To the Graduate Council:

I am submitting herewith a thesis written by Jessica Marie Christman entitled "Antimicrobial Activity of Casein Hydrolysates against *Listeria monocytogenes* and *Escherichia coli* O157:H7." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

David A. Golden
Major Professor

We have read this thesis
and recommend its acceptance:

P. Michael Davidson

Svetlana Zivanovic

Accepted for the council:

Carolyn R. Hodges
Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Antimicrobial Activity of Casein Hydrolysates against *Listeria monocytogenes* and *Escherichia coli* O157:H7

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Abstract

Listeriosis has the highest fatality and hospitalization rate among foodborne illnesses. *Listeria monocytogenes* causes listeriosis and is a difficult bacterium for ready-to-eat food processors to eliminate because of its ability to grow in the absence of oxygen and under refrigeration. Recently, milk and its proteins have gained recognition as the largest source of biologically active peptides, and, it stands reason that several antimicrobial peptides (AMP) can be released from casein as it is the most abundant milk protein. AMPs are commonly obtained by cutting the whole protein into peptide fragments using enzymes or by acidification. The objective of this study was to predict potential AMPs through computer aided tools, improve hydrolysate preparation, and determine trypsin and pepsin-casein hydrolysate antimicrobial activity in growth media and on frankfurters against two strains of *Listeria monocytogenes* (Scott A and 310) and *Escherichia coli* O157:H7 (Salami strain).

The prediction study procedure was to identify the most common variants of primary peptide sequences. The sequences were analyzed for greatest possible enzyme cuts on the protein, peptide masses, isoelectric point, net charge and percent hydrophilic residues using online proteomics programs. The fragments were explored for AMP commonalities: fragment length of 3 to 50 amino acids, positive (cationic) net charge, and hydrophilic residues between 25 and 50%. This technique identified 16 potential AMPs which proved that it is possible to screen for AMPs.

The method used to determine the trypsin-casein hydrolysate (TCH) and pepsin-casein hydrolysate (PCH) antimicrobial activity was to hydrolyze sodium caseinate with pepsin or trypsin. *L. monocytogenes* (strains Scott A and 310) were incubated in 0, 10, 20, and 40% PCH and 0 and 50% TCH concentrations over a 24 hour period. PCH suppressed growth of *L.*

monocytogenes Scott A by 1.76 log CFU/mL and reduced initial populations of *L. monocytogenes* 310 and *E.* coli O157:H7 by 0.52 and 0.62 log CFU/ml, respectively. TCH had little or no effect on growth suppression of any of the three test organisms.

The frankfurter study was conducted by spot inoculating frankfurters with *L. monocytogenes* Scott A and then dipping frankfurters into one of five treatments (deionized water, pH 2.7 buffer, pH 5.1 buffer, pH 2.7 PCH, and pH 5.1 PCH) for 30 seconds; inoculated frankfurters that were not dipped served as controls. Frankfurters were incubated at 32°C for seven days. The results showed that there was no significant difference ($p>0.05$) in antimicrobial effectiveness among the treatments and control.

This study demonstrated that enzymatically derived casein hydrolysates somewhat inhibit growth of *L. monocytogenes* and *E. coli* O157:H7 in culture media, but were ineffective when applied to frankfurters. Casein hydrolysate solutions can be easily made in a processing facility for application in fluid systems such as an antimicrobial spray on beef carcasses and in milk, juice, sports drinks, soda, soups, and yogurt. It also could be used in solid systems such as frankfurters, cheese, ground beef, and processed or RTE foods.

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Introduction: Literature Review

According to the National Hot Dog and Sausage Council, Americans spent more than $4.1 billion on frankfurters and sausages in retail stores in 2008, amounting to more than 1.5 billion pounds of product *(26)*. Frankfurters are well received by Americans; however, they have been associated with listeriosis, a foodborne illness. On January 5, 2007, the United States Department of Agriculture's (USDA) Food Safety and Inspection Service (FSIS) announced that the Golden Star Sausage Company recalled 15,514 pounds of sausage product because of *Listeria monocytogenes* contamination *(37)*. Fortunately, no illnesses were reported *(37)*, but controlling *L. monocytogenes* in these products is very important. Casein hydrolysates may prove to be useful for post-processing control *of L.* monocytogenes on frankfurters. In this literature review, frankfurters, their relationship to *L. monocytogenes*, and antimicrobial casein peptides will be discussed.

Frankfurters

Frankfurters are cylindrical shaped meat products made by extruding comminuted meat into a casing. *(10)*. Frankfurters are made of meat and/or poultry, back fat and rind, water, binder, seasoning, farina, cure, liquid smoke, tomato powder, and coloring.

There are four types of sausages: fresh, cooked, fermented, and emulsion. Fresh sausages are uncooked, uncured, and filled with coarsely comminuted products *(10)*. An example of a fresh sausage is sweet Italian sausage. Fresh sausages are sometimes cooked to enhance safety as well as convenience. Fermentation is one of the oldest preservation methods for foods, including sausages. The two styles of fermented sausages are dry and semi-dry, such as salami and summer sausage, respectively *(10)*. Fermented sausages have starter cultures added and are filled into casings to be dried *(10)*. As they dry, bacteria produce lactic acid that lowers the pH allowing for extended storage *(10)*. Semi-dry fermented sausages are usually smoked, dried, and then cooked

(10). Frankfurters are examples of emulsion sausage. Emulsion sausage meat products are finely comminuted *(10)* and have the appearance of baby food before cooking. Emulsion sausages are typically cooked with steam and are sometimes smoked *(10)*.

There are several steps in producing frankfurters. First, raw materials with acceptable qualities must be procured and stored under proper conditions before processing can take place. The second step is to temper, slice, and weigh the meat according to a recipe *(10)*. Next, the ingredients are added in precise quantities and at specific times into the bowl chopper that comminutes the meat products and mixes in the other ingredients *(10)*. Ice is added during chopping to keep the mixture cold *(10)*. From the bowl chopper, the meat batter is mechanically pumped into casings *(10)*. Casings are either natural, such as pig or sheep intestines, or artificially made from collagen, cellulose, and plastic materials; frankfurters are typically stuffed into artificial casings, which are more easily removed after processing *(10)*. After the casings are filled with meat product and twisted, they are cooked in a steam oven to an internal temperature between 85°C and 95°C followed by a shower or soak in water for 20 minutes *(10)*. Frankfurter strings next are feed through a peeler as most franks are sold with the casings removed *(10)*. Then they are chilled, vacuum packaged in sanitized or sterilized packaging units *(10)*, and sometimes pasteurized after packaging *(23)*. The packages are passed through a metal detector and placed into a secondary packaging unit such as a cardboard box for distribution *(10)*. Many manufacturers chill or freeze and hold the franks for 24 h before distribution so the results of microbiological tests confirm the absence of *L. monocytogenes (10)*.

