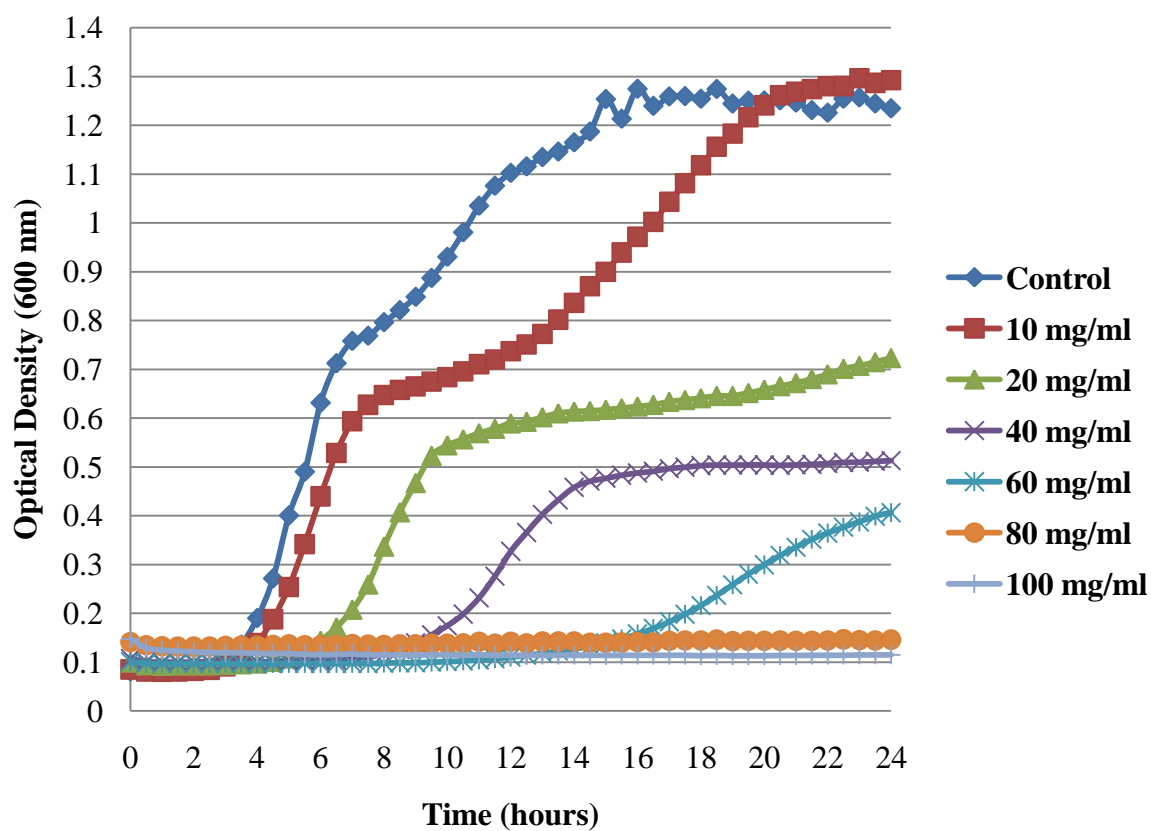


Figure 2. Growth of *Escherichia coli* O157:H7 in BHI broth (35 °C) supplemented with various concentrations of phosvitin.

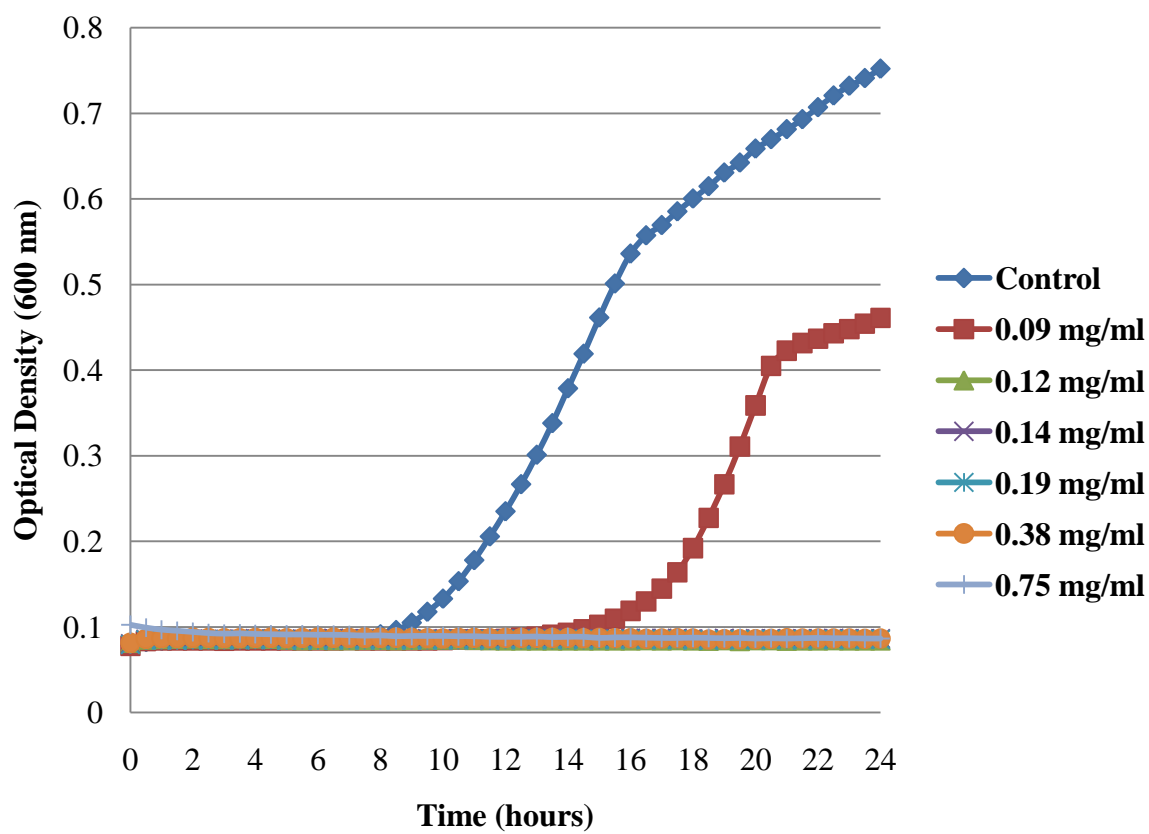


Figure 3. Growth of *Staphylococcus aureus* in BHI broth (35 °C) supplemented with various concentrations of carvacrol.

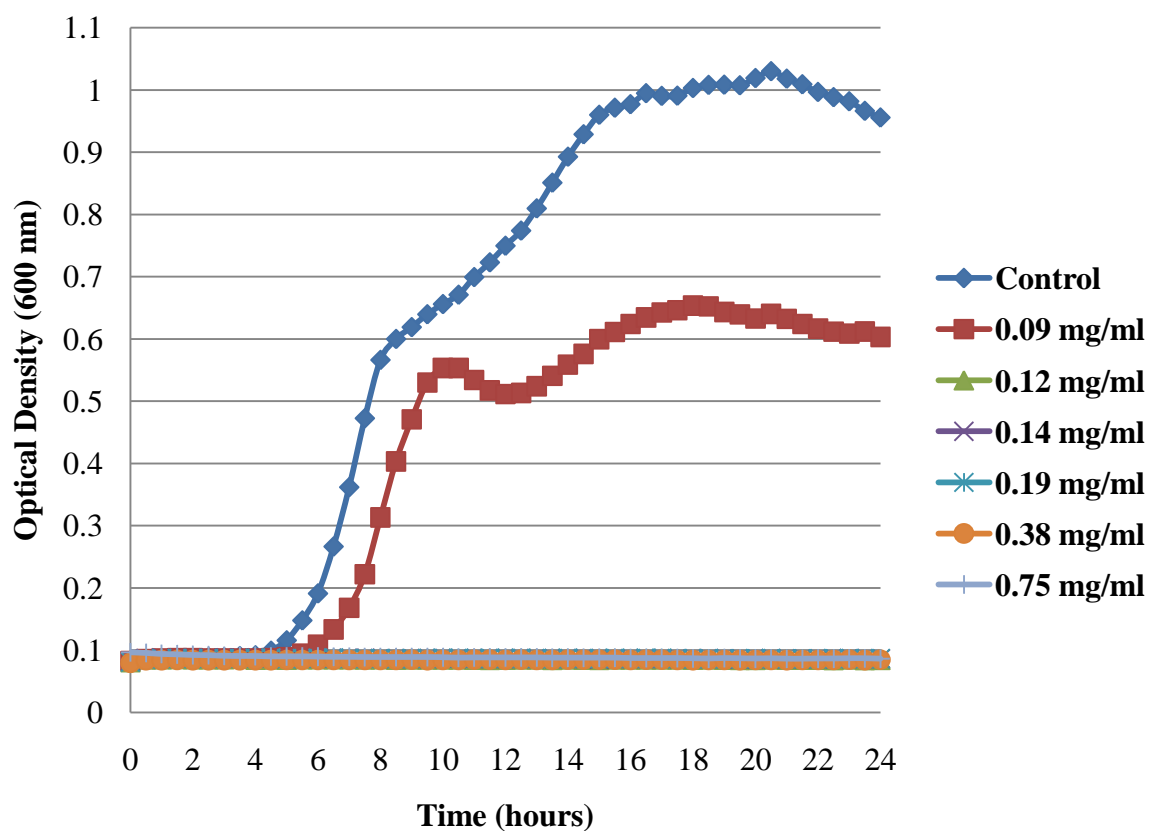


FIGURE 4. Growth of *Escherichia coli* O157:H7 in BHI broth (35 °C) supplemented with various concentrations of carvacrol.

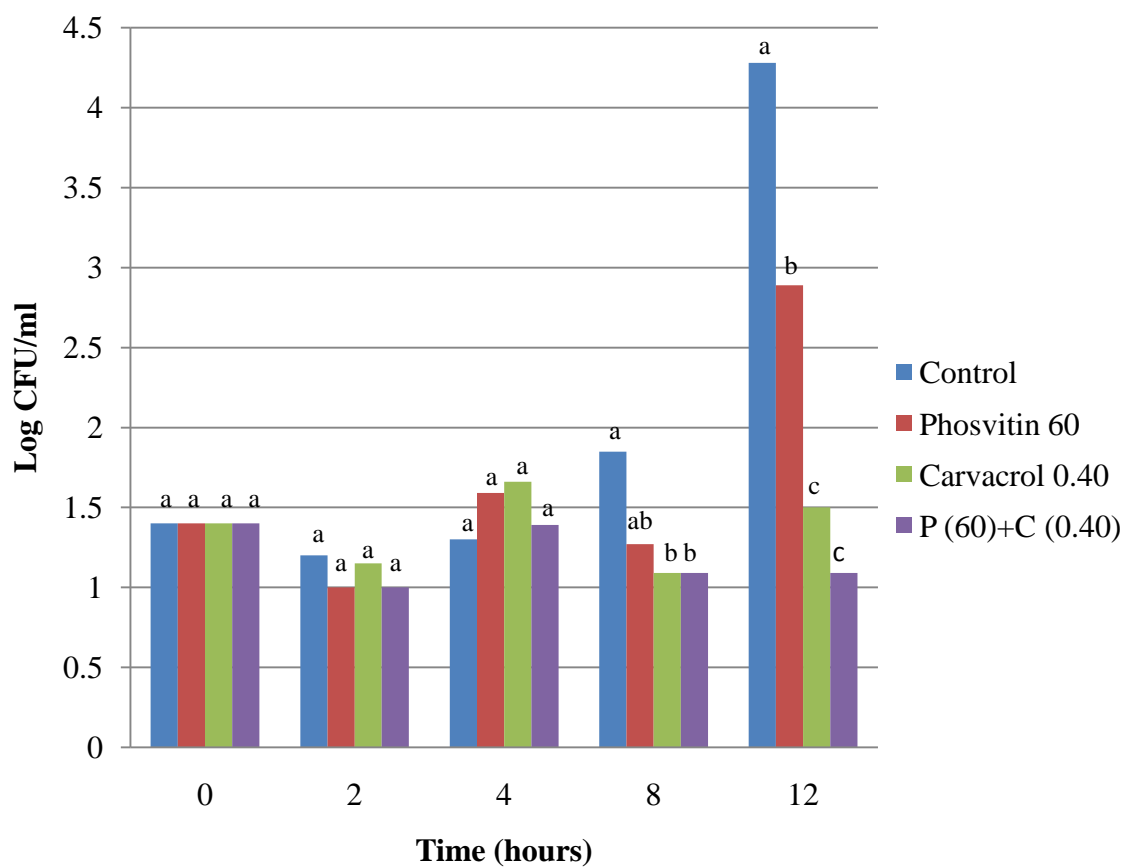


FIGURE 5. The effect of phosvitin (60 mg/ml), carvacrol (0.40 mg/ml) and their combinations against background microflora in soup at 35 °C.

TABLE 2. Antibacterial effectiveness of phosvitin (60 mg/ml) alone or combined with carvacrol against *Staphylococcus aureus* in onion mushroom soup held at 12 °C for 8 days.

Treatment (mg/ml)	Viable count (Log ₁₀ CFU/ml) ^x			
	2d	4d	6d	8d
Control	5.02 ± 0.54ax	4.16 ± 0.20aby	4.29 ± 0.05axy	4.32 ± 0.25axy
Phosvitin (60)	3.76 ± 0.67abx	3.79 ± 0.44abx	3.60 ± 0.48ax	3.40 ± 0.32abx
Carvacrol (0.20)	4.55 ± 0.05ax	4.36 ± 0.92ax	3.97 ± 0.51ax	3.88 ± 0.71abx
Carvacrol (0.40)	2.70 ± 0.49bx	2.59 ± 1.03bx	2.05 ± 0.63bx	0.80 ± 0.72cx
Phos + Car (0.20)	3.81 ± 0.60abx	3.47 ± 0.49abx	3.19 ± 0.63abx	2.68 ± 0.77bx
Phos + Car (0.40)	0.68 ± 1.18cx	0.00 ± 0.00cx	0.00 ± 0.00cx	0.00 ± 0.00cx

^xEach reported value for viable count represents the mean (standard deviation) of three independent replications of the experiment

^{a,b,c}Means with a different letter within a column differ significantly (P<0.05)

^{x,y,z}Means with a different letter within a row differ significantly (P<0.05)

TABLE 3. Antibacterial effectiveness of phosvitin (60 mg/ml) alone or combined with carvacrol against *Escherichia coli* O157:H7 in onion mushroom soup held at 12 °C for 8 days.

Treatment (mg/ml)	Viable count (Log ₁₀ CFU/ml) ^x			
	2d	4d	6d	8d
Control	4.80 ± 0.20ax	5.66 ± 1.18ax	6.74 ± 3.37ax	6.90 ± 3.47ax
Phosvitin (60)	4.45 ± 0.74ax	4.54 ± 0.38abx	4.50 ± 0.32abx	4.62 ± 0.84ax
Carvacrol (0.20)	3.82 ± 0.36abx	3.67 ± 1.53abx	3.63 ± 3.71abx	3.09 ± 5.35ax
Carvacrol (0.40)	2.41 ± 0.74bx	0.00 ± 0.00cy	0.00 ± 0.00by	0.00 ± 0.00ay
Phos + Car (0.20)	3.79 ± 0.52abx	2.99 ± 0.36bx	1.70 ± 1.87abx	1.20 ± 2.08ax
Phos + Car (0.40)	0.75 ± 0.77cx	0.00 ± 0.00cx	0.00 ± 0.00bx	0.00 ± 0.00ax

^xEach reported value for viable count represents the mean (standard deviation) of three independent replications of the experiment

^{a,b,c}Means with a different letter within a column differ significantly (P<0.05)

^{x,y,z}Means with a different letter within a row differ significantly (P<0.05)

TABLE 4. Antibacterial effectiveness of phosvitin (60 mg/ml) alone or combined with carvacrol against *Staphylococcus aureus* in onion mushroom soup held at 35 °C for 24 hours.