Meat processing establishments are required to implement a hazard analysis and critical control points (HACCP) plan in conjunction with their entire process. There are several critical

control points in a HACCP plan for cooked sausages, especially concerning biological hazards such as *L. monocytogenes*. Control points start at procurement and intake of ingredients *(10)*. Pathogens could grow if ingredients are stored at improper temperature and humidity conditions. Other control points are placed at the chopping and mixing in the bowl chopper, cooking, post-cook chilling, and post-packaging for distribution *(10)*. These control points primarily deal with controlling temperature and making sure that raw and cooked products never meet. While these critical control points are important, according to the FSIS, post-processing steps such as peeling, sorting, slicing, and packaging serve as the greatest potential sources for *L. monocytogenes* contamination *(12, 23, 40)*. Between 1998 and 2002, there were three major *L. monocytogenes* outbreaks linked to frankfurters in the United States *(31)*. Selby et al. *(31)* reported that, in all three cases, frankfurters and ready-to-eat (RTE) meat products were contaminated after cooking and before packaging *(31, 32, 39)*. Therefore, essential critical controls for *L. monocytogenes* also should be placed close to the packaging process to have lasting effects.

Frankfurter-*Listeria monocytogenes* Relationship

Listeriosis is a foodborne illness caused by *L. monocytogenes*. *L. monocytogenes* is ubiquitious in nature and has been isolated from soils, vegetation, water *(20, 25, 39)*, silage, fecal material, sewage *(39)*, decaying vegetation *(20)*, and food processing environments *(25)*. This bacterium causes two types of illness manifestations. One is non-invasive, which is commonly called listerial gastroenteritis, where symptoms range from nothing to fever, vomiting, and diarrhea in healthy adults *(11, 14)*. Invasive listeriosis, the second and most serious form of the disease, affects infants, pregnant women including their fetuses, immunocompromised individuals, such as AIDS and cancer patients, children, and the elderly *(11, 14, 19)*. The

illnesses most often experienced by non-pregnant adults are sepsis, meningitis, meningoencephalitis, or bacteremia *(11)*. Symptoms include fever, malaise, ataxia, seizures, and altered mental status that may not show up for several weeks *(11)*. Pregnant women usually present with flu-like symptoms when infected with *L. monocytogenes (11)*. It is believed that fetal listeriosis is acquired transplacentally or from an intrauterine infection that leads to death or spontaneous abortion of the fetus *(11)*. In 1985, the USDA Food Safety and Inspection Service (FSIS) announced that an outbreak associated with *L. monocytogenes*-contaminated Mexican-style cheese caused 29 deaths *(13)*. Of these deaths, eight were neonates, 13 were still births, and eight were non-neonatal *(13)*. Listeriosis is a sporadic illness but has the highest fatality rate of 20 - 30% and hospitalization requirements of approximately 90% of foodborne illnesses *(20, 25, 39)*.

L. monocytogenes is a formidable foe for food processors, especially the RTE sector. Frankfurters are RTE products. RTE products are defined by 9 CFR Part 430.1 as meat or poultry products that are in an edible form without need for additional preparation to achieve food safety *(36)*. *L. monocytogenes* is a Gram-positive, non-sporeforming bacillus that is highly motile *(25, 40),* facultatively anaerobic, and psychrotrophic, which allows it to grow in the absence of oxygen and at refrigeration temperatures *(40)*. Also, *L. monocytogenes* can grow in curing salt solution up to 13% *(40)*, in nitrate solutions *(40)*, and over a pH range of approximately 4.5 to 9.6 *(40)*. Frankfurters are often consumed with minimal reheating which can lead to acquired *L. monocytogenes* infection.

For the food processing industry, perhaps the biggest problem with *L. monocytogenes* is its ability to incorporate into biofilms on food processing equipment and other surfaces *(20)*.

Biofilms are notoriously difficult to remove. *L. monocytogenes* reportedly can persist in food processing environments for months and up to 10 years *(12)*. Between 1993 and 1996, the USDA-FSIS monitored samples of frankfurters and reported that 5.3, 4.8, 4.1, 3.7 % (1993-1996) of frankfurters tested positive for *L. monocytogenes (20)*. Frankfurters are considered nearly *L. monocytogenes* free before peeling, but once franks are exposed to the environment and surfaces, they can become contaminated. Once *L. monocytogenes* comes in contact with the surface of the frankfurter, it can irreversibly attach itself within five minutes *(11)*. One example of frankfurter associated *L. monocytogenes* outbreak was in 1998. Thirty-eight patients contracted listeriosis from Bil Mar Foods hot dog and other meat products *(6)*. Of the thirty-eight patients who were infected, four died: one was an infant and the other three were elderly *(6)*. Interestingly, over half of the patients were women and six were newborns *(6)*. This shows the importance of sanitation as another control for *L. monocytogenes* on frankfurters, but if it fails as it did at Bil Mar Foods, there must be other controls built into the frankfurters or packaging.

In response to outbreaks caused by *L. monocytogenes*, the USDA-FSIS has established a "zero tolerance" policy for *L. monocytogenes* in RTE products *(11, 12, 13, 31, 40)*. On October 6, 2003, an interim final rule by the USDA-FSIS was established to prevent product recontamination with *L. monocytogenes (31)*. It requires RTE food processors, which produce post-lethality exposed products, to adopt one of three alternatives into their HACCP, SSOPs, or other prerequisite plans *(31)*. If an establishment chooses Alternative 1, they are required to use an anti-listerial agent and a post-lethatlity treatment *(40)*. That establishment then is subject to verification of post-lethality effectiveness by FSIS *(40)*. When Alternative 2 is chosen, either an anti-listerial agent or post-lethality treatment is expected *(40)*. The establishment that chooses this alternative would be open to more frequent verification activity by FSIS *(40)*. The third

alternative only requires the establishment to use sanitation measures *(40)*. However, this option would receive the greatest scrutiny and the most frequent visits from FSIS inspectors *(40)*.

The sanitation-only option is the most difficult to achieve control of *L. monocytogenes*. *L. monocytogenes* can survive in adverse conditions and persist in food processing environments for months to years in biofilms *(12, 40)*. HAACP plans are USDA-FSIS mandated and are integral components of sanitation in a food processing establishment *(25, 34)*. These plans help identify, evaluate, monitor, and provide follow-up procedures for optimal sanitation. Biofilms are particularly problematic, because they produce a polysaccharide matrix that traps microorganisms, including *L. monocytogenes*. Since biofilms containing *L. monocytogenes* can form on stainless steel tables, processing equipment, walls, floors, and air handling systems they must be washed and scrubbed frequently. Some sanitizers used in food processing plants are Sterilix (a biofilm remover) *(15)*, sodium hypochlorite, quaternary ammonium compounds, peroxyacetic acid *(20, 40)*, and iodophores *(20)*. However, biofilms are very difficult to remove, almost impossible to see, and difficult to penetrate by sanitizers.

There are some current methods being used to fulfill the anti-listerial agent, post-lethality treatment, and sanitization rules of the USDA-FSIS interim final rules. Some anti-listerial agents being used are organic salts and acids, such as sodium chloride *(34)*, sodium lactate, sodium diacetate *(12, 40)*, sodium nitrate, sodium nitrite *(10)*, citric acid, lactic acid *(34)*, and benzoic acid *(34)*. Consumers are becoming more health conscience and see these additives as detrimental to their health. Other antibacterial additives include bateriocins such as nisin *(15, 30, 31, 34)* and pediocin *(27)*. Nisin is the most widely used and approved to be used in meat products in the United States*(40)*. Essential oils such as rosemary *(34, 40)*, garlic, and grape seed

extract *(31)* have been used as anti-listerial agents, also. However, these essential oils impart a flavor profile that may not be acceptable to the consumer. These are usually incorporated into the meat batter of frankfurters to provide inhibition or bactericidal effects long after the product is distributed. A mixture of bacteriophage that are specific to *L. monocytogenes* are sprayed onto the final product, rather than being incorporated in to the batter *(34)*. This bacteriophage mixture has gained FDA approval for the use on RTE meats *(34)*.

Post-lethality treatments, such as post-processing pasteurization by steam, hot water, or radiant heat, are applied prior to or after packaging *(23, 25, 40)*. Other post-lethality treatments include irradiation *(27, 40)*, high pressure processing *(40)*, and hydrostatic pressure *(20, 27)*. Most of these treatments can be expensive and detrimental to product color, flavor, and texture, which would be undesirable to the manufacturer and consumer.

Antimicrobial Casein Hydrolysates

Milk is naturally antimicrobial. It is designed to provide the neonate protection from its initial ingestion throughout digestion. As milk is formed in the mammary glands, it incorporates immunity factors, such as immunoglobulins, from the mother's blood *(33)*. This provides the neonate with its first acquired immunity and indirectly influences the neonates fight against microbial infection *(7)*. Lactoferrin and enzymes such as lysozyme and lactoperoxidase are antimicrobial compounds present in raw milk *(7)*. Lactoferrin sequesters essential iron, making it unavailable for microbial utilization; lysozyme and lactoperoxidase disrupt the microbial cell wall *(33)*. Researchers have discovered that enzymatic digestion of milk proteins releases antimicrobial peptides such as isracidin *(17)*.

Most milk commercially produced in the United States is from cattle *(33)*. Bovine milk is mainly comprised of water, lactose, fat, and proteins. Milk proteins account for approximately 3.6% of milk composition *(33)*. There are two categories of milk proteins: casein and whey. Traditionally, caseins are defined as the solid curd protein, while whey is the protein liquid waste of cheese making. Caseins are the most abundant milk proteins making up nearly 80% of the total protein content of milk; whey makes up the remaining 20% *(33)*. Caseins are further categorized as α_S-caseins ($\alpha_{S1}$and α_{S2}), β-casein, κ-casein, and γ-casein. γ-casein is not a major protein because it is cleaved from the C and N-terminal of β-casein by a naturally occurring enzyme in milk, plasmin *(9, 33)*.

Caseins require calcium for their specialized formation. Casein proteins undergo post-transitional phosphorylation of seryl residues *(7)*, which results in anionic clusters for calcium-sensitive protein *(33)*. α_{S1}, α_{S2}, and β-caseins are considered calcium-sensitive because they have low stability in the presence of calcium ions *(33)*. When the anionic clusters bind with calcium, it discharges and dehydrates these regions altering the balance of hydrophobic interactions and electrostatic repulsive forces *(33)*. This results in calcium-sensitive casein carrying a large negative charge at pH 6.6 - 6.8 in milk and influences the hydrophobic/hydrophilic regions in sub-micelle and micelle formation *(33)*. Furthermore, primary structure analysis suggests that calcium-sensitive caseins have specific polar and hydrophobic regions creating amphipathic structures *(33)*. α_{S2}-casein is the most hydrophobic of caseins, whereas κ-casein is the most hydrophilic and sensitive to temperature *(33)*. κ-casein is different from the other caseins in that it has only one phosphorylated residue, which makes it calcium-insensitive and does not have anionic clusters to bind calcium *(33)*. It is amphipathic with very specific hydrophobic and polar

domains *(33)*. These characteristics play an important role in the formation and stabilization between caseins in sub-micelles and the complete micelle.

Although the micelle and sub-micelle structures are not fully understood, there are several aspects of these structures that have been discovered. Micelles are highly hydrated, spherical, spongy, and are made of many subunits of casein called sub-micelles *(33)*. A sub-micelle interior is rich in calcium-sensitive caseins and the surface is rich in κ-casein *(33)*. The predominate force keeping these individual sub-micellular caseins together are hydrophobic interactions *(33)*. Casein micelles have a raspberry-like appearance in micrographs and are made of 92% protein *(33)*. They also contain 8% milk salts and have the mole ratio α_{S1}:α_{S2}:β: κ of 3:1:3:1 *(33)*. The milk salts are mostly calcium phosphates, but also contain Mg^{2+} and citrate in moderate concentrations *(33)*. Micelles are hardy and can endure harsh processing conditions because of their highly solvated and unreactive surfaces *(33)*. κ-casein is susceptible to the chymosin enzyme that targets the Phe 105 – Met 106 position on the N-terminus which is described as a "hairy extension" of the micelle surface *(33)*. The proteolytic cleavage destabilizes the surface of the micelle which activates it and results in aggregation of casein micelles into curds found in cheese-making *(33)*. It appears that sub-micelles stick together more by salt-bridge interactions than protein-protein interactions *(33)*.

Researchers have discovered that naturally occurring antimicrobial peptides (AMPs), such as cathelicidins and defensins, are widely used as biochemical defense agents against pathogens by animal, plant, and insect cells *(28)*. These peptides are usually cell bound or secreted *(28)*, as is the case with the bactericin, nisin, which is secreted by *Lactoccocus lactis* ssp. *lactis (8)*. In the desire to provide the demands of a health conscience public with minimally processed foods, researchers have sought to find food sources that contain bioactive peptides

(BAP) that may be used as biopreservatives. BAPs have been commonly found in bovine and human milk, fish, eggs, and cereals *(16)*. So far, the greatest amounts of BAPs have been isolated from milk *(19, 29)*. BAPs are different from the naturally occurring AMPs in that they only become active when enzymatically cleaved from a parent protein by gastrointestinal enzymes *(16, 29)*.