Treatment (mg/ml)	Viable count (Log ₁₀ CFU/ml) ^x			
	4h	8h	12h	24h
Control	5.60 ± 0.58az	7.2 ± 0.58ay	8.28 ± 0.74axy	8.83 ± 0.25ax
Phosvitin (60)	4.38 ± 0.88ax	4.78 ± 1.22bx	5.07 ± 1.54bcx	6.06 ± 1.44abx
Carvacrol (0.20)	4.96 ± 0.69ay	6.08 ± 0.62abx	6.77 ± 0.57abx	7.48 ± 0.24abx
Carvacrol (0.40)	4.15 ± 0.86ax	4.05 ± 0.80bx	3.85 ± 0.94cx	2.50 ± 0.35cdx
Phos + Car (0.20)	4.11 ± 0.88ax	4.43 ± 0.96bxy	4.03 ± 1.09bcx	5.00 ± 1.94bcx
Phos + Car (0.40)	3.69 ± 1.03ax	3.72 ± 0.85bx	3.51 ± 1.07cx	2.05 ± 0.56dx

^xEach reported value for viable count represents the mean (standard deviation) of three independent replications of the experiment

^{a,b,c}Means with a different letter within a column differ significantly (P<0.05)

^{x,y,z}Means with a different letter within a row differ significantly (P<0.05)

TABLE 5. Antibacterial effectiveness of phosvitin (60 mg/ml) alone or combined with carvacrol against *Escherichia coli* O157:H7 in onion mushroom soup held at 35 °C for 24 hours.

Treatment (mg/ml)	Viable count (Log ₁₀ CFU/ml) ^x			
	4h	8h	12h	24h
Control	7.59 ± 0.07az	9.62 ± 0.30ay	9.99 ± 0.12ay	10.69 ± 0.27ax
Phosvitin (60)	4.54 ± 0.95bcy	5.29 ± 1.51bcy	6.12 ± 0.05aby	9.23 ± 0.17ax
Carvacrol (0.20)	4.98 ± 0.36z	8.32 ± 0.05aby	9.23 ± 0.11ax	8.89 ± 0.08ax
Carvacrol (0.40)	2.35 ± 0.82cdx	3.89 ± 2.16cdx	4.37 ± 3.79bcx	5.43 ± 4.71abx
Phos + Car (0.20)	4.52 ± 0.81bcx	4.63 ± 0.84cdx	5.44 ± 1.43abcx	5.11 ± 4.43abx
Phos + Car (0.40)	1.58 ± 1.37dx	1.32 ± 1.22dx	1.26 ± 1.20cx	0.80 ± 0.72bx

^xEach reported value for viable count represents the mean (standard deviation) of three independent replications of the experiment

^{a,b,c}Means with a different letter within a column differ significantly (P<0.05)

^{x,y,z}Means with a different letter within a row differ significantly (P<0.05)

CHAPTER 5

THE ANTIMICROBIAL EFFECT OF PHOSVITIN, NISIN AND COMBINATION AGAINST *SALMONELLA ENTERICA*, *ESCHERICHIA COLI* O157:H7 AND *STAPHYLOCOCCUS AUREUS* IN A COMMERCIAL TYPE SOUP AT 12 °C AND 35 °C

Shecoya White¹, Aubrey Mendonca^{1*}, Aura Daraba³, and Dong Ahn²

¹Department of Food Science and Human Nutrition, and ²Department of Animal Science, Iowa State University, Ames IA, USA 50011; ³Faculty of Food Science and Engineering, University “Dunarea de Jos” Galati, Romania.

*Corresponding author: Department of Food Science and Human Nutrition, 2312 Food Sciences Bldg., Iowa State University, Ames, IA, USA 50011.

Phone: (515) 294-9078. Fax: (515) 294-8181. Email: amendon@iastate.edu.

Key words: *Salmonella*, *Escherichia*, *Staphylococcus*, phosvitin, nisin, soup

Abstract

The objective of this research was to identify antimicrobial activities of phosvitin, or nisin alone, or in combination against *Salmonella enterica*, *Escherichia coli* O157:H7 and *Staphylococcus aureus* in brain heart infusion (BHI) broth and abused soup held at unsafe temperatures. Broth supplemented with phosvitin (10 - 100 mg/ml), nisin (500 or 1000 IU) or combinations was inoculated (10^5 CFU/ml) with *S. enterica*, *E. coli* O157:H7 or *S. aureus*. Broth or soup without antimicrobials served as control. Growth of *S. enterica*, *E. coli*, and *S. aureus* in BHI (35 °C, 24 hours) was monitored using a Bioscreen C turbidometer (OD 600nm). Growth of the pathogens in soup (12 °C, 8 days and 35 °C, 24 hours) were monitored by surface plating. In BHI broth minimum inhibitory concentration (MIC) of phosvitin was 80 mg/ml for both *S. enterica* and *E. coli* O157:H7 and greater than 100 mg/ml for *S. aureus*. Phosvitin at the concentration range of 20-100 mg/ml inhibited growth of *S. enterica* and *E. coli* O157:H7; 80 mg/ml was bacteriostatic for both organisms. *S. aureus* was less sensitive to phosvitin compared to the two Gram-negative pathogens. For *S. enterica* and *E. coli* O157:H7 control cultures grew rapidly in soup and reached about 6.90 log CFU/ml in 8 days.

Phosvitin inhibited growth of *S. enterica* and *E. coli* O157:H7 for 6 and 8 days, respectively whereas, nisin (1000 IU) was ineffective in preventing growth of those pathogens. No growth of *S. aureus* occurred in control or treated soup at 12 °C. Irrespective of storage temperature (12 °C or 35 °C), phosvitin combined with either concentration of nisin did not offer enhanced antibacterial effect above that provided by phosvitin used alone.

Introduction

Foodborne illnesses caused by human enteric pathogens such as *Salmonella enterica*, *Escherichia coli* O157:H7 and *Staphylococcus aureus* are a significant problem and cause major health concerns annually (Mead et al., 1999). For several decades synthetic food preservatives such as nitrites, sulfites, and parabens have been used to control foodborne pathogens and spoilage microorganisms (Jay, 2005). The extensive use of synthetic chemicals for antimicrobial or other functions has resulted in the widespread movement towards use of natural antimicrobials as consumers become more health conscious. The food industry is looking to use “natural”, cost efficient antimicrobials from plant, microbial and/or animal sources, as replacements. The use of hurdle technology will allow natural antimicrobials to be combined, increasing microbial safety, decreasing high individual concentrations and widening the spectrum of antimicrobial activity (Leistner, 2000).

Phosvitin is a metal chelating protein found in egg yolk. Egg yolk participates in embryonic development by providing essential nutrients for the developing embryo. Phosvitin, a 35 kDa phosphoglycoprotein, is the major egg yolk protein. It is currently recognized as the most phosphorylated protein in nature. Over half of the amino acids present in phosvitin are serine, of which 90% are phosphorylated (Taborsky, 1983; Byrne et al., 1984; Clark, 1985). The numerous phosphorylated serine residues have a strong affinity for divalent metals and contribute to the chelating ability of phosvitin (Osaki et al., 1975; Grogan and Taborsky, 1987). This property allows binding to nutrients in the environment and binding to membranes of bacteria. To date there is only one published paper addressing

the antimicrobial properties of phosvitin (Sattar Khan et al., 2000). These authors found that the combination of phosvitin and heat (50 °C) inactivated *Escherichia coli* O157:H7.

Nisin, is produced as a defense response to some lactic acid bacteria (LAB), specifically *Lactococcus lactis* subspecies *lactis*. To date, nisin is the only Generally Recognized As Safe (GRAS) commercially available bacteriocin approved for use as a preservative additive in over fifty countries (FAO/WHO, 1969; FDA, 1988; Jay, 2000). Nisin has been used as a natural antimicrobial peptide and is bactericidal against some strains of following Gram-positive bacteria: *Alicyclobaccillus* (Komitopoulou et al., 1999), *Bacillus* (Jaquette and Beuchat, 1998), *Brochothrix* (Cutter and Siragusa, 1996), *Clostridium* (Okereke and Montville, 1992), *Enterococcus* (Laukova, 1995), *Lactobacillus* (Chun and Hancock, 2000) *Listeria* (Knight et al., 1999), *Micrococcus* (Dutreux et al., 2000), and *Staphylococcus* (Thomas and Wimpenny, 1996). Nisin is unable to penetrate the cell wall of yeast and Gram-negative bacteria (Kordel et al., 1989; Boziaris and Adams, 1999). In susceptible vegetative cells the primary site of action by nisin is the cytoplasmic membrane (Abee et al., 1995; Kuwano et al., 2005). This bacteriocin has the ability to bind to the membrane and form pores that destroy the membrane integrity. Pore formation leads to leakage of K⁺ ions and ATP, depletion of the proton motive force, and depolarization of transmembrane potential, resulting in cell death (Sahl, 1991; Bruno and Montville, 1993; Millette et al., 2004).

While there is an extensive knowledge base on the antimicrobial effects of nisin against various foodborne pathogens, such published research involving phosvitin is scarce. In fact there is only one published paper on the effects of phosvitin on *E. coli* O157:H7 (Sattar Khan, 2000). Also, to our knowledge there is no published research involving the

combined use of phosvitin and nisin against *Salmonella enterica*, *Escherichia coli* O157:H7, or *Staphylococcus aureus*. The first objective of the present study was to establish the minimum inhibitory concentration of phosvitin and nisin for *S. enterica*, *E. coli* O157:H7 and *S. aureus* in laboratory broth medium. The second objective was to evaluate the antimicrobial effectiveness of phosvitin, nisin, and their combination against those same foodborne pathogens in a commercial-type onion mushroom soup.

Materials and Methods

Bacterial cultures and culture conditions. Five serotypes of *Salmonella enterica* (Enteritidis-ATCC13076, Heidelberg, Typhimurium-ATCC 14802, Gaminara-8324, 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 used in the present study. All cultures were obtained from the culture collection of the Microbial Food Safety Laboratory, Iowa State University, Ames, IA. Stock cultures were kept frozen (-70 °C) in Brain Heart Infusion (BHI) broth (Difco, Becton, Dickinson, Sparks, MD) supplemented with 10% (vol/vol) glycerol. Stock cultures were activated in BHI broth (pH 7.2) and incubated at 35 °C. At least two consecutive 18 to 22-h transfers of each stock culture were carried out before using the cells as inocula in each experiment.

Preparation of inoculum. An equal volume of the individual working cultures of *L. monocytogenes* or *S. enterica* 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 (American

Laboratory Trading, Inc., East Lyme, CT) and washed once in 0.1% (wt/vol) peptone. The pelleted cells were suspended in fresh 0.1% (wt/vol) peptone to obtain a final viable cell concentration of approximately 10^9 CFU ml⁻¹. Viable counts of the washed cell suspensions were evaluated by surface plating serially diluted (10-fold) samples on tryptic soy agar supplemented with 0.6% yeast extract. The individual cell suspensions were used to inoculate BHI broth or commercial onion mushroom soup.

Antimicrobials. Phosvitin was supplied by Dr. Dong Ahn from the Animal Science Department at Iowa State University, Ames, IA. Nisin was purchased from Danisco (Nisaplin, Danisco Cultor USA) and was reported to be 2.5% pure (1000 IU/mg).

Total phenolic content of carvacrol. The concentration of total phenolics of carvacrol was determined using the method described by Waterman and Mole (1994). The assay involves reduction of ferric iron to the ferrous state by phenolic compounds that results in the formation of the Prussian blue complex $\text{Fe}_4[\text{Fe}(\text{CN})_6]_3$ with a potassium ferricyanide reagent. The Prussian blue complex is determined colorimetrically (Khiyami et al., 2005). Two separate 1% (vol/vol) solutions of carvacrol in 10% (vol/vol) ethanol and deionized water were prepared. The absorbance of three sub- samples from each solution was measured at 720 nm and the total phenolic content expressed as the number of catechin equivalents x 100.

Preparation of treatment solutions for Bioscreen C assay. BHI broth with added phosvitin (10, 20, 40, 60, 80, 100 mg/ml) or nisin (500 or 1000 IU) were filter sterilized using 0.22 μm pore size Millipore filters (Fisher Scientific, Pittsburgh, PA). Samples (2.5-ml) of the treatment solutions and control (BHI with no added antimicrobial) were each inoculated with 25 μl of diluted (1:100) *S. enterica*, *E. coli* O157:H7 or *S. aureus* cell

suspension to obtain a final concentration of approximately 10^5 CFU/ml of sample.

Bioscreen C assay. Aliquots (200- μ l) of inoculated samples were added in triplicate to the wells of microtiter plate for the Bioscreen C Turbidometer (Growth Curves, Piscataway, NJ), an automated microbial growth analyzer and incubator. Plates were incubated in the Bioscreen C at 35 °C for 24 h and the instrument was programmed to take optical density (OD) measurements at 600 nm every 30 min, with shaking of samples before each OD reading. Minimum inhibitory concentration (MIC) was defined as the lowest treatment concentration that completely inhibited microbial growth for 24 hr (< 0.05 OD unit increase).

Preparation and inoculation of soup. Dehydrated onion mushroom soup/dip mix was purchased from a local grocery store. The soup mix was hydrated with sterile distilled water and prepared according to the directions stated on the package. Soup ingredients are shown in Table 1. After boiling, the soup was tempered to ~ 35 °C and 10-ml portions of the soup were aseptically transferred into sterile 30 ml screw cap pyrex tubes. Phosvitin, nisin or combinations were added to each tube to give the following concentrations: phosvitin (60 mg/ml), nisin (500 IU or 1000IU), phosvitin (60 mg/ml) + nisin (500 IU) (*S. aureus* only) and phosvitin (60 mg/ml) + nisin (1000 IU). Phosvitin was added to the tube prior to the addition of the soup to prevent aggregation and facilitate its dissolving in the soup. Tubes of soup without added antimicrobial served as control. The treatments were then warmed or cooled to the target temperature. Tubes of soup with added antimicrobials were mixed by vortexing and inoculated with a suspension of washed cells of the foodborne pathogens to give a final cell concentration of approximately 10^5 CFU/ml for each pathogen. After

inoculation, each tube of soup was gently mixed by vortexing and held at either 12 °C or 35 °C.

Microbiological analysis. Inoculated tubes of soup held at 12 °C were tested for survivors of the pathogens every 2 days for a total of 8 days whereas, soup held at 35 °C were tested at 4, 8, 12 and 24 h. Ten-fold serial dilutions of the soup were prepared using 0.1% (vol/vol) peptone with added Tween 80. Aliquots (1.0 ml or 0.1ml) of the soup or diluted samples of soup were surface plated (in duplicate) on xylose lysine desoxycolate (XLD) agar (for *S. enterica*), sorbitol MacConkey agar (for *E.coli*) and mannitol salt agar (for *S. aureus*). All inoculated plates were incubated at 35 °C for 48h before counting bacterial colonies.

Measurement of pH and water activity. The initial pH and water activity of each sample of soup including controls was measured at day 0 (prior to inoculation). Measurements of pH were also taken periodically during storage of the soup at 12 °C or 35 °C. 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).