Lactoferricin is a probably the most studied BAP that is isolated from milk. Lactoferricin is derived from the whey protein, lactoferrin, by enzymatic digestion with pepsin *(19, 38)*. Lactoferrin is a cationic, iron-binding glycoprotein that has a bacteriostatic effect on the growth of bacteria *(16)*. Lactoferricin loses the ability to sequester iron, but gains the ability to be bactericidal. Lactoferricin B (B for bovine) has been found to be nine to 25 times more potent than lactoferrin, needing only 0.5 to 500 mg/mL to inhibit Gram-positive and Gram-negative bacterial growth *(4)*. Yeast, mold, and parasite growth have also been inhibited by lactoferricin *(4)*. Lactoferricin is active over a wide pH range and is heat resistant, but activity seems to dwindle in the presence of cations and salts *(38)*. The structure is amphipathic, quasicyclic from the disulfide bonds between its cysteine residues, and is a low molecular weight peptide consisting of 25 amino acids *(38)*. As a point of interest, lactoferricin takes on a twisted β-sheet conformation in water that is reminiscent of prion proteins that cause Creutzfeldt-Jakob disease *(7)*.

Lactoferricin has an interesting mode of action. It is attracted to the negatively charged membranes and binds at the lipopolysaccharide and teichoic acid sites in Gram-positive and Gram-negative bacteria *(18)*. This action is believed to cause a depolarization of the cytoplasmic membrane resulting in the fusion of negatively charged liposomes *(7)*. The cytoplasmic membrane is not lysed by lactoferricin, as is the case with other AMPs; instead lactoferricin

transverses the membrane into the cytoplasm and apparently blocks bacterial protein synthesis, although the exact inhibition of macromolecule biosynthesis is unknown *(18)*.

AMPs utilize similar mechanisms to kill bacteria. All AMPs interact with the cell wall or membrane of bacteria. AMPs have an affinity for the anionic phospholipids and lipopolysaccharides found in cell walls and membranes of bacteria *(2)*. Lopez-Exposito et al. *(18)* reported that α_{S2}-casein f(183 – 207) peptide initially binds with lipoteichoic acid of Gram-positive bacteria and lipopolysaccharides in Gram-negative bacteria. As noted previously, lactoferricin also is capable of this. It is thought that the cationic side chains of arginine, lysine, and histidine mediate binding *(2)*. After the initial binding, many AMPs fold into their final configuration, possibly with α-helixes *(2, 7)*.

Once attached, an AMP inserts its hydrophobic domain into the bacterial membrane forming pores *(7)*, as is the case with nisin *(8)*, by lateral diffusion and forming complexes with other AMPs *(28)*. AMPs, such as lactoferricin, α_{S2}-casein f(183 – 207) *(18)*, and nisin *(8)* are capable of transversing the cell wall and membrane. α_{S2}-casein f(183 – 207) was found to transverse both the inner and outer membranes into the cytoplasm of Gram-negative bacteria and cause leakage of the cytoplasmic contents in Gram-positive bacteria after initial attachment *(18)*. In addition to the anionic cell wall and membrane constituents being targets, AMPs also target DNA, RNA, and proteins and inhibits macromolecular synthesis and enzymes. For example, nisin forms pores and also interferes with cell wall biosynthesis by binding to lipid II, a peptidoglycan precursor *(8)*. The formation of pores allows the leakage of cellular contents and the loss of membrane potential. As an example, when nisin forms pores it disrupts the proton motive force and the pH equilibrium *(8)*. This causes the leakage of ions, hydrolysis of ATP, and finally, cell death *(8)*.

Almost all AMPs have less than 100 amino acid residues with most falling into the 3 - 50 residue range *(2, 28, 29)*. The peptide chain of AMPs contain positively charged domains with two or more positively charged residues *(2, 18)* resulting in the AMP being cationic at neutral pH *(28)*. Typically, AMPs have 50% or more of its peptide chain containing hydrophobic residues *(2)*. AMP chains are usually amphipathic and can form structures ranging from linear, random coil molecules that have α-helixes in hydrophobic environments, to β-sheets that contain multiple disulfide bonds without α-helical components *(28)*. It seems that all AMPs have the ability to associate with cell membranes/walls and do not induce antibiotic resistance *(2)*.

As of recent, milk has provided the largest amount of BAPs and it stands reason that several of them can be released from casein as it is the most abundant milk protein. There are some casein-derived antimicrobial that have been researched. One such BAP is casocidin-I. Casocidin-I was isolated from acidified milk and determined to be segment 165 – 203 of bovine α_{S2}-casein *(41)*. Zucht et al. *(41)* also found that casocidin-I has a pI of 8.9, molecular weight of 4870 Daltons and an amphipathic structure. Synthesized casocidin-I was proven to be antibacterial against *Escherichia coli* and *Staphylococcus carnosus (41)*.

Another antimicrobial casein peptide is isracidin. It is bovine α_{S1}-casein segment #1 – 23 that has been lysed by chymosin and has a molecular weight of 2770 Daltons *(17)*. Isracidin has antibacterial activity against lactobacilli, Gram-positive, and Gram-negative bacteria *(17)*. Various Gram-positive bacteria were affected by a concentration between 0.1 and 1 mg/mL *(17)*. Furthermore, Birkemo et al. *(3)* showed that isracidin was effective against *E. coli* DPC6053 with a minimum inhibitory concentration (MIC) of 0.2 mg/mL. However, this AMP has mostly been tested *in vivo* as immunoprotectants for animal infections against such bacteria as *L. monocytogenes* and *Staphylococcus aureus (17)*. Isracidin and other milk-derived peptides have

not gained FDA approval as clinically useful compounds, because they are typically isomeric mixtures that vary from batch to batch, and the FDA requires homogeneity *(17)*.

A third antimicrobial casein peptide that has been well researched is glycomacropeptide (GMP). GMP is the "hairy extension" on the casein micelle that is lysed from κ-casein by chymosin in cheese making. The GMP segment (residues 106 – 169) is hydrophilic and accounts for 15 – 20% of the total whey protein *(1)*. GMP has shown to be antimicrobial against cariogenic Gram-positive *Streptococcus mutans* and Gram-negative *Porphyromonas gingivitis* and *E.coli (21)*. Interestingly, *Vibrio cholerae* and *E. coli* enterotoxins can be bound by GMP, which can mimic enterotoxin carbohydrate structures that bind to cell receptors *(29)*. Currently, GMP is being used in chewing gum and oral health care products.

Other antimicrobial casein peptides recently discovered include casecidin 15 and 17 and caseicin A, B, and C. Casecidin 15 and 17 are derived from acidified colostrum and are found on the C-terminal of bovine β-casein *(3)*. They both had MICs of 0.4 mg/mL against *E. coli* DPC6053 *(3)*. The other antimicrobial casein peptides, caseicin A, B, and C, in contrast, were produced by *Lactobacillus acidophilus* DPC6026 fermentation *(14)*. All three were derived from bovine α_{S1}-casein and had MIC values against *E. coli* DPC5063 of 52 μg/ml, 0.22 μg/ml, and 1.48 mg/ml, respectively *(14)*.

Although the aforementioned and other casein-derived peptides were found to be antimicrobial, the literature is devoid of reference to the control of *L. monocytogenes* on frankfurters or meat with antimicrobial casein peptides. Most studies are limited to lactic acid bacteria bacteriocins being added as a single peptide *(22)*, in combination with chemical antimicrobials *(35)*, or bacteriocin producing bacteria as a hurdle step *(22)*. Antimicrobial casein

peptides may prove to be a useful for post-processing control of *L. monocytogenes* on frankfurters.

The objectives of this thesis were to:

1. further the prediction of antimicrobial peptides and improve casein hydrolysate preparation.
2. use minimally processed casein hydrolysates to challenge *L. monocytogenes* and *E. coli* O157:H7 survival in microdilution and biobroth assays.
3. use minimally processed casein hydrolysates as an antimicrobial dip for processed frankfurters inoculated with *L. monocytogenes*.

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Chapter 1: Pepsin and Trypsin-Casein Hydrolysate: Theoretical Digestion and Preparation

Abstract

Antimicrobial peptides (AMPs) are commonly obtained by cutting the intact protein into peptide fragments by enzymes or acidification. By knowing the action of an enzyme, primary structure of a protein, and common characteristics of AMPs, potential peptide fragments can be predicted and screened for their antimicrobial activity. The most common variants of α_{S1}, α_{S2}, β, and κ casein primary peptide sequences were analyzed for greatest possible enzyme cuts on the protein, peptide masses, isoelectric charge, net charge, and percent hydrophilic residues using online proteomics programs. The fragments were explored for AMP commonalities: fragment length of 3 – 50 amino acids, positive (cationic) net charge, and hydrophilic residues between 25 and 50%. This technique identified16 potential AMPs which proved that it is possible to screen for AMPs. The technique will help researchers determine the plausibility of finding AMPs before conducting research projects.

Introduction

Antimicrobial peptides (AMPs) potentially can be derived from any protein source. One protein source for AMPs that has become of interest is casein. As of recent, milk has provided the largest amount of bio-active peptides and it stands reason that several of them can be released from casein as it is the most abundant milk protein. There are some casein-derived antimicrobial that have been researched. One such BAP is casocidin-I. Casocidin-I was isolated from acidified milk and determined to be segment 165 – 203 of bovine α_{S2}-casein *(10)*. Zucht et al. *(10)* also found that casocidin-I has a pI of 8.9, molecular weight of 4870 Daltons and an amphipathic structure. Synthesized casocidin-I was proven to be antibacterial against *Escherichia coli* and *Staphylococcus carnosus (10)*.

Another antimicrobial casein peptide that has been well researched is glycomacropeptide (GMP). GMP is the "hairy extension" on the casein micelle that is lysed from κ-casein by chymosin in cheese making. The GMP segment (residues 106 – 169) is hydrophilic and accounts for 15 – 20% of the total whey protein *(1)*. GMP has shown to be antimicrobial against cariogenic Gram-positive *Streptococcus mutans* and Gram-negative *Porphyromonas gingivitis* and *E. coli (6)*. Interestingly, *Vibrio cholerae* and *E. coli* enterotoxins can be bound by GMP, which can mimic enterotoxin carbohydrate structures that bind to cell receptors *(8)*. GMP is currently being used in chewing gum and oral health care products.

Almost all AMPs have less than 100 amino acid residues with most falling into the 3 - 50 residue range *(2, 7, 8)*. The peptide chain of AMPs contain positively charged domains with two or more positively charged residues *(2, 5)* resulting in the AMP being cationic at neutral pH *(7)*. Typically, AMPs have 50% or more of its peptide chain containing hydrophobic residues *(2)*. AMP chains are usually amphipathic and can form structures ranging from linear, random coil

molecules that have α-helixes in hydrophobic environments, to β-sheets that contain multiple disulfide bonds without α-helical components *(7)*. It seems that all AMPs have the ability to associate with cell membranes/walls and do not induce antibiotic resistance *(2)*.

AMPs are commonly obtained by cutting the intact protein into peptide fragments with enzymes or by acidification. By knowing the action of an enzyme, primary structure of a protein, and common characteristics of AMPs, potential peptide fragments can be predicted and screened for their antimicrobial activity. Burris has shown predicted cutting sites on the casein molecule by trypsin and pepsin and also developed a digestion protocol *(3)*. The purpose of this study was to expand on Burris' research on both of these subjects *(3)*.

Materials and Methods

Theoretical digest of casein with pepsin and trypsin. The most common variants of α_{S1}, α_{S2}, β, and κ casein primary peptide sequences were gathered from Eigel et al *(4)*. The primary sequences were catalogued by enzyme and casein type. Then each casein type was analyzed for greatest possible enzyme cuts on the protein, peptide masses, and pI, taking into account all cysteines in reduced form, methionines not oxidized, and masses in monoisotopic form. The pepsin enzyme selection was set for pH 1.3. This analysis was conducted at the ExPasy website with the Peptide Mass program found at http://us.expasy.org/tools/. Further analysis was conducted to determine the net charge and percent hydrophilic residues for all peptide fragments using the Peptide Property Calculator at the Innovagen website (http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp). The fragments were explored for AMP commonalities: fragment length

of 3 – 50 amino acids, positive (cationic) net charge, and hydrophilic residues between 25 and 50%.

Casein digest protocol. A 10% sodium caseinate solution was prepared by slowly adding sodium caseinate (Sigma Product # C8654; St. Louis, MO) to deionized water to avoid large clumps. It was stirred until completely dissolved for about one hour at ambient temperature. For the pepsin digestion, the sodium caseinate solution was adjusted to pH 2.0 ± 0.5 with 6N HCl. When the solution was pH stable for 10 minutes, a 0.2% pepsin (Sigma Product # P6887; St. Louis, MO) solution was added for final solution concentration of 5% casein and 0.1% pepsin. For trypsin digestion, the 10 % caseinate solution was adjusted to pH 8.0 with 1N NaOH. When the solution was pH stable for 10 minutes, a 0.2% trypsin (Sigma Product # T1426; St. Louis, MO) solution was added for final solution concentration of 5% casein and 0.1% trypsin. The solutions were allowed to digest on a stir plate for 2 h at ambient temperature. After digestion, solutions was placed into a 95°C water bath until it reached 95°C for 5 minutes to deactivate the enzymes and then cooled to 25°C in an ice water bath. Solutions were centrifuged in a Sorvall RC-B5 Plus (ThermoEletric; Waltham, MA) at 45,000 x *g* for 30 minutes at 10°C. The supernatants were decanted, filter sterilized (0.22 μm), and stored at 4°C.