Statistical analysis. Three independent replications of each experiment were performed. Mean numbers of survivors *S. enterica*, *E. coli* O157:H7, or *S. aureus* were statistically analyzed using SAS statistical software version 8.1 (SAS Institute Inc., Cary, N.C.). Treatment means were evaluated for statistically significant differences using Tukey's test. Significant differences were defined at $P < 0.05$ for all the experimental data.

Results

Inhibitory effect of phosvitin in BHI broth. Figures 1, 2 and 3 show the effect of various concentrations of phosvitin on the growth of *S. enterica*, *E. coli* O157:H7 and *S. aureus*, respectively, in Brain Heart Infusion (BHI) broth at 35 °C. The initial OD for all the cultures at time 0 was approximately 0.1. Phosvitin at the concentration range of 20 -100 mg/ml inhibited growth of *S. enterica* and *E. coli* O157:H7; 80 mg/ml was bacteriostatic for both organisms (Figures 1 and 2). Also increased lag phase and lower maximum OD values were observed with increased phosvitin concentrations. Compared to the two Gram-negative pathogens, *S. aureus* was less sensitive to phosvitin. Absolutely no growth inhibition of this pathogen occurred at phosvitin concentrations of 10 and 20 mg/ml. Also, growth of the pathogen occurred at 80 and 100 mg/ml which were bacteriostatic for *S. enterica* and *E. coli* O157:H7.

Inhibitory effect of phosvitin/nisin combinations in BHI broth. Figures 4, 5, and 6 show the effect of phosvitin and phosvitin/nisin combinations on growth of *S. enterica*, *E. coli* O157:H7, and *S. aureus* in BHI. Nisin (500 or 1000 IU) was ineffective against *S. enterica* and *E. coli* O157:H7; both pathogens grew equally well in control and in BHI with added nisin. All combinations of phosvitin and nisin were more inhibitory than nisin but similar in inhibitory action to phosvitin alone at the concentration corresponding to the phosvitin in the combination (Figures 4 and 5). For *S. aureus*, nisin (500 and 1000 IU) completely inhibited growth of the organism. Except for 20 mg/ml phosvitin, all other phosvitin concentrations (40 and 60 mg/ml) were growth inhibitory. Combinations of

phosvitin and nisin were less effective than nisin alone but similar in inhibitory action to phosvitin alone at the concentration corresponding to the phosvitin in the combination.

Antibacterial effect of phosvitin and nisin in soup at 12 °C. The total phenolic content of the 1% solution of carvacrol was 0.98 catechin equivalents. This result coincides with the manufacturer's claim of "up to 98% total polyphenols". The initial pH of the soup was 6.1 and water activity 0.994. Tables 2, 3, and 4 show viable counts of *S. enterica*, *E. coli* O157:H7 and *S. aureus*, respectively, in onion mushroom soup (12 °C) with phosvitin, nisin, or combinations of those antimicrobials. The initial viable count for *S. enterica*, *E. coli* O157:H7, or *S. aureus*, in soup was $\sim 10^5$ CFU/ml. For *S. enterica* and *E. coli* O157:H7 control cultures grew rapidly and reached about 6.90 log CFU/ml in 8 days. Phosvitin inhibited growth of *S. enterica* and *E. coli* O157:H7 for 6 and 8 days, respectively whereas, nisin (1000 IU) was ineffective in preventing growth of those pathogens. Viable counts of the pathogens in soup with the combination of phosvitin and nisin were not significantly different from those of control or other treatments (Figures 2 and 3).

No growth of *S. aureus* occurred in control or treated soup at 12 °C. In control soup viable numbers of *S. aureus* decreased and survivors remained constant throughout the storage period. Viable counts of the pathogen in soup with phosvitin (60 mg/ml) were not significantly ($P>0.05$) different from the control after 2 days. Both nisin (500 and 1000 IU) concentrations alone were significantly different from the control. At day 2 a log reduction of 2.75 and 3.19 was seen for 500 IU and 1000 IU, viable cells continued to decrease a through 8 days. Phosvitin combined with either concentration of nisin similarly increased the effectiveness of phosvitin alone.

Antibacterial effect of phosvitin and nisin in soup at 35 °C. Tables 5, 6, and 7 show the effect of phosvitin, nisin, and combinations against *S. enterica*, *E. coli* O157:H7 and *S. aureus* in onion mushroom soup (35 °C). In control soup viable numbers of *S. enterica* increased from 7.63 log at 4 hours to 11.31 log CFU/ml at 24 hours of incubation. Viable counts of the pathogen in soup with phosvitin alone were significantly ($P<0.05$) different from those of control for the first 12 hours of the study. Viable counts in soup with nisin (1000 IU) alone were not significantly ($P>0.05$) different from those of the control. Phosvitin plus nisin (1000 IU) also extended the lag phase of growth and was significantly different from other treatments and control at 24 hours of incubation (Table 5). At 24 hours the viable count of *S. enterica* was 2.16 log CFU/ml less than that of the control.

In control soup viable numbers of *E. coli* O157:H7 increased from 7.59 log at 4 hours to 10.69 log CFU/ml at 24 hours of incubation. Phosvitin (60 mg/ml) was bacteriostatic, limiting the growth of the microorganisms for the first 8 hours. Phosvitin was significantly ($P<0.05$) different from the control the entire duration of the study. Nisin (1000 IU) alone was similar to the control and was ineffective in growth suppression of *E. coli* O157:H7. The combination of phosvitin and nisin was similar to phosvitin alone for the first 12 hours. At 24 hours, this combination was the most effective reducing cell viability by 2.59 log CFU/ml.

In control soup viable numbers of *S. aureus* increased from 5.60 log at 4 hours to 8.83 log CFU/ml at 24 hours of incubation. Phosvitin alone was bacteriostatic for the first 12 hours, and was significantly ($P<0.05$) different from the control at 8 and 12 hours of the study. Both nisin (500 and 1000 IU) concentrations and combinations were significantly ($P<0.05$) different from the control for up to 12 hours. Nisin (500 and 1000) initially reduced

viable cells by 4 hours, and gradually increased as time progressed over the remaining 18 hours. After 12 hours both nisin concentrations were no longer different from the control, however the two combinations were significantly ($P < 0.05$) different from the control at 24 hours. Phosvitin combined with nisin 500 IU and nisin 1000 IU reduced survivors by 3.15 and 4.31, respectively, at 24 hours compared to the c-control.

Discussion

Naturally derived antimicrobials for food preservation may be extracted from plant, animal or microbial sources. Examples of such antimicrobials are phosvitin and nisin. Phosvitin is a glycoprotein from egg yolk (Mecham and Olcott, 1949). It is currently known for being the most phosphorylated protein in nature (Taborsky, 1983). Also it is known to have a very strong chelating ability, can bind up to 60 mol of Fe^{2+} per phosvitin molecule (Mecham and Olcott, 1949; Taborsky, 1963; Webb et al., 1973; Albright et al., 1984). Nisin, is a bacteriocin, produced by *Lactococcus lactis* spp. *lactis*. Currently it is the only bacteriocin approved for use in foods. The antimicrobial spectrum of nisin is limited to some species of gram-positive bacteria. It is generally ineffective against Gram-negative bacteria. However, when combined with chelating agents such as ethylenediametetraacetic acid (EDTA) enhanced bactericidal effects have been observed (Gill and Holly, 2000; Branen and Davidson, 2004).

In the present study we exploited the metal chelating ability of phosvitin to enhance the activity of nisin. We hypothesized that phosvitin, would destabilize the outer membrane of Gram-negative activity to allow nisin to penetrate the bacterial cell. In this regard, we used a combination of phosvitin and nisin to inhibit growth of pathogens in a laboratory

broth medium and in a commercial-type soup held at abusive temperatures. Phosvitin was inhibitory in a dose dependent manner against *S. enterica*, *E. coli O157:H7* and *S. aureus* in a laboratory broth medium. Nisin alone was ineffective against the Gram-negative bacteria, as expected. Interestingly the combination of phosvitin and nisin was not effective in inactivating the pathogens. In fact the antimicrobial effect of the combination was quite similar to the effect of phosvitin used alone. The results of the study suggest that the combination of phosvitin and nisin under these circumstances would not be cost efficient, due to possible requirements for higher concentrations of phosvitin to achieve the desired antimicrobial control. However, future studies should be conducted to determine phosvitin mechanism of action and test the efficacy of phosvitin peptides.

References

- Abee, T., Krockel, L., and Hill, C. 1995. Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. *Int J Food Microbiol.* 28: 169-185.
- Boziaris, I. S., and Adams, M. R. (1999). Effect of chelators and nisin produced in situ on inhibition and inactivation of gram negatives. *Int J Food Microbiology.* 53:105-113.
- Branen, J. K. and Davidson, P.K. 2004. Enhancement of nisin, lysozyme, and monolaurin antimicrobial activities by ethylenediaminetetraacetic acid and lactoferrin. *Int J Food Microbiol.* 90:63-74.
- Bruno, M.E. and Montville, T.J. 1993. Common mechanistic action of bacteriocins from lactic Acid bacteria. *Appl Environ Microbiol.* 59:3003-10.

- Byrne, B.,M., van het Schip, A.D., van de Klundert, J.A.M., Arnberg, A.C., Gruber, M., and Ab, G. 1984 Amino acid sequence of phosvitin derived from the nucleotide sequence of part of the chicken vitellogenin gene. *Biochemistry*. 23: 4275-4279.
- Chun, W. and Hancock, R.E. 2000. Action of lysozyme and nisin mixtures against lactic acid bacteria. *Int J Food Microbiol*. 60:25-32.
- Clark, R.C. 1985. The primary structure of avian phosvitins (contributions through the edman degradation of methylmercaptovitins prepared from the constituent phosphoproteins). *Int J Biochem*. 17:983-988.
- Cutter C.N. and Siragusa G.R. 1996. Reduction of *Brochothrix thermosphacta* on beef surfaces following immobilization of nisin in calcium alginate gels. *Lett App Microbiol*. 23:9-12.
- Dutreux, N., Notermans, S., Góngora-Nieto, M.M., Barbosa-Cánovas, G.V., and Swanson B.G. 2000. Effects of combined exposure of *micrococcus luteus* to nisin and pulsed electric fields. *Int J Food Microbiol*. 60:147-152.
- FAO/WHO Expert Committee on Food Additives. 1969. Specifications for identity and purity of some antibiotics. 12th Report. WHO Technical Report Series, No. 430.
- FDA (Food and Drug Administration). 1988. Nisin preparation: Affirmation of GRAS status as a direct human food ingredient. *Fed Reg*. 53:11247.
- Gill, A. O. and Holley, R. A. 2000. Inhibition of bacterial growth on ham and bologna by lysozyme, nisin and EDTA. *Food Res Int*. 33:83-90.

- Grogan, J., and Taborsky, G. 1987. Iron binding by phosvitin: variable mechanisms of release by phosvitin of diverse species characterized by different degrees of phosphorylation. *J. Inorg. Biochem.* 29:33-47.
- Jaquette C.B. and Beuchat L.R. 1998. Combined effects of pH, nisin, and temperature on growth and survival of psychrotrophic *Bacillus cereus*. *J Food Prot.* 61:563-570.
- Jay, J. M., Loessner, M. J., and Golden, D. A. 2005. *Modern Food Microbiology*, 7th edition, Springer Science + Business Media, LLC, Pubs., New York, NY.
- Jay, J.M., 2000. *Modern Food Microbiology*, sixth ed. Aspen Publishers, Inc., Gaithersburg, MD. p. 679.
- Knight K.P., Bartlett F.M., McKellar R.C., and Harris L.J. 1999. Nisin reduces the thermal resistance of *Listeria monocytogenes* Scott A in liquid whole egg. *J Food Prot.* 62:999-1003.
- Komitopoulou, E., Boziaris, I. S., Davies, E. A., Delves-Broughton, J. and Adams, M. R. 1999. *Alicyclobacillus acidoterrestris* in fruit juices and its control by nisin. *Int J Food Sci Technol.* 34:81-85.
- Kuwano, K., Tanaka, N., Shimizu, T., Nagatoshi, K., Nou, S., and Sonomoto, K. 2005. Dual antibacterial mechanisms of nisin Z against Gram-positive and Gram-negative bacteria. *Int J Antimicrob Agents.* 26:396-402.
- Lauková A. 1995. Inhibition of ruminal staphylococci and enterococci by nisin in vitro. *Lett Appl Microbiol.* 20:34-36.

Leistner, L. 2000. Basic aspects of food preservation by hurdle technology. *Int J Food Prot.* 55:181-186.

Mead, P.S., Slutsker, Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., and Tauxe, R.V. 1999. Food-related illnesses and death in the United States. *Emerg Infect Dis.* 5:607-625.

Millette, M., Smoragiewicz, W., and Lacroix, M. 2004. Antimicrobial potential of immobilized *Lactococcus lactis subsp. lactis* ATCC 11454 against selected bacteria. *J Food Prot.* 67:1184-1189.

Mulders, J. W. M., Boerrigter, I. J., Rollema, H. S., Siezen, R. J., and de Vos, W. M. 1991. Identification and characterization of the lantibiotic nisin Z, a natural nisin variant. *Eur J Biochem.* 201:581-584.

Okereke, A., and Montville, T.J. 1992. Nisin dissipates the proton motive force of the obligate anaerobe *Clostridium sporogenes* PA 3679. *Appl Environ Microbiol.* 58: 2463-2467.

Osaki, S., Sexton, R.C., Pascual, E., and Frieden, E. 1975. Iron oxidation and transferrin formation by phosvitin. *J Biochem.* 151:519-525.

Sahl, H.-G. 1991. Pore formation in bacterial membranes by cationic lantibiotics. In *Nisin and Novel Lantibiotics*, Jung, G. and Sahl, H.G., Eds., Escom, Leiden, The Netherlands. 347-358.

- Sattar Khan, M.A, Nakamura, S., Ogawa, M., Akita, E., Azakami, H., and Kato, A. 2000. Bactericidal action of egg yolk phosvitin against *Escherichia coli* under thermal stress. J Agric Food Chem. 48:1503-1506
- Taborsky, G. 1983. Phosvitin. Adv. Inorg. Biochem. 5:235-279.
- Thomas L.V. and Wimpenny J.W. 1996. Investigation of the effect of combined variations in temperature, pH, and NaCl concentration on nisin inhibition of *Listeria monocytogenes* and *Staphylococcus aureus*. Appl Environ Microbiol. 62:2006-2012.

TABLE 1. ^aIngredients in dehydrated onion and mushroom soup/dip mix.

Cornstarch
Dehydrated onions
Salt
Monosodium glutamate
Maltodextrin
Wheat flour
Dehydrated mushrooms
Partially hydrogenated soybean oil
Hydrolyzed protein (corn, soy, wheat)
Autolyzed yeast extract
Caramel color
Onion powder
Guar gum
Garlic powder
Natural flavors
Disodium guanylate
Disodium inosinate

*Sodium 700 mg- 29%

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

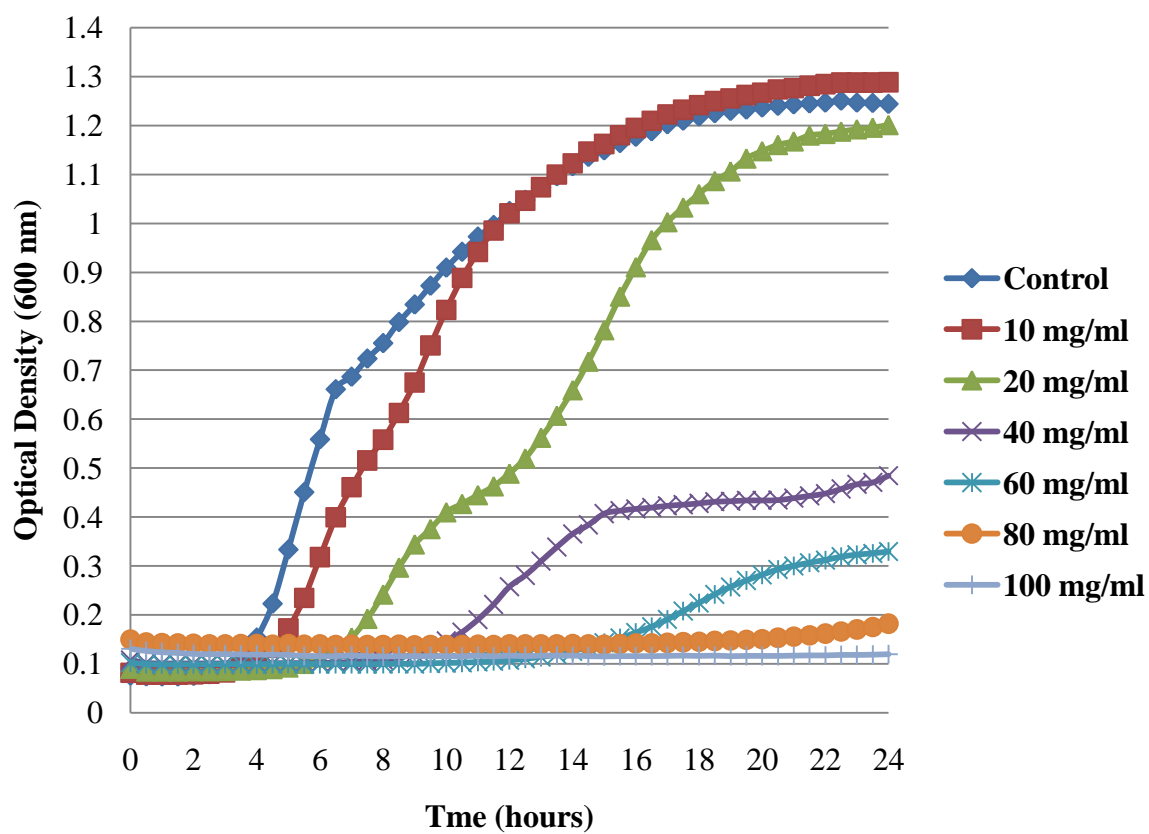


Figure 4. Growth of *Salmonella enterica* in BHI broth (35 °C) supplemented with various concentrations of phosvitin.

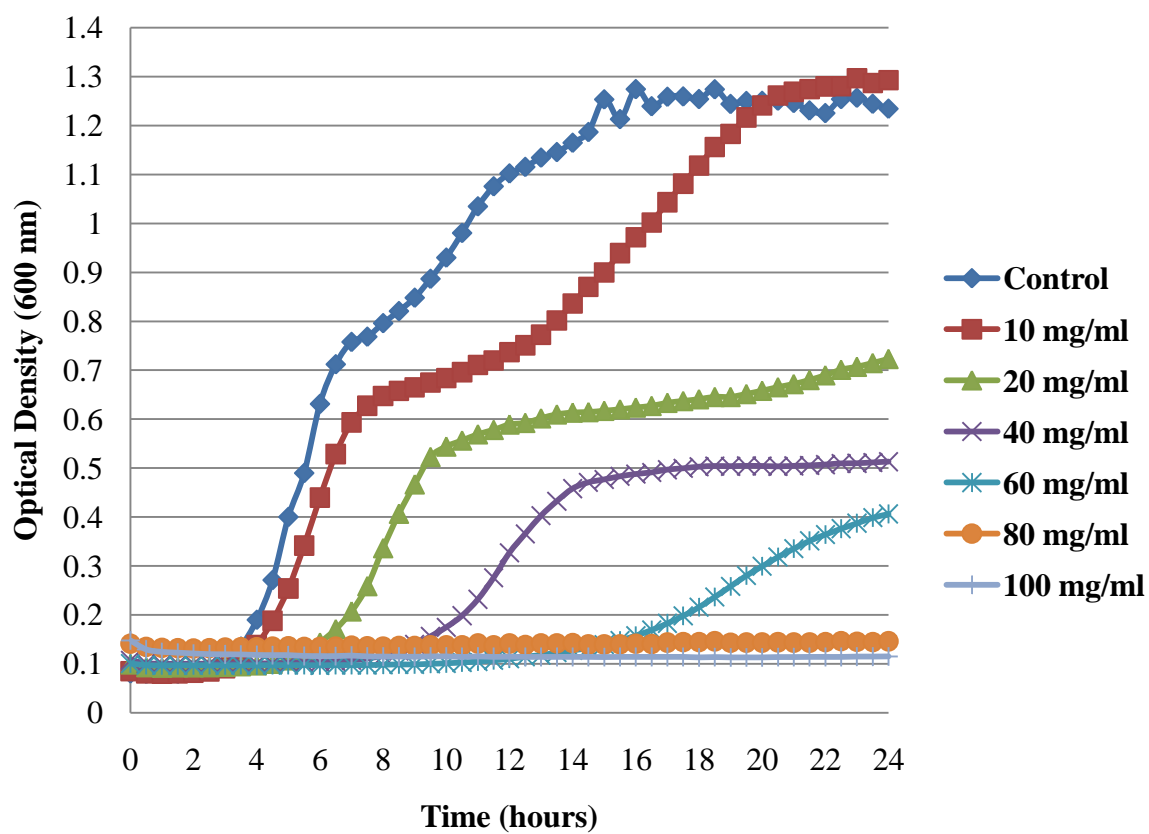


Figure 2. Growth of *Escherichia coli* O157:H7 in BHI broth (35°C) supplemented with various concentrations of phosvitin.

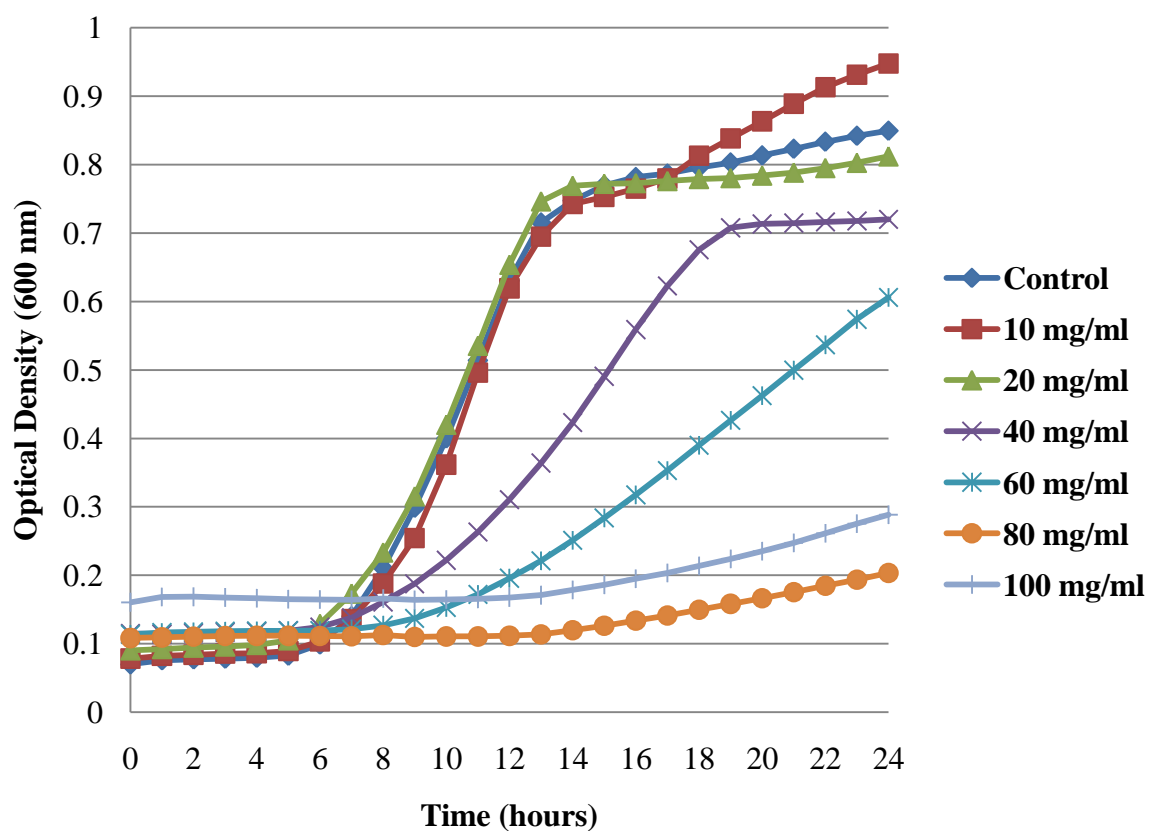


Figure 3. Growth of *Staphylococcus aureus* in BHI broth (35 °C) supplemented with various concentrations of phosvitin.

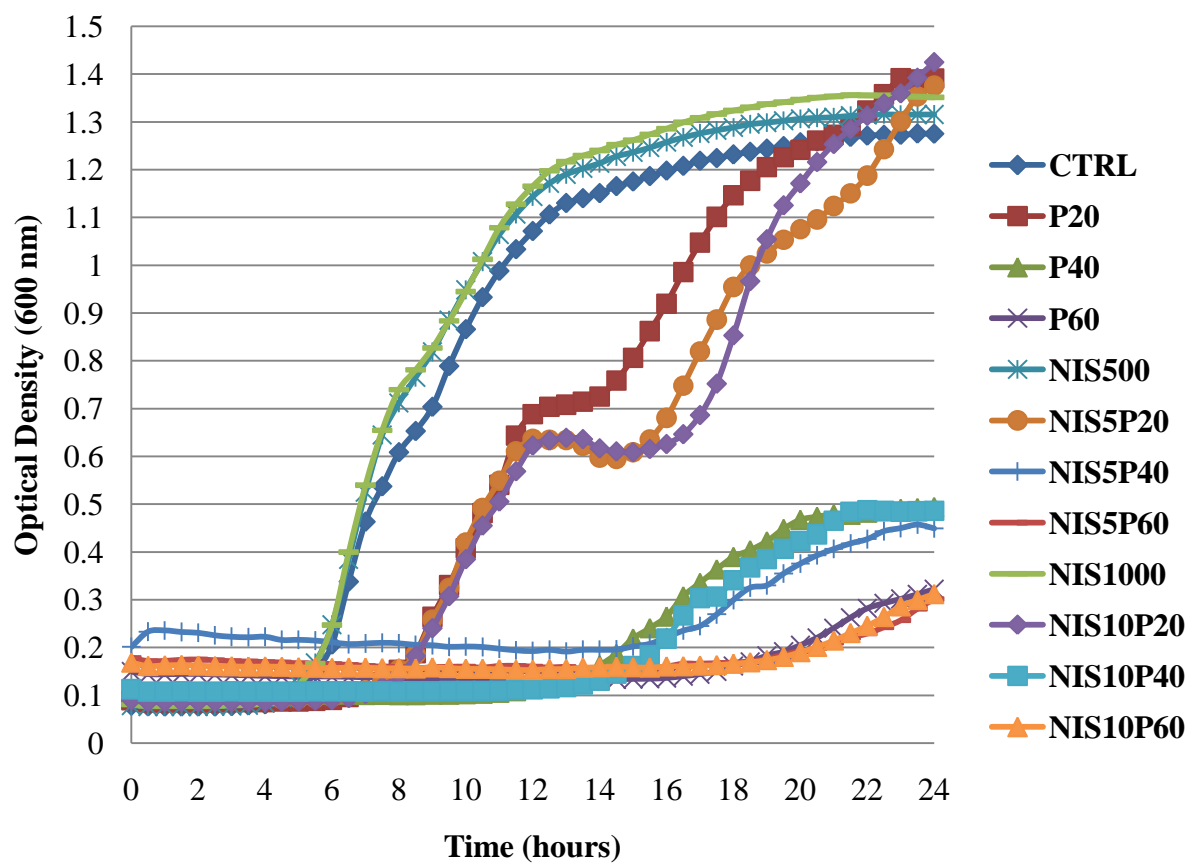


Figure 4. The effect of phosvitin combined with nisin against *Salmonella enterica* in BHI broth at 35 °C.

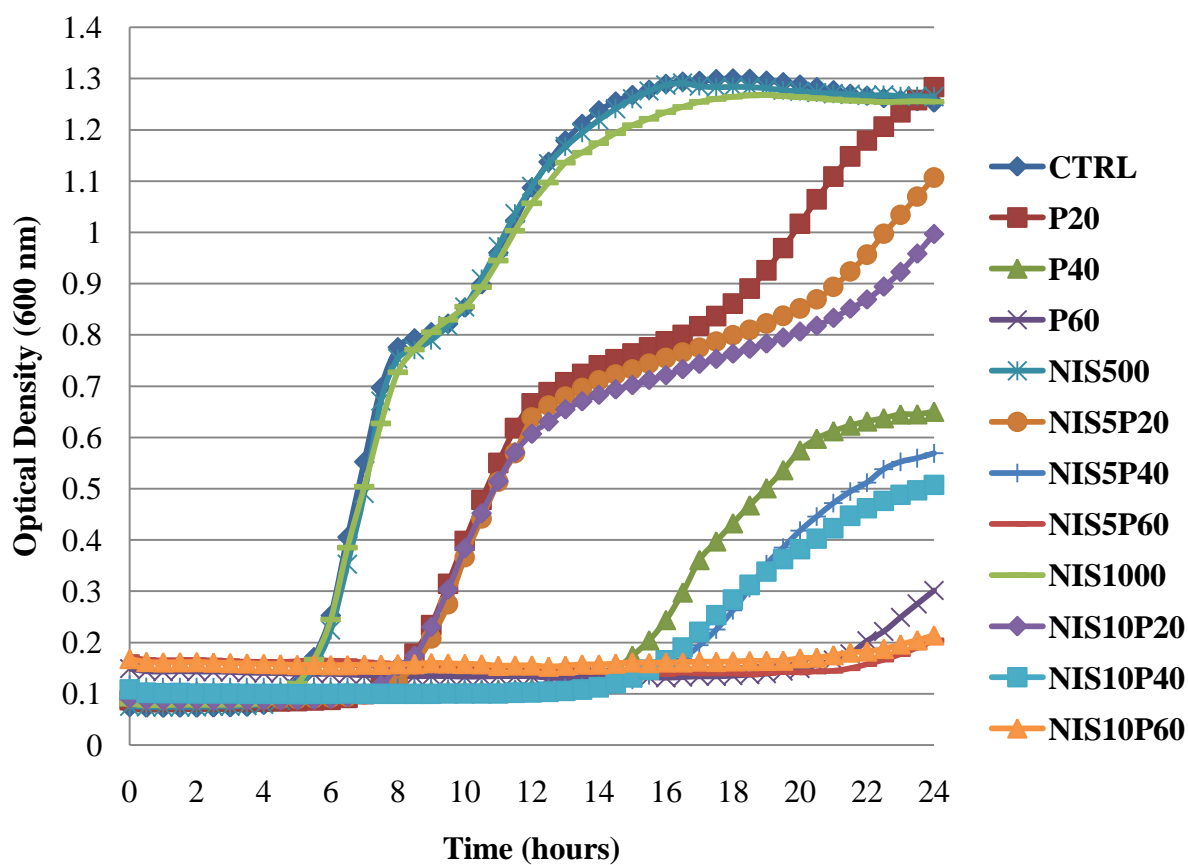


Figure 5. The effect of phosvitin combined with nisin against *Escherichia coli* O157:H7 in BHI broth at 35 °C.