Results and Discussion

Theoretical digest of casein with pepsin and trypsin. Table 2.1 (Appendix contains all tables and figures) lists the 16 identified potential AMPs and their theoretical properties. All digests except Trypsin *Bos* β–Casein A^2-5P, produced AMPs. Each enzyme yielded eight potential AMPs. The results show that it is possible to screen for potential AMPs using known primary structure of proteins, enzyme specificity, and AMP commonalities.

Burris reported a very basic view of peptide fragments produced by pepsin and trypsin digested casein *(3)*. Since it was basic, certain enzyme specificity rules were not used and theoretical peptide fragment properties were not found. Trypsin cleaves on the carboxyl side of lysine and arginine, but not when arginine and lysine are followed by proline; this is an example of a rule not observed by Burris *(3)*. To improve on the research, this project utilized online proteomic programs that include enzyme cutting rules and theoretical characteristics of peptide fragments. These programs require a single letter amino acid code, which makes it easy to miss or mistype a letter while entering peptide sequences. Any changes in the primary structure sequence can have consequences such as missed cutting sites, imprecise theoretical characteristics, and ultimately the inability to identify potential AMPs. To minimize this type of error, it is recommended to enter the code sequentially in sets of 10 and then double-check the codes.

This technique could be used for any enzyme with known cutting specificities and protein substrate that has a known primary structure to screen for AMPs prior to conducting full research projects. However, further research is necessary to determine if the identified potential AMPs actually prove to be antimicrobial. AMPs found to be antimicrobial should be placed into a database such as the one described by Wang and Wang *(9)* to continue study on AMP properties. Understanding what makes a strong AMP will allow the synthesis of AMPs that can be used to control pathogens. An improvement to this technique should include structure analysis of amphipacity, because most AMPs have this characteristic *(7)*. Current programs are not geared toward small peptide segments. Development of a software suite for this technique would cut down on the many hours of cataloguing and entering each individual fragment for analysis. Furthermore, such a program could aid in selecting enzymes that produce desired AMPs.

Casein digest protocol. The final digestion solutions are clear and slightly straw colored. On average, the final pH was 7.1 for trypsin digest and 2.7 for pepsin digest. This study incorporated several modifications of Burris' casein hydrolysate protocol (Method 2) *(3)*. The first change was to the casein product used. Originally, the casein used was a granular powder that did not dissolve into solution. The granules persisted even after digestion, which means that the solution never reached a 5% concentration. The switch was to sodium caseinate, which dissolves completely into water. This product must be added slowly, because the powder is so fine that it clumps excessiveoly. The clumps eventually dissolve, but it can take hours of continual stirring. Additionally, if the solution is stirred too rapidly, it creates foam that can overflow the container.

Another product exchange was the pepsin and trypsin enzymes. The original enzymes chosen were without any enzymatic activity indication, which was a sign of low purity. Having a low purity increases digestion time and impurities to the final product. In this case trypsin has an activity rating of ≥10,000 BAEE units/mg of protein, where 1 BAEE unit produces a change in absorbance (253 nm) of 0.001 per minute with BAEE as a substrate. Trypsin was also treated with TPCK to inhibit the activity of chymotrypsin. Pepsin was rated at 3200 – 4500 units/mg of protein, where one unit produces a change in absorbance (280 nm) of 0.001 per minute as TCA soluble products using hemoglobin as substrate. Although, the activity ratings are protein specific, they give a comparison within the specific enzyme of its speed to hydrolyze proteins. A higher activity rating is an indication of its purity. The enzymes are very fluffy and stick to weigh boats. It is wise to cover the weigh boat while moving from balance to workbench, because the slightest air movement disperses the material, wasting precious enzyme. Weigh boats and papers should be rinsed into the vessel used for dissolving the enzyme because of their

static retention of the powder. Burris *(3)* utilized each enzyme at 1:100 w/w (enzyme:casein) and a 5 h incubation. For the present study, a 1:50 w/w (enzyme:casein) ratio was used, which allowed for a decrease in incubation to 2 h.

A fourth change was to indicate the acid and base used to adjust pH of the casein solution. Burris *(3)* failed to indicate the acid or base used and the concentration used to obtain the appropriate pH for digestion. In this study, 6N HCl and 1N NaOH were used for pepsin and trypsin digests, respectively. Reaching the correct pH for pepsin digests proved to be difficult. The solution became thick and clumped and required swirling by hand after each drop of acid. Additionally, the thick solution coated the pH probe, which may have contributed to slow pH readings and inaccuracies. Another problem was reaching a stable pH. The sodium caseinate solution behaved much like a buffer. As soon as the target pH was reached, it would slowly decrease towards the starting pH of around 4.6. The trypsin digest was much easier to obtain optimal pH for digestion. An improvement to this protocol would be to decrease the casein concentration, thereby decreasing the viscosity of the solution.

The fifth adjustment was to change the centrifugation of 4000 x *g* for 10 minutes *(3)* to 45,000 x *g* for 30 minutes. The solids did not pelletize well at the lower force and shorter time. By increasing centrifugation force and time, solids compacted better for decanting the supernatant.

In order to prevent precipitation of the peptides, the final solution was not adjusted to pH 7, as Burris did *(3)*. Trypsin digest did not require adjustment because the pH was already about 7.1, but the pepsin digest was very acidic at pH 2.7. Unfortunately, using base to increase pH of the pepsin digest may create salts in the solution causing peptides to precipitate out of solution.

Lastly, for added sterility, a 0.22 μm filter was used instead of the 0.45 μm filter used by Burris *(3)*.

Conclusion

This study demonstrates that it is possible to screen for AMPs using known enzyme specificities, primary protein structures, and AMP commonalities. This will help researchers determine the appropriate AMPs in enzymatic digestions of proteins before undergoing a full research project. The casein digestion protocol has proven to be difficult, but the improvements should increase yields of peptide fragments and possibly AMPs.

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Appendix

TABLE 1.1. Potential antimicrobial peptides identified from theoretical bovine casein digests with pepsin or trypsin and their properties. (See Figure 1.1 and Tables 1.2 – 1.10 for further information.)

Peptide Sequence[1]	Fragment Position[1]	Number of Amino Acids[1]	Mass[1]	pI[1]	Net Charge (pH 7)[2]	% Hydrophilic Residues[2]
Trypsin *Bos* α_{S1}-Casein B-8P Digest						
HPIK	4 - 7	4	494.31	8.76	+1	25%
HIQK	80 - 83	4	525.31	8.76	+1	50%
LHSMK	120 - 124	5	615.33	8.76	+1	40%
QGIHAQQK	125 - 132	8	909.49	8.76	+1	50%
Pepsin *Bos* α_{S1}-Casein B-8P Digest						
RPKHPIKHQGL	1 - 11	10	1310.78	11.17	+3	36%
KKYKVPQL	102 - 109	8	1003.63	10	+3	50%
Trypsin *Bos* α_{S2}-Casein A-11P Digest						
HYQK	77 - 80	4	575.29	8.6	+1	50%
TVYQHQK	182 - 188	7	903.47	8.29	+1	43%
AMKPWIQPK	189 - 197	9	1098.61	10	+2	33%
Pepsin *Bos* α_{S2}-Casein A-11PDigest						
NPWDQVKRNAVPITPTL	107 - 123	17	1949.06	8.75	+1	35%
TKKTKL	148 - 153	6	718.48	10.3	+3	50%
KTVYQHQKAMKPWIQP KTKVIPYVRYL	181 - 207	27	3343.89	10.22	+6	33%
Pepsin *Bos* β-Casein A^2-5P Digest						
QPEVMKVSKVKEAMAP KHKEMPF	89 - 111	23	2669.4	9.4	+2	43%
Trypsin *Bos* κ-Casein B-1P Digest						
YPSYFLNYYQQKPVALI NNQFLPYPYYAKPAAVR	35 - 68	34	4100.11	8.38	+3	29%
Pepsin *Bos* κ-Casein B-1P Digest						
SDKIAKYIPIQYVL	19 - 32	14	1650.95	8.15	+1	36%
SDTVPAKSCQAQPTTMA RHPHPHL	80 - 103	24	2610.27	8.01	+1	29%

[1]Analyzed at Expasy with Peptide Mass program found at http://us.expasy.org/tools/.
[2]Analyzed at Innovagen with Peptide Property Calculator found at http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp.

Trypsin *Bos* α_{S1}-Casein B-8P Digest

R_1PK●HPIK●HQGLPQEVLNENLLR●FFVAPFPQVFGK●EK●VNELSK●DIGSESTEDQAMEDIK●EMEAESIS SSEEIVPNSVEQK●HIQK●EDVPSER●YLGYLEQLLR●LK●K●YK●VPQLEIVPNSAEER●LHSMK●QGIHAQ QK●EPMGVNNQELAWFYPELFR●QFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSEK●TTMPL W_{199}

Pepsin *Bos* α_{S1}-Casein B-8P Digest

R_1PKHPIKHQGL●PQEVL●NENL●L●RF●F●VAPF●PQVF●GKEKVNEL●SKDIGSESTEDQAMEDIKEMEAE SISSSEEIVPNSVEQKHIQKEDVPSERYL●GYL●EQL●L●RL●KKYKVPQL●EIVPNSAEERL●HSMKQGIHA QQKEPMGVNNQEL●AWF●YPEL●F●RQF●YQL●DAYPSGAWYYVPL●GTQYTDAPSF●SDIPNPIGSENSE KTTMPL●W_{199}

Trypsin *Bos* α_{S2}-Casein A-11P Digest

K_1●NTMEHVSSSEESIISQQTTK●EEK●NMAINPSK●ENLCSTFCK●EVVR●NANEEEYSIGSSSEESAEVATE EVK●ITVDDK●HYQK●ALNEINEFYQK●FPQYLQYLYQGPIVLNPWDQVK●R●NAVPITPTLNR●EQLSTSE ENSK●K●TVDMESTEVFTK●K●TK●LTEEEK●NR●LNFLLK●ISQR●TQK●FALPQYLK●TVYQHQK●AMK PWIQPK●TK●VIPYVR●YL_{207}

Pepsin *Bos* α_{S2}-Casein A-11P Digest

K_1NTMEHVSSSEESIISQQTTKEEKNMAINPSKENL●CSTF●CKEVVRNANEEEYSIGSSSEESAEVATEEVKI TVDDKHYQKAL●NEINEF●YQKF●PQYL●QYL●YQGPIVL●NPWDQVKRNAVPITPTL●NREQL●STSEENS KKTVDMESTEVF●TKKTKL●TEEEKNRL●NF●L●L●KISQRTQKF●PQYL●AL●KTVYQHQKAMKPWIQPK TKVIPYVRYL$_{207}$

Trypsin *Bos* β-Casein A^2-5P Digest

R_1●ELEELNVPGEIVESLSSSEESITR●INK●K●IEK●FQSEEQQQTEDELQDK●IHPFAQTQSLVYPFPFPIPNSL PQNIPPLTQPPVVVPPFLQPEVMK●VSK●VK●EAMAPK●HK●EMPFPK●YPVQPFTESQSLTLTDVENLHLP PLLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSK●VLPVPEK●AVPYPQR●DMPIQAFLLYQQPVLGPVR●GP FPIIV$_{209}$

Pepsin *Bos* β-Casein A^2-5P Digest

R_1EL●EEL●NVPGEIVESL●SSSEESITRINKKIEKF●QSEEQQQTEDEL●QDKIHPF●AQTQSL●VYPF●PF●PIP NSL●PQNIPPL●TQPPVVVPPF●L●QPEVMKVSKVKEAMAPKHKEMPF●PKYPVQPF●TESQSL●TL●TDVE NL●HL●PPL●L●L●QSWMHQPHQPL●PPTVMF●PPQSVL●SL●SQSKVL●PVPEAVPYPQRDMPIQAF●L●L● YQQPVL●GPVRGPF●PIIV$_{209}$

Trypsin *Bos* κ-Casein B-1P Digest

E_1EQNQEQPIR●CEK●DER●FFSDK●IAK●YIPIQYVLSR●YPSYFLNYYQQKPVALINNQFLPYPYYAKPAA VR●SPAQILQWQVLSDTVPAK●SCQAQPTTMAR●HPHPHLSFMAIPPK●K●NQDK●TEIPTINTIASGEPTST PTTEAVESTVATLEDSPEVIESPPEINTVQVTSTAV$_{169}$

Pepsin *Bos* κ-Casein B-1P Digest

E_1EQNQEQPIRCEKDERF●F●SDKIAKYIPIQYVL●SRYPSYF●L●NYYQQKPVAL●INNQF●L●PYPYYAKPA AVRSPAQIL●QWQVL●SDTVPAKSCQAQPTTMARHPHPHL●SF●MAIPPKKNQDKTEIPTINTIASGEPTSTP TTEAVESTVATL●EDSPEVIESPPEINTVQVTSTAV$_{169}$

FIGURE 1.1. Theoretical trypsin or pepsin digestion of each casein component, where "●" represents potential sites for enzymatic cuts into casein fragments. The enzymatic cuts were generated at Expasy with Peptide Mass program found at http://us.expasy.org/tools/. The highlighted fragments have been identified as potential antimicrobial peptides.

TABLE 1.2. Theoretical trypsin *Bos* α_{S1}-Casein B-8P digest properties.