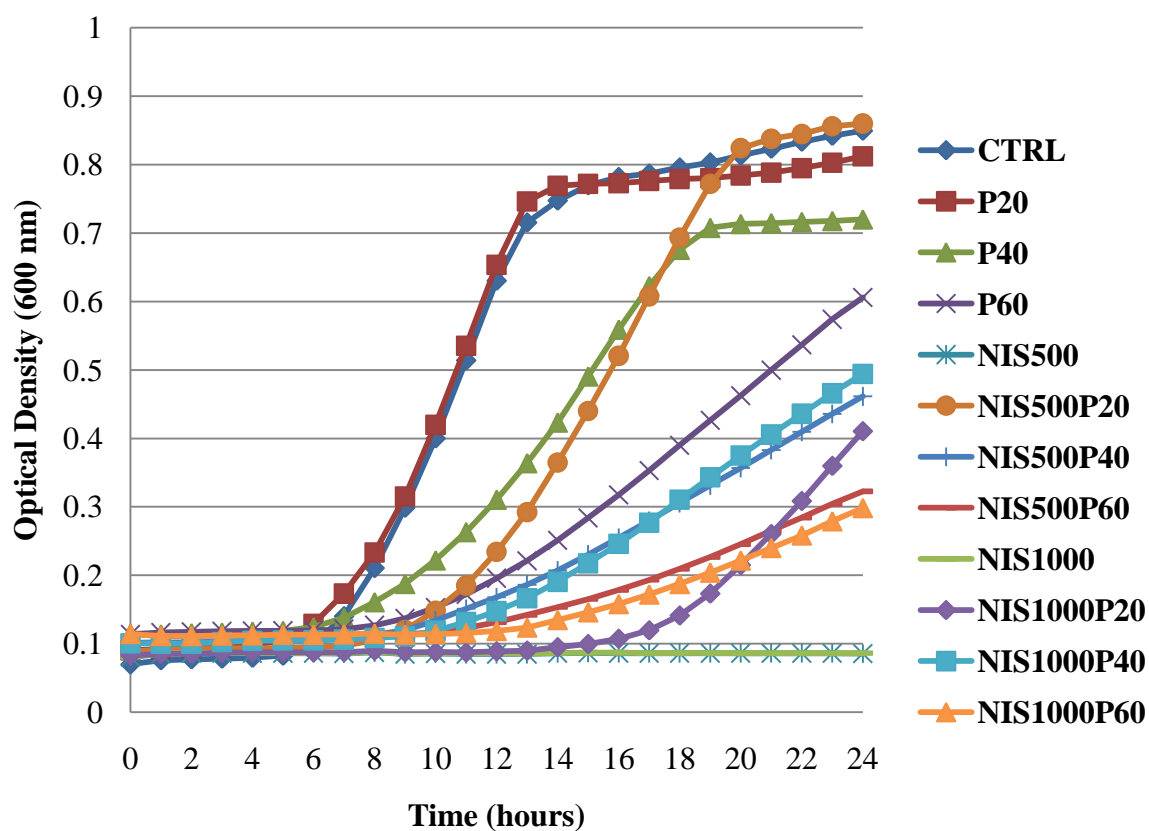


Figure 6. The effect of phosvitin combined with nisin against *Staphylococcus aureus* in BHI broth at 35 °C

TABLE 2. Antibacterial effectiveness of phosvitin (60 mg/ml) alone or combined with nisin against *Salmonella enterica* in onion mushroom soup held at 12 °C for 8 days.

Treatment	Viable count (Log ₁₀ CFU/ml) ^x			
	2d	4d	6d	8d
Control	4.60 ± 0.05ax	6.26 ± 2.51ax	6.02 ± 3.44ax	6.95 ± 3.62ax
Phosvitin (60mg/ml)	4.42 ± 0.34ax	4.53 ± 0.14ax	4.49 ± 0.42ax	5.28 ± 1.59ax
Nisin (1000 IU)	4.43 ± 0.14ax	5.08 ± 0.80ax	6.35 ± 2.93ax	6.76 ± 3.80ax
Phos + Nis (1000 IU)	4.49 ± 0.55ax	4.34 ± 0.13ax	4.29 ± 0.41ax	4.35 ± 0.57ax

^xEach reported value for viable count represents the mean (standard deviation) of three independent replications of the experiment

^{a,b,c}Means with a different letter within a column differ significantly (P<0.05)

^{x,y,z}Means with a different letter within a row differ significantly (P<0.05)

TABLE 3. Antibacterial effectiveness of phosvitin (60 mg/ml) alone or combined with nisin against *Escherichia coli* O157:H7 in onion mushroom soup held at 12 °C for 8 days.

Treatment	Viable count (Log ₁₀ CFU/ml) ^x			
	2d	4d	6d	8d
Control	4.80 ± 0.20ax	5.66 ± 1.18ax	6.74 ± 3.37ax	6.90 ± 3.47ax
Phosvitin (60mg/ml)	4.45 ± 0.74ax	4.54 ± 0.38ax	4.50 ± 0.32ax	4.62 ± 0.84ax
Nisin (1000 IU)	4.09 ± 0.09ax	6.07 ± 2.29ax	6.14 ± 3.23ax	6.49 ± 3.81ax
Phos + Nis (1000 IU)	4.52 ± 0.31ax	4.10 ± 0.22ax	4.16 ± 0.46ax	4.26 ± 0.21ax

^xEach reported value for viable count represents the mean (standard deviation) of three independent replications of the experiment

^{a,b,c}Means with a different letter within a column differ significantly (P<0.05)

^{x,y,z}Means with a different letter within a row differ significantly (P<0.05)

TABLE 4. Antibacterial effectiveness of phosvitin (60 mg/ml) alone or combined with nisin against *Staphylococcus aureus* in onion mushroom soup held at 12 °C for 8 days.

Treatment	Viable count (Log ₁₀ CFU/ml) ^x			
	2d	4d	6d	8d
Control	5.02 ± 0.54ax	4.16 ± 0.20ay	4.29 ± 0.05axy	4.32 ± 0.25axy
Phosvitin (60mg/ml)	3.76 ± 0.67bx	3.79 ± 0.44ax	3.60 ± 0.48abx	3.4 ± 0.32abx
Nisin (500 IU)	2.27 ± 0.52cx	1.9 ± 0.64bcx	2.01 ± 0.31cdx	1.85 ± 0.33cdx
Nisin (1000 IU)	1.83 ± 0.34cx	1.23 ± 1.06cx	1.15 ± 0.99dx	0.93 ± 0.81dx
Phos + Nis (500 IU)	3.05 ± 0.27bcx	2.76 ± 0.28abcx	2.82 ± 0.52bcx	2.65 ± 0.53bcx
Phos + Nis (1000 IU)	3.02 ± 0.17bcx	2.85 ± 0.20abx	2.69 ± 0.27bcx	2.48 ± 0.19bcx

^xEach reported value for viable count represents the mean (standard deviation) of three independent replications of the experiment

^{a,b,c}Means with a different letter within a column differ significantly (P<0.05)

^{x,y,z}Means with a different letter within a row differ significantly (P<0.05)

TABLE 5. Antibacterial effectiveness of phosvitin (60 mg/ml) alone or combined with nisin against *Salmonella enterica* in a mushroom/onion soup held at 35 °C for 24 hours.

Treatment	Viable count (Log ₁₀ CFU/ml) ^x			
	4h	8h	12h	24h
Control	7.63 ± 0.31 ^{az}	9.46 ± 1.29 ^{ay}	10.28 ± 0.26 ^{axy}	11.31 ± 0.02 ^{ax}
Phosvitin (60mg/ml)	5.52 ± 0.95 ^{bcz}	6.95 ± 0.51 ^{byz}	8.12 ± 0.50 ^{by}	10.99 ± 0.03 ^{ax}
Nisin (1000 IU)	7.06 ± 0.28 ^{aby}	10.37 ± 0.63 ^{ax}	10.62 ± 0.68 ^{ax}	11.07 ± 0.34 ^{ax}
Phos + Nis (1000 IU)	5.08 ± 0.69 ^{cz}	6.80 ± 0.77 ^{by}	7.38 ± 0.01 ^{by}	9.15 ± 0.48 ^{bx}

^xEach reported value for viable count represents the mean (standard deviation) of three independent replications of the experiment

^{a,b,c}Means with a different letter within a column differ significantly (P<0.05)

^{x,y,z}Means with a different letter within a row differ significantly (P<0.05)

TABLE 6. Antibacterial effectiveness of phosvitin (60 mg/ml) alone or combined with nisin against *Escherichia coli* O157:H7 in onion mushroom soup held at 35 °C for 24 hours.

Treatment	Viable count (Log ₁₀ CFU/ml) ^x			
	4h	8h	12h	24h
Control	7.59 ± 0.07az	9.62 ± 0.30ay	9.99 ± 0.12ay	10.69 ± 0.27ax
Phosvitin (60mg/ml)	4.54 ± 0.95by	5.29 ± 1.51by	6.12 ± 1.51by	9.23 ± 0.17bx
Nisin (1000 IU)	8.15 ± 0.39ay	9.58 ± 0.88ax	10.13 ± 0.07ax	10.65 ± 0.51ax
Phosvitin + Nisin	4.48 ± 0.51by	5.00 ± 0.86by	5.76 ± 0.48by	8.10 ± 0.20cx

^xEach reported value for viable count represents the mean (standard deviation) of three independent replications of the experiment

^{a,b,c}Means with a different letter within a column differ significantly (P<0.05)

^{x,y,z}Means with a different letter within a row differ significantly (P<0.05)

TABLE 7. Antibacterial effectiveness of phosvitin (60 mg/ml) alone or combined with nisin against *Staphylococcus aureus* in onion mushroom soup held at 35 °C for 24 hours.

Treatment	Viable count (Log ₁₀ CFU/ml)*			
	4h	8h	12h	24h
Control	5.6 ± 0.58az	7.20 ± 0.58ay	8.28 ± 0.74axy	8.83 ± 0.25ax
Phosvitin (60mg/ml)	4.38 ± 0.88abx	4.78 ± 1.22abx	5.07 ± 1.54abx	6.06 ± 1.44abx
Nisin (500 IU)	3.01 ± 0.98bcy	4.13 ± 1.23bxy	4.69 ± 1.30bxy	6.75 ± 0.92abx
Nisin (1000 IU)	1.97 ± 0.94cy	2.83 ± 1.20by	4.05 ± 1.27bxy	5.76 ± 0.59abx
Phos + Nis (500 IU)	3.76 ± 0.82abcx	3.76 ± 0.76bx	4.05 ± 1.21bx	5.68 ± 1.19abx
Phos + Nis (1000 IU)	3.46 ± 0.65abcx	3.46 ± 0.67bx	3.18 ± 0.79bx	4.52 ± 1.96bx

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

^{a,b,c}Means with a different letter within a column differ significantly (P<0.05)

^{x,y,z}Means with a different letter within a row differ significantly (P<0.05)

CHAPTER 6

THE DEATH RATE OF FOODBORNE PATHOGENIC BACTERIA EXPOSED TO CARVACROL INCREASES WITH RATE OF CELLULAR LEAKAGE

Shecoya White¹, Aubrey Mendonca^{1*}, Byron Brehm-Stecher¹

¹Department of Food Science and Human Nutrition, Iowa State University, Ames IA, USA 50011.

*Corresponding author: Department of Food Science and Human Nutrition, 2312 Food Sciences Bldg., Iowa State University, Ames, IA, USA 50011.

Phone: (515) 294-9078. Fax: (515) 294-8181. Email: amendon@iastate.edu.

Key words: carvacrol, foodborne pathogens, membrane disruption

Abstract

Carvacrol is a plant-derived antimicrobial compound that has strong antibacterial activity. In spite of numerous published research reports on the antimicrobial effectiveness of carvacrol, only few have attempted to elucidate its mechanism of bactericidal action. Washed cells of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica*, and *Staphylococcus aureus* were suspended to obtain $\sim 1 \times 10^8$ CFU/ml in 0.6% NaCl (37 °C) containing carvacrol at 0.0 (control), 0.2, and 0.4 mg/ml. At 5-min intervals, suspensions were sterile filtered and each filtrate analyzed for material with A_{260} . Viability of the cell suspensions was evaluated by enumeration on both non-selective and selective agars. The cellular death rate was determined by using absorbance and viability data. Cell morphology was evaluated by transmission electron microscopy. For all organisms, A_{260} increased significantly with increased carvacrol concentration and exposure time. Within 0.2 min of exposure to carvacrol (0.4 mg/ml) initial viable numbers of *E. coli* O157:H7 and *L. monocytogenes* decreased by 8.90 and 6.64 Log CFU/ml, respectively. There was a very strong correlation between the initial rate of release of A_{260} and death rate of the pathogens ($r = 0.998$). At 0.4 mg/ml carvacrol, *E. coli* O157:H7 cells appeared collapsed and showed signs of lysis when observed by transmission electron microscopy. At 0.4 and 0.6 mg/ml carvacrol, *L. monocytogenes* cells showed no physical damage; however, loss of intracellular material was observed by transmission electron microscopy. It was concluded that carvacrol-induced death of foodborne pathogens involves disruption of the cytoplasmic membrane.

Introduction

Changing consumer attitude to the use of synthetic chemicals in foods has fueled

current interest of food manufacturers to seek naturally derived antimicrobial for food applications (Ultee et al., 1998). However, the adoption of naturally-derived antimicrobials by the food industry has been slow due to several issues. The major issues include precise identification of active ingredients and the use of relatively high antimicrobial concentrations that may negatively affect sensory qualities of foods (Nychas, 2003; Roller and Board, 2003). The antimicrobial activity of essential oils has been associated with substituted aromatic molecules such as cinnamaldehyde, eugenol, and carvacrol (Moleyar and Narasimhan, 1992).

To circumvent the issue of high concentrations plant essential oils or their components may be used in a manner consistent with the hurdle technology concept (Adams and Smid, 2003; Blaszyk and Holley, 1998). The production of multicomponent antimicrobial systems for foods calls for a very good understanding of the mechanism of action of specific components. This knowledge can help in focusing on potentially effective combinations.

Antimicrobial effects of essential oils are believed to involve interactions with the cell membrane; however, the actual mechanism of inhibitory action needs to be clarified (Roller and Board, 2003). Experiments on the antimicrobial activity of carvacrol revealed that it possesses a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria (Fenaroli, 1995). Carvacrol's antimicrobial activity encompasses inactivation of fungi (Chami et al., 2005; Tampieri et al., 2005), parasites (Lindberg et al., 2000), and insects (Panella et al., 2005). In spite of the large amount of research on carvacrol performed in recent years little is understood in regard to its antibacterial mechanism of action. Based on carvacrol's hydrophobicity, the bacterial membrane seems to be a likely initial target of this naturally-derived antimicrobial. Ultee et al. (1999) demonstrated that

carvacrol at 0.25 and 1.0 mM increased cytoplasmic membrane permeability for protons and potassium ions, respectively, in *Bacillus cereus*. Xu et al. (2008) reported that both carvacrol and thymol increased the permeability of the cytoplasmic membrane of *Escherichia coli*. More recently La Storia et al. (2011), using atomic force microscopy analysis, demonstrated alterations in surface structure of several carvacrol-treated foodborne bacteria. The main objective of the present study was to determine if the bactericidal effect of carvacrol is linked to cytoplasmic membrane damage in selected foodborne pathogenic bacteria.

Materials and Methods

Bacterial cultures and culture conditions. *Salmonella enterica* (Typhimurium-ATCC 14802), *Escherichia coli* O157:H7 (ATCC 438952) and *Listeria monocytogenes* (Scott A - NADC-2045 – 4b) and *Staphylococcus aureus* (ATCC 6538) were used in the present study. All cultures were obtained from the culture collection of the Microbial Food Safety Laboratory, Iowa State University, Ames, IA. Stock cultures were kept frozen (-70 °C) in Brain Heart Infusion (BHI) broth (Difco, Becton, Dickinson, Sparks, MD) supplemented with 10% (vol/vol) glycerol. Stock cultures were activated in BHI broth (pH 7.2) and incubated at 35 °C. At least two consecutive 18 to 22-h transfers of each stock culture were carried out before using the cells as inocula in each experiment.

Chemicals. A commercial preparation of carvacrol (Aldrich W224502) and sodium chloride (NaCl) was purchased from Sigma-Aldrich.

Preparation of treatments. Stock solutions of carvacrol (0.20 mg/ml, 0.40 mg/ml) were prepared in 0.6% saline. Each treatment (30 ml) was transferred aseptically into separate, sterile tubes and held at 37 °C for 30 min in a water bath prior to inoculation.

Preparation of cell suspension. Pathogens were grown separately in Erlenmeyer flasks containing 100ml of BHI at 35 °C. After 18 h the cells were harvested by centrifugation in a Beckman J2-21 centrifuge (Beckman Instruments Inc., Palo Alto Calif.) operating at 10,000 x g for 10 min at 4 °C. The cell pellets were resuspended in 0.6% saline to give a final viable cell concentration of approximately 2.0×10^{10} CFU/ml as determined by plate counts on tryptic soy agar plus yeast extract (TSAYE).

Exposure to treatment. Each tube of prewarmed treatment (carvacrol) was inoculated with 500 µl of cell suspension to give a final concentration of 5.2×10^8 CFU/ml. Cells were mixed immediately by vortexing and then held at 37 °C. Note that 12 s elapsed from the time of inoculation of the buffer to the time cells were plated or diluted in buffered peptone water (Difco Laboratories, Detroit, Mich.); time zero represented approximately 12 s of exposure. At time 0, 5, 10, 15 min, 5ml aliquots of treated cell suspension were removed from the treatment tubes. Of the treated cell suspension 1 ml was added to 9 ml of buffered peptone water and vortexed, then plated onto selective and nonselective agar. Simultaneously, the remaining 4.0 ml of treated cell suspension was filtered through a 25mm diameter Luer Lock plastic syringe. Filtrates were then examined for material with A_{260} (206 nm AM).

Measurement of cellular leakage. Leakage of cytoplasmic contents was determined by the detection of 260 nm absorbing material (AM) in the cell filtrates. Quartz cuvettes containing 3 ml of cell filtrate were scanned at wavelengths ranging from 400 to 230 nm at a

scan speed of 500 nm per min using a UV spectrophotometer (Beckman Instruments). All treatments were replicated 3 times.

Cell viability. Cell viability was determined by serially diluting treated cells in buffered peptone water followed by surface plating 1.0 ml onto nonselective and selective agar media. TSAYE was used as the nonselective medium. Sorbitol MacConkey agar (Difco Laboratories, Detroit, Mi) and modified Oxford agar (Oxoid/Unipath, Columbia, Md.) were used as selective media for *E. coli* O157:H7 and *L. monocytogenes*.

Transmission electron microscopy. Pelletted cells were resuspended in fresh fixative containing 2.5% (v/vol) glutaraldehyde in 0.1 M SCB and then held at 4 °C for 2 h. The cells were pelleted by centrifugation, washed three times with 0.1 M SCB, and fixed with 1% osmium tetroxide (OsO₄) in 0.1 M SCB for 3 h at 25 °C. After three buffer washes in 0.1 M SCB, the cells were negatively stained and prepared for transmission electron microscopy.

Statistical analysis. Three independent replications of each experiment were performed. Mean numbers of survivors *E. coli* O157:H7 or *L. monocytogenes* were statistically analyzed using SAS statistical software version 8.1 (SAS Institute Inc., Cary, N.C.). Treatment means were evaluated for statistically significant differences using Tukey's test. Significant differences were defined at $P < 0.05$ for all the experimental data.

Results

Effect of carvacrol on cell viability. The viability of *E. coli* O157:H7 and *L. monocytogenes* decreased as time of exposure to carvacrol increased. Viable counts of *E. coli* O157:H7 in 0.6% saline with added carvacrol (0.4 mg/ml) declined faster than those of

L. monocytogenes at that same concentration of carvacrol (Fig. 1 and 2). After 0.2 min, the death rate (Log_{10} reductions per min) of both *E. coli* O157:H7 and *L. monocytogenes* decreased (Fig. 1 and 2).

Leakage of cytoplasmic constituents. At carvacrol concentrations of 0.2, 0.4, and 0.8 mg/ml leakage of 260 nm absorbing material (AM) from *E. coli* O157:H7, *L. monocytogenes*, *S. enterica*, and *S. aureus* increased with time. The amount of 260 nm AM released from those organisms also increased as carvacrol concentration increased (Table 1). There was a strong correlation ($R^2 = 0.9981$) between leakage of 260 nm AM and death rate of the cells.

Effect of carvacrol on cell microtopography. Cells of *E. coli* O157:H7 that were exposed to 0.4, and 0.6 mg/ml carvacrol appear wrinkled and collapsed (Fig. 4 C and D) whereas *L. monocytogenes* cells exposed to those same carvacrol concentrations retained their shape (Fig. 5 C and D). Loss of intracellular material was seen in cells of *E. coli* O157:H7 (Figure 6 B and C) and *L. monocytogenes* exposed to 0.4 and 0.6 mg/ml carvacrol.

Discussion

There is rapidly increasing trend towards the use of plant-derived antimicrobials. One such antimicrobial is carvacrol, which is part of the essential oil fractions of oregano (60 to 70% carvacrol) and thyme (45% carvacrol). The antimicrobial effectiveness of carvacrol has been previously reported (Lambert et al., 2001; Valero and Frances, 2006). Carvacrol's mechanism of antibacterial action has been purportedly associated with damage of the cytoplasmic membrane (Ultee et al., 1999, 2002; Ousslah et al., 2006). While various

carvacrol concentrations have been reported to cause cytoplasmic membrane damage, to date no research has correlated the extent of carvacrol induced membrane damage (as evidenced by leakage of 260 nm AM) with bacterial death rate. The rapid death of both *E. coli* O157:H7 and *L. monocytogenes* in 0.0.6% saline during exposure to relatively low concentrations of carvacrol (0.2 or 0.4 mg/ml) reflects the bactericidal characteristic of carvacrol. While the viable numbers of both pathogens rapidly decreased within 0.2 min, *E. coli* O157:H7 exhibited a greater sensitivity to carvacrol compared to *L. monocytogenes*. *E. coli* O157:H7's loss of viability seemed greater than that of *L. monocytogenes* at both concentrations of carvacrol tested in the present study. La Storia et al. (2011) also reported that gram negative *E. coli* O157:H7 was less resistant to carvacrol exposure. The phenolic component of carvacrol has prompted research focused on its effect on structural and functional damage to cellular membranes (Sikkema et al., 1995, Sivropoulou et al., 1996, Ultee et al., 1999). Several methods have been used to investigate the antibacterial mechanism of action of carvacrol: spectrofluorometry (Ultee et al., 1999, 2002; Lambert, 2001), scanning electron microscopy (Kwon et al., 2003; Di Pasqua et al., 2007), confocal laser scanning microscopy (Gill and Holly, 2006) and flow cytometry (Xu et al., 2008). It is well known that the membrane damaging effects of certain disinfectants such as triclosan, cresol, hexachloaphene against bacteria is due to their phenolic constituents (Bean and Das, 1966; Heath et al., 2000). Damage to the bacterial cytoplasmic membrane can result in leakage of 260 nm absorbing material (AM). In the present study the increased leakage of 260 nm AM from cells of *E. coli* O157:H7, *L. monocytogenes*, *S. enterica*, and *S. aureus* during their exposure to carvacrol indicated that their cytoplasmic membranes endured damage and were unable to retain cytoplasmic constituents. Damage to the cytoplasmic

membrane was consistent with release of 260 nm AM (Fig. 3). The absorption spectra for nucleic acids have a maximum at 260 nm (Ulitzur, 1972); therefore, the 260 nm AM released by carvacrol-treated cells most likely consisted of purines and pyrimidines from the metabolic pool.

Negative stain and transmission electron microscopy provided further evidence for cytoplasmic membrane damage in cells exposed to carvacrol. Carvacrol-treated *E. coli* O157:H7 cells (as seen in transmission electron microscopy photographs) appeared wrinkled, collapsed, and showed loss of cellular constituents compared to control cells (Fig. 4 and Fig. 6). Interestingly, although carvacrol-treated *L. monocytogenes* cells endured damage to the cytoplasmic membrane no signs of wrinkled or collapsed cells were observed in the electron micrographs; however, loss of cellular material was observed in Fig. 7. A plausible explanation for this observation is that *L. monocytogenes* is a Gram-positive bacterium and has a very thick cell wall (peptidoglycan) that better maintain the shape of the cell compared to Gram-negative bacteria such as *E. coli*.

Based on the results of the present study, particularly the very strong correlation ($R^2 = 0.9981$) between leakage of 260 nm AM from carvacrol-treated cells and their death rate, we conclude that carvacrol induced death of foodborne pathogenic bacteria involves disruption of the cytoplasmic membrane.

Acknowledgments

The authors thank the Microscopy and NanoImaging Facility (MNIF) staff (Tracy Pepper) at Iowa State University.

References

1. Adams, M., and Smid, E., 2003. Nisin in multifactorial food preservation, p. 11-33. In S. Roller (ed.), Natural antimicrobials for the minimal processing of foods. Woodhead Publishing Ltd., Cambridge, United Kingdom.
2. Bean, H. S. and Das, A. 1966. The absorption by *Escherichia coli* of phenols and their bactericidal activity. J. Pharm. Pharmacol. 18:107S-113S.
3. Blaszyk, M., and Holley, R. A. 1998. Interaction of monolaurin, eugenol and sodium citrate on growth of common meat spoilage and pathogenic organisms. Int. J. Food Microbiol. 39:175-183.
4. Chami, N., Bennis, S., Chami, F., Aboussekhra, A., Remmal, A. 2005. A study of anticandidal activity of carvacrol and eugenol in vitro and in vivo. Oral Microbiol. Immunol. 20:106-111.
5. Di Pasqua, R., Betts, G., Hoskins, N., Edwards, M., Ercolini, D., and Mauriello, G. 2007. Membrane toxicity of antimicrobial compounds from essential oils. J. Agric. Food Chem. 55:4863-4870.
6. Fenaroli, G. 1995. Fenaroli's handbook of flavor ingredients, third ed. CRC Press, Boca Raton, Fla.
7. Fernandez, A., Salmeron, C., Fernandez, P.S. and Martinez, A. 1999. Application of a frequency distribution model to describe the thermal inactivation of two strains of *Cacillus cereus*. Trends Food Sci. Technol. 10:158-162.
8. Gill, A.O., and Holley, R.A. 2006. Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant oil aromatics. Int. J. Food Microbiol. 108:1-9.

9. Heath, R. J., Li, J., Roland, G. E., and Rock, C.O. 2000. Inhibition of the *Staphylococcus aureus* NADPH-dependent enoyl-acyl carrier protein reductase by triclosan and hexachlorophene. *J. Biol. Chem.* 275:4654-4659.
10. Kwon, J.A., Yu, C.B., and Park, H.D. 2003. Bactericidal effects and inhibition of cell separation of cinnamic aldehyde on *Bacillus cereus*. *Lett. Appl. Microbiol.* 37:61-65.
11. La Storia, A., Ercolini, D., Marinello, F., Di Pasqua, R., Villani, F., Mauriello, G. 2011. Atomic force microscopy analysis shows surface structure changes in carvacrol-treated bacterial cells. *Res. Microbiol.* 162:164-172.
12. Lambert, R.J.W., Skandamis, P.N., Coote, P.J., and Nychas, G.-J.E. 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J. Appl. Microbiol.* 91:453-462.
13. Nychas, G. -J., E., and Skandamis, P.N. 2003. Antimicrobials from herbs and spices, p. 176-200. *In* S. Roller (ed.), *Natural antimicrobials for the minimal processing of foods*. Woodhead Publishing Ltd., Cambridge, United Kingdom.
14. Ousslah, M., Caillet, S., and Lacroix, M. 2006. Mechanism of action of Spanish oregano, Chinese cinnamon, and savory essential oils against cell membranes and walls of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *J. Food Prot.* 69: 1046-1055.
15. Roller, S., and Board, R. G. 2003. Naturally occurring antimicrobial systems, p. 262-290. *In* N. J. Russell and G. W. Gould (ed.), *Food preservatives*, 2nd ed. Kluwer Academic, New York, N.Y.
16. Sikkema, J., de Bont, J.A.M. and Poolman, B. 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.* 59:201-222.

17. Sivropoulou, A., Papanikolaou, E., Nikolaou, C., Kokkini, S., Lanaras, T., and Arsenakis, M. 1996. Antimicrobial and cytotoxic activities of origanum essential oils. *J. Agric. Food Chem.* 44:1201-1205.
18. Tampieri, M., Galuppi, R., Macchioni, F., Carelle, M.S., Falcioni, L., Cioni, P.L., Morelli, I. 2005. The inhibitin of *Candida albicans* by selected essential oils and their major components. *Mycopathologia.* 59:339-345.
19. Ulitzur, S. 1972. Rapid determination of DNA base composition by ultraviolet spectroscopy. *Biochim. Biophys. Acta.* 272:1-11.
20. Ultee, A., Bennik, M.H.J., and Moezelaar, R. 2002. The phenolic hydroxyl group of
21. Ultee, A., Gorris, L. G. M., and Smid, E. J. 1998. Bactericidal activity of carvacrol on the food-borne pathogen *Bacillus cereus*. *J. Appl. Microbiol.* 85:211-218.
22. Ultee, A., Kets, E.P.W. and Smid, E.J. 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. *Appl. Environ. Microbiol.* 65:4606-4610.
23. Xu, J., Zhou, F., Ji, B.-P., Pei, R.-S., and Xu, N., 2008. The antibacterial mechanism of carvacrol and thymol against *Escherichia coli*. *Lett. Appl. Microbiol.* 47:174-179.

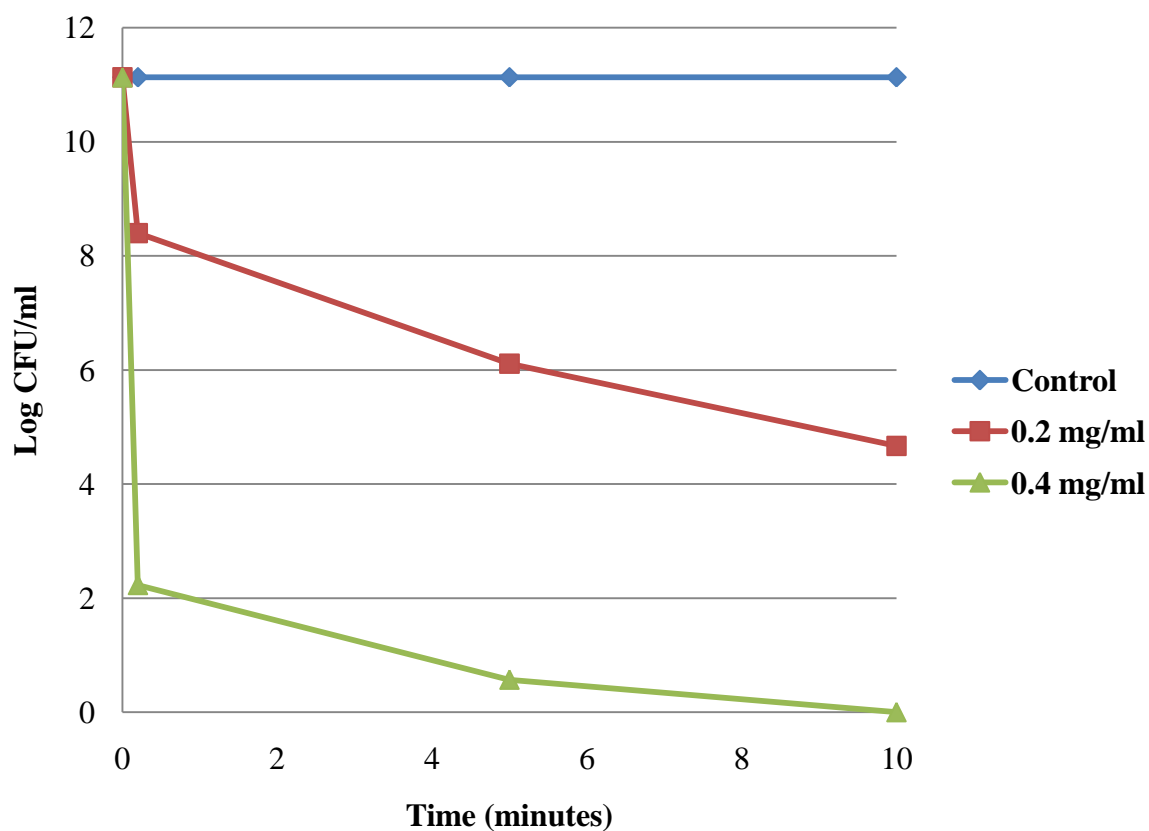


FIGURE 1. *E. coli* O157:H7 survivors after exposure to carvacrol in 0.6% saline^x.

^xEach reported value for viable count represents the mean (standard deviation) of three independent replications of the experiment.

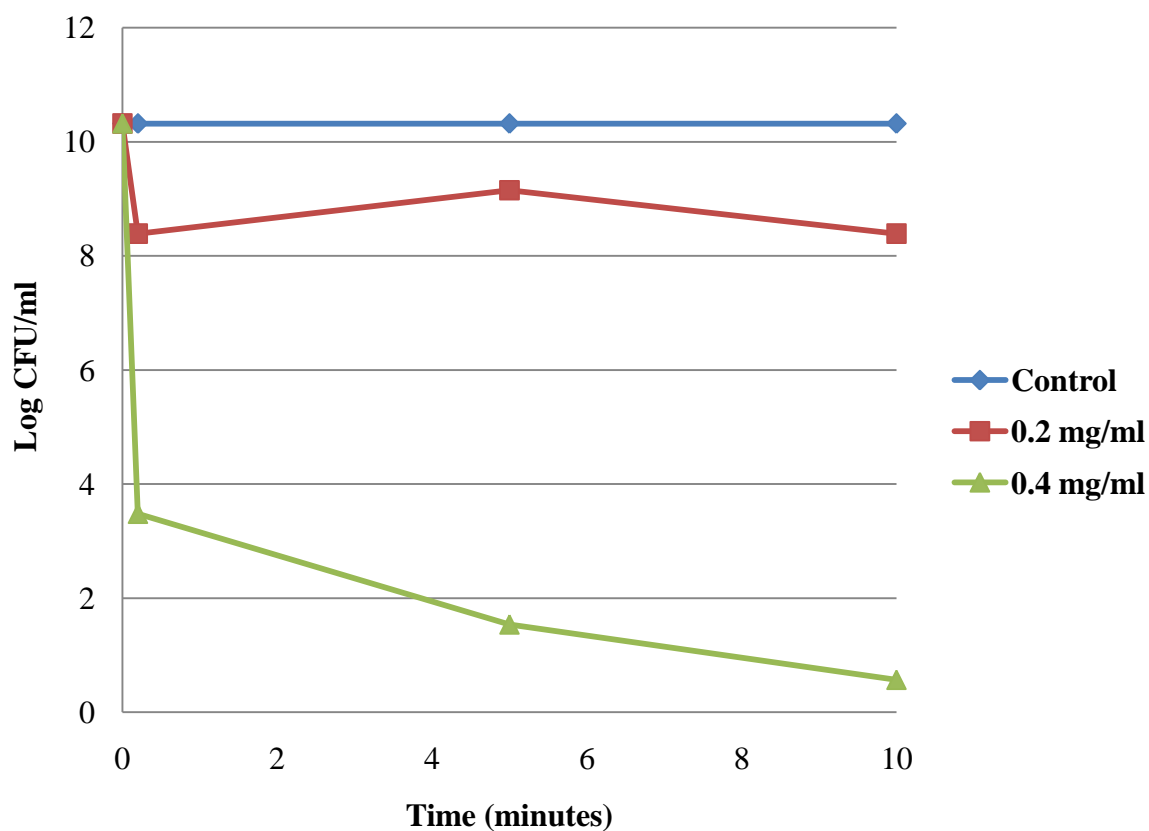


FIGURE 2. *L. monocytogenes* survivors after exposure to carvacrol in 0.6% saline^x.

^xEach reported value for viable count represents the mean of three independent replications of the experiment.

TABLE 1. Effect of varying concentration of carvacrol on DNA leakage of *Escherichia coli* O157:H7 (ATCC 43895), *Listeria monocytogenes* (Scott A), *Salmonella enterica* (Typhimurium) and *Staphylococcus aureus* (ATCC 6538) in 0.6% saline solution for 15 minutes.

Treatment	260 nm AM ^x			
	0.2 m	5m	10m	15m
<i>E. coli</i> O157:H7				
Control	0.04 ± 0.00a	0.04 ± 0.00b	0.04 ± 0.00b	0.04 ± 0.00b
Carvacrol 0.2 mg/ml	0.13 ± 0.08a	0.19 ± 0.14ab	0.34 ± 0.21ab	0.27 ± 0.17b
Carvacrol 0.4 mg/ml	0.20 ± 0.09a	0.28 ± 0.11a	0.33 ± 0.13ab	0.31 ± 0.17ab
Carvacrol 0.8 mg/ml	0.17 ± 0.04a	0.23 ± 0.02ab	0.48 ± 0.12a	0.62 ± 0.12a
<i>L. monocytogenes</i>				
Control	0.03 ± 0.00b	0.03 ± 0.00c	0.03 ± 0.00c	0.03 ± 0.00b
Carvacrol 0.2 mg/ml	0.23 ± 0.03a	0.21 ± 0.11b	0.20 ± 0.09bc	0.21 ± 0.16b
Carvacrol 0.4 mg/ml	0.18 ± 0.06a	0.26 ± 0.04ab	0.33 ± 0.13b	0.21 ± 0.70b
Carvacrol 0.8 mg/ml	0.18 ± 0.02a	0.40 ± 0.03a	0.61 ± 0.08a	0.61 ± 0.03a
<i>S. enterica</i>				
Control	0.04 ± 0.00a	0.04 ± 0.00b	0.04 ± 0.00b	0.04 ± 0.00c
Carvacrol 0.2 mg/ml	0.23 ± 0.17a	0.25 ± 0.15ab	0.30 ± 0.22ab	0.25 ± 0.15bc
Carvacrol 0.4 mg/ml	0.17 ± 0.07a	0.27 ± 0.12ab	0.38 ± 0.13a	0.47 ± 0.04b
Carvacrol 0.8 mg/ml	0.18 ± 0.07a	0.42 ± 0.02a	0.60 ± 0.02a	0.95 ± 0.23a
<i>S. aureus</i>				
Control	0.05 ± 0.00a	0.05 ± 0.00b	0.05 ± 0.00b	0.05 ± 0.00b
Carvacrol 0.2 mg/ml	0.23 ± 0.10a	0.22 ± 0.13ab	0.30 ± 0.15a	0.35 ± 0.22a
Carvacrol 0.4 mg/ml	0.14 ± 0.05a	0.29 ± 0.10a	0.26 ± 0.08ab	0.24 ± 0.01ab
Carvacrol 0.8 mg/ml	0.20 ± 0.11a	0.20 ± 0.02ab	0.24 ± 0.05ab	0.38 ± 0.05a

^xEach reported value for viable count represents the mean (standard deviation) of three independent replications of the experiment

^{a,b,c}Means with a different letter within a column differ significantly (P<0.05)

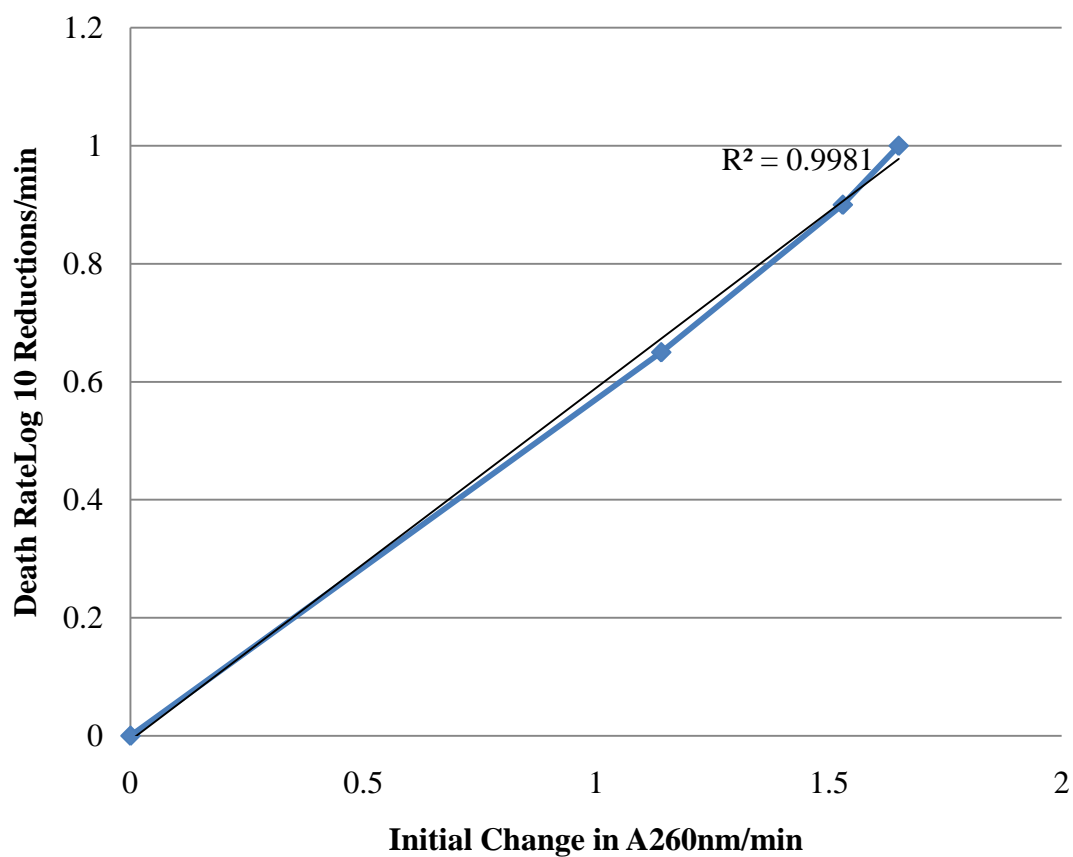


FIGURE 3. Relationship between initial rate of leakage of 260 nm AM and death rate for *E. coli* O157:H7, exposed to 0.20 mg/ml and 0.40 mg/ml carvacrol in 0.6% saline.

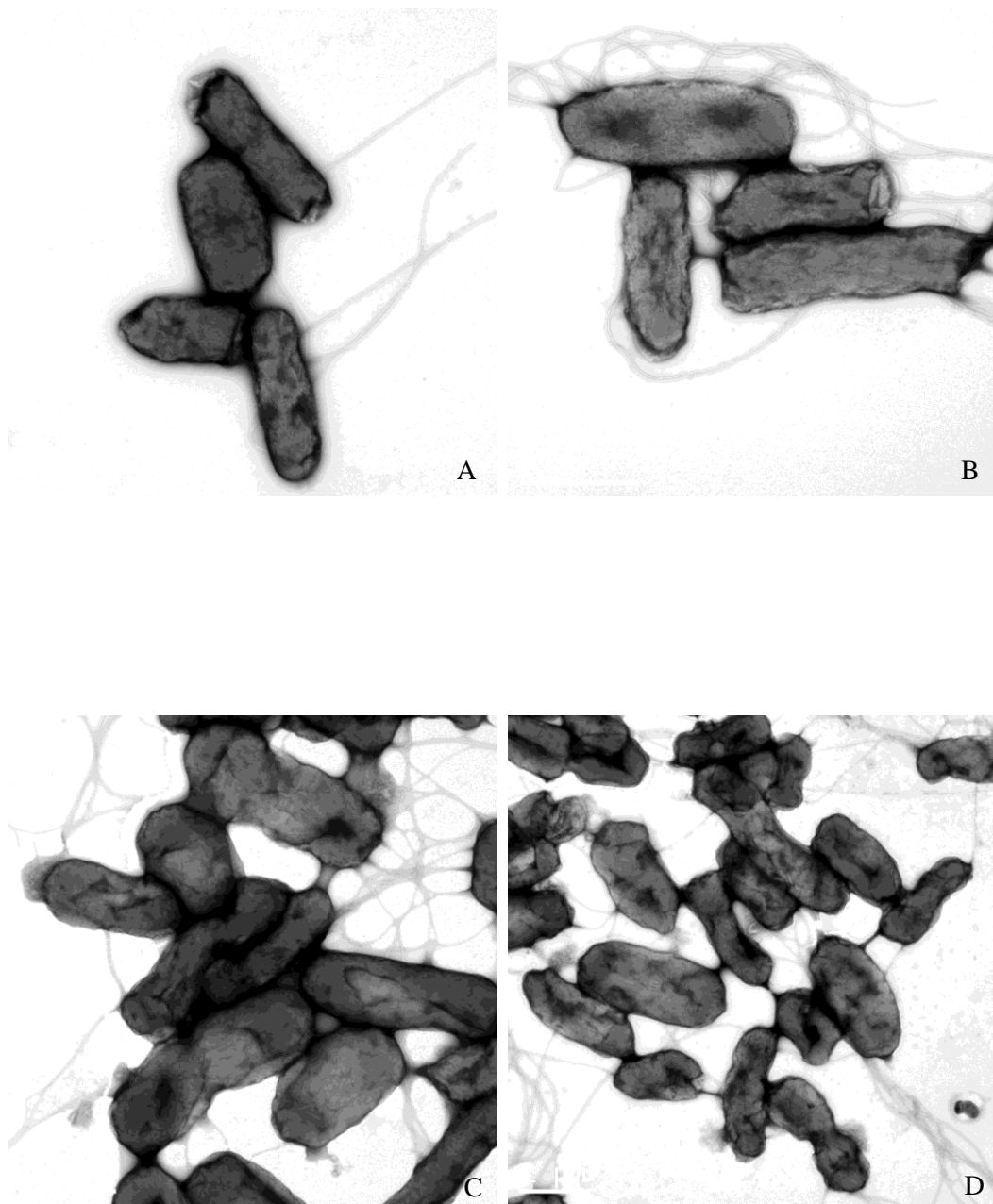


FIGURE 4. *Negative staining of Escherichia coli O157:H7 control (A) and cells exposed to carvacrol 0.20 mg/ml (B), 0.40 mg/ml (C), and 0.60 mg/ml (D) in 0.6% saline for 15 min.*

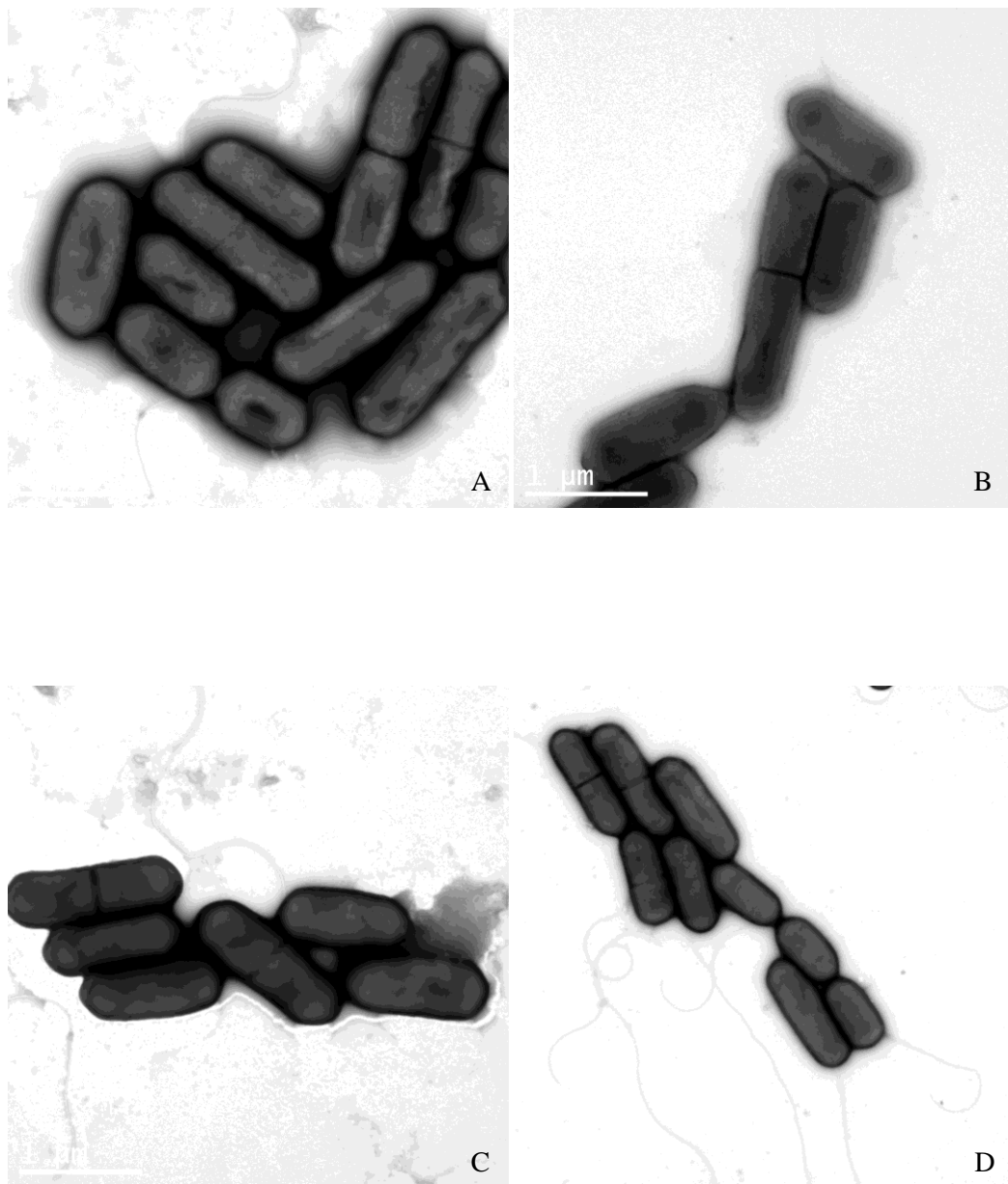


FIGURE 5. *Negative staining of Listeria monocytogenes control (A) and cells exposed to carvacrol, 0.20 mg/ml (B), 0.40 mg/ml (C), and 0.60 mg/ml (D) in 0.6% saline for 15 min.*

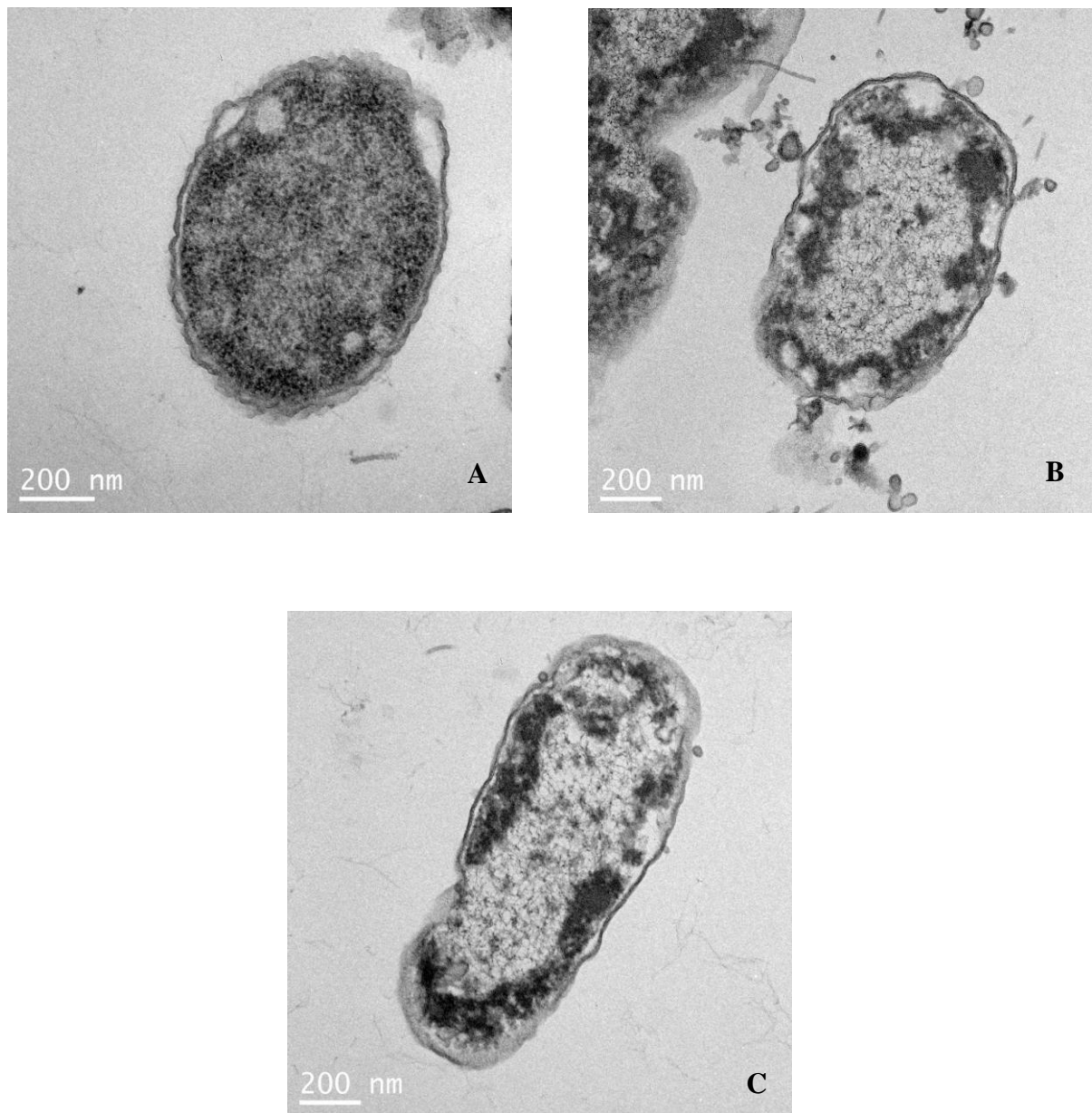


FIGURE 6. *Transmission Electron Microscopy (TEM) of Escherichia coli* O157:H7 exposed to carvacrol 0.0 mg/ml (control) (A), 0.4 mg/ml (B), and 0.60 mg/ml (C) in 0.6% saline for 15 min.

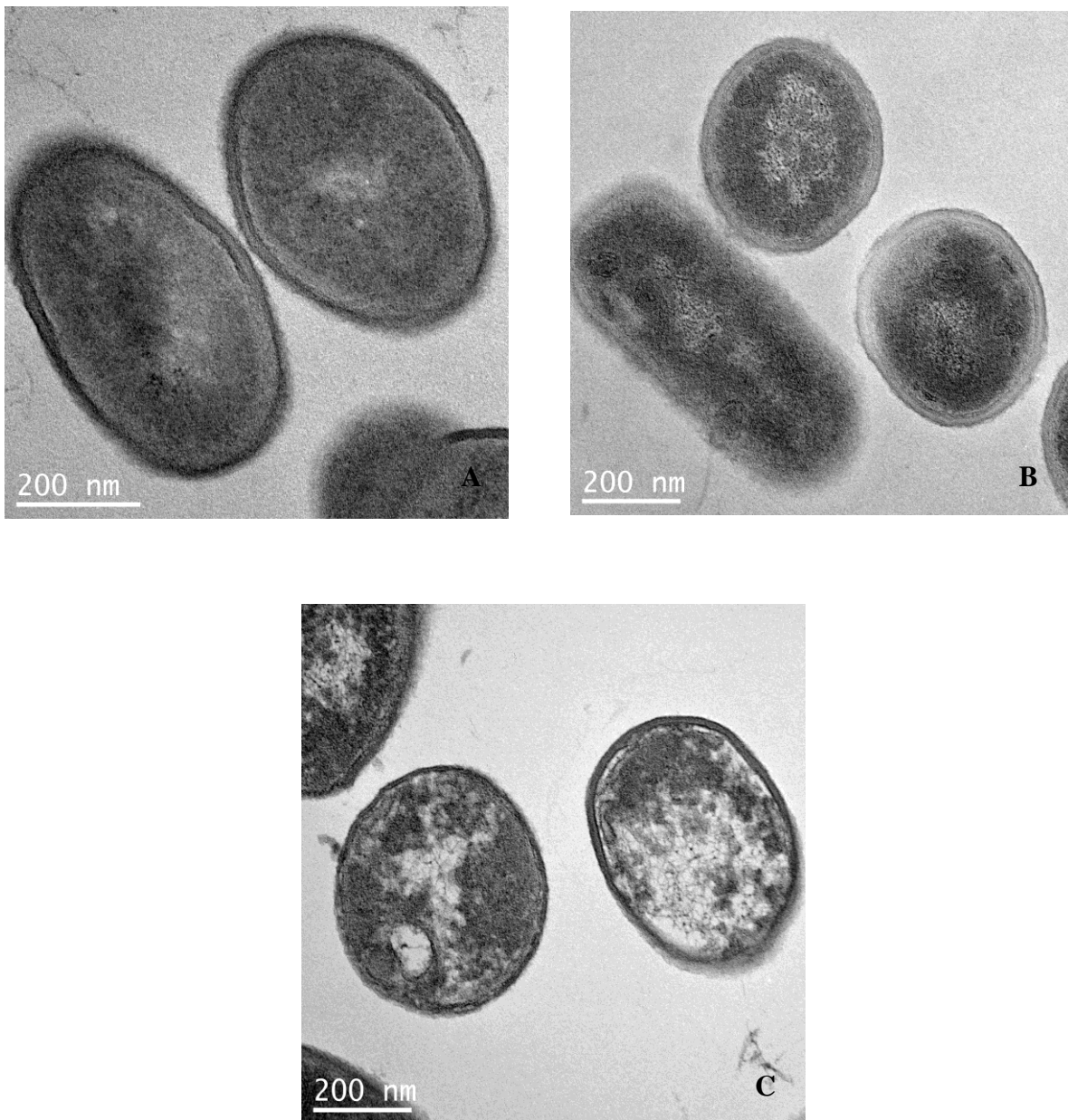


FIGURE 7. *Transmission Electron Microscopy (TEM) of Listeria monocytogenes* exposed to *carvacrol* 0.0 mg/ml (control) (A), 0.4 mg/ml (B), and 0.60 mg/ml (C) in 0.6% saline for 15 min.

CHAPTER 7. GENERAL CONCLUSIONS

Bacterial pathogens present in food and beverages can infect humans and cause foodborne illnesses. Naturally-derived antimicrobials from plant, animal or microbial sources are gaining increased attention from the food industry as consumers demand more natural food products with no synthetic chemical preservatives.

The present research indicated that combinations of two naturally-derived antimicrobials namely carvacrol (from oregano or thyme) and phosvitin (from egg yolk) are strongly inhibitory to *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica*, and *Staphylococcus aureus* in both laboratory broth media and in onion mushroom soup stored at abusive temperatures (12 and 35 °C). Phosvitin (60 mg/ml) combined with carvacrol (0.4 mg/ml) proved to be the most effective combination for controlling the foodborne pathogens used in the present study. Phosvitin and carvacrol exert a bacteriostatic and bactericidal effect, respectively, against foodborne pathogenic bacteria. Possible mechanism of antibacterial action of carvacrol is linked to cytoplasmic membrane damage.

Phosvitin (60 mg/ml) combined with nisin (1000 IU) does not offer any greater antimicrobial effect than phosvitin used alone against Gram-negative foodborne pathogens in onion mushroom soup held at 12 °C and 35 °C. Further research is needed to address the following: sensory characteristics (organoleptic properties), possible combinations with other hurdle techniques (antimicrobials, high pressure), and optimize combinations in various food products as a natural source of food safety and food preservation.

ACKNOWLEDGMENTS

First and foremost I would like to thank God for all of my blessings. I would like to thank my parents Edward and Barbara White; brother, Brian Little; sister Chantell White; nephew Sean White-Mcperson and extended family for their continued love and support throughout the course of my degrees.

My journey through Iowa State has been a meaningful one, starting from a summer internship ending at a Ph.D. degree. I would like to thank Mary de Baca for accepting me and allowing me to participate in the George Washington Carver Internship Program (formerly College of Agriculture Summer Internship Program) twice. Prior to this experience I had never conducted research or considered attending graduate school. The experience made such a great impression on me; it brought me back to attend graduate school. I appreciate you Mary you have always been there for me, as a mentor, a friend, and as a source for educational funding.

Words could not express how grateful I am to have had Dr. Aubrey F. Mendonca as my major professor. He not only has been a wonderful mentor but a friend and father figure throughout this three year process. I appreciate you always having time and going above and beyond the duty of being a major professor. You have always believed in me; pushing me to be my best. Dr. Mendonca I can finally say I'm not worried about disappointing you. Under your guidance and wisdom I completed this degree. I would also like to acknowledge and extend appreciation to my committee members Dr. Ruth MacDonald, Dr. Dong Ahn, Dr. Dennis Olsen and Dr. Byron Brehm-Stecher. All of you have challenged me in a way I could

never imagine, thanks. I can't forget to thank Dr. Aura Daraba, Dr. Stephanie Clark and Linda Wild for their assistance.

Last but not least I would like to thank my friends, colleagues and the Mendonca Microbial Food Safety Lab.