Peptide Sequence[1]	Fragment Position[1]	Number of Amino Acids[1]	Mass[1]	pI[1]	Net Charge (pH 7)[2]	% Hydrophilic Residues[2]
RPK	1 - 3	3	400.26	11.00	2	67%
HPIK	4 - 7	4	494.31	8.76	1	25%
HQGLPQEVLNENLLR	8 - 22	15	1759.94	5.40	-1	47%
FFVAPFPQVFGK	23 - 34	12	1383.75	8.75	1	17%
LK	35 - 36	2	260.2	8.75	1	50%
VNELSK	37 - 42	6	689.38	5.97	0	67%
DIGSESTEDQAMEDIK	43 - 58	16	1767.76	3.71	-5	63%
EMEAESISSSEEIVPNSVEQK	59 - 79	21	2322.07	3.90	-5	67%
HIQK	80 - 83	4	525.31	8.76	1	50%
EDVPSER	84 - 90	7	831.38	4.14	-2	71%
YLGYLEQLLR	91 - 100	10	1267.7	6.00	0	30%
EK	101 - 102	2	276.15	6.10	0	100%
K	103	1	147.11	8.75	1	100%
YK	104 - 105	2	310.18	8.59	1	50%
VPQLEIVPNSAEER	106 - 119	13	1580.83	4.25	-2	50%
LHSMK	120 - 124	5	615.33	8.76	1	40%
QGIHAQQK	125 - 132	8	909.49	8.76	1	50%
EPMGVNNQELAWFYPELFR	133 - 151	19	2340.11	4.25	-2	37%
QFYQLDAYPSGAWYYVPLGTQ YTDAPSFSDIPNPIGSENSEK	152 - 193	42	4716.17	3.85	-4	38%
TTMPLW	194 - 199	6	748.37	5.19	0	0%

[1]Analyzed at Expasy with Peptide Mass program found at http://us.expasy.org/tools/.
[2]Analyzed at Innovagen with Peptide Property Calculator found at http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp.

TABLE 1.3. Theoretical pepsin *Bos* α_{S1}-Casein B-8P digest properties.

Peptide Sequence[1]	Fragment Position[1]	Number of Amino Acids[1]	Mass[1]	pI[1]	Net Charge (pH 7)[2]	% Hydrophilic Residues[2]
RPKHPIKHQGL	1 - 11	10	1310.78	11.17	3	36%
PQEVL	12 - 16	5	585.32	4.00	-1	40%
NENL	17 - 20	4	489.23	4.00	-1	75%
L	21	1	132.1	5.52	0	0%
RF	22 - 23	2	322.19	9.75	1	50%
F	24	1	166.09	5.52	0	0%
VAPF	25 - 28	4	433.24	5.49	0	0%
PQVF	29 - 32	4	490.26	5.96	0	25%
GKEKVNEL	33 - 40	8	916.51	6.14	0	63%
SKDIGSESTEDQAMEDIKEMEA ESISSSEEIVPNSVEQKHIQKED VPSERYL	41 - 92	52	5880.74	4.18	-10	63%
GYL	93 - 95	3	352.19	5.52	0	0%
EQL	96 - 98	3	389.2	4.00	-1	67%
L	99	1	132.1	5.52	0	0%
RL	100 - 101	2	288.2	9.75	1	50%
KKYKVPQL	102 - 109	8	1003.63	10.00	3	50%
EIVPNSAEERL	110 - 120	11	1256.65	4.25	-2	55%
HSMKQGIHAQQKEPMGVNNQ EL	121 - 142	22	2504.21	6.92	0	50%
AWF	143 - 145	3	423.2	5.57	0	100%
YPEL	146 - 149	4	521.26	4.00	-1	25%
F	150	1	166.09	5.52	0	0%
RQF	151 - 153	3	450.25	9.75	1	67%
YQL	154 - 156	3	423.22	5.52	0	33%
DAYPSGAWYYVPL	157 - 169	13	1501.7	3.80	-1	15%
GTQYTDAPSF	170 - 179	10	1086.47	3.80	-1	30%
SDIPNPIGSENSEKTTMPL	180 - 198	19	2029.97	4.14	-2	47%
W	199	1	205.1	5.15	0	0%

[1]Analyzed at Expasy with Peptide Mass program found at http://us.expasy.org/tools/.
[2]Analyzed at Innovagen with Peptide Property Calculator found at http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp.

TABLE 1.4. Theoretical trypsin *Bos* α_{S2}-Casein A-11P digest properties.

Peptide Sequence[1]	Fragment Position[1]	Number of Amino Acids[1]	Mass[1]	pI[1]	Net Charge (pH 7)[2]	% Hydrophilic Residues[2]
K	1	1	147.11	8.75	1	100%
NTMEHVSSSEESIISQQTTK	2 - 21	20	2236.04	4.75	-2	60%
EEK	22 - 24	3	405.2	4.53	-1	100%
NMAINPSK	25 - 32	8	874.45	8.75	1	50%
ENLCSTFCK	33 - 41	9	1044.45	6.08	0	44%
EVVR	42 - 45	4	502.3	6.10	0	50%
NANEEEYSIGSSSEESAEVATEEVK	46 - 70	25	2688.16	3.77	-7	64%
ITVDDK	71 - 76	6	690.37	4.21	-1	50%
HYQK	77 - 80	4	575.29	8.60	1	50%
ALNEINEFYQK	81 - 91	11	1368.68	4.53	-1	55%
FPQYLQYLYQGPIVLNPWDQVK	92 - 113	22	2709.41	5.83	0	32%
R	114	1	175.12	9.75	1	100%
NAVPITPTLNR	115 - 125	11	1195.68	9.75	1	27%
EQLSTSEENSK	126 - 136	11	1251.57	4.25	-2	82%
K	137	1	147.11	8.75	1	100%
TVDMESTEVFTK	138 - 149	12	1386.65	4.14	-2	42%
K	150	1	147.11	8.75	1	100%
TK	151 - 152	2	248.16	8.41	1	50%
LTEEEK	153 - 158	6	748.37	4.25	-2	67%
NR	159 - 160	2	289.16	9.75	1	100%
LNFLLK	161 - 166	6	747.48	8.75	1	33%
ISQR	167 - 170	4	503.29	9.75	1	75%
TQK	171 - 173	3	376.22	8.41	1	67%
FALPQYLK	174 - 181	8	979.56	8.59	1	25%
TVYQHQK	182 - 188	7	903.47	8.29	1	43%
AMKPWIQPK	189 - 197	9	1098.61	10.00	2	33%
TK	198 - 199	2	248.16	8.41	1	50%
VIPYVR	200 - 205	6	746.46	8.72	1	17%
YL	206 - 207	2	295.17	5.52	0	0%

[1]Analyzed at Expasy with Peptide Mass program found at http://us.expasy.org/tools/.
[2]Analyzed at Innovagen with Peptide Property Calculator found at http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp.

TABLE 1.5.Theoretical pepsin *Bos* α_{S2}-Casein A-11P digest properties.

Peptide Sequence[1]	Fragment Position[1]	Number of Amino Acids[1]	Mass[1]	pI[1]	Net Charge (pH 7)[2]	% Hydrophilic Residues[2]
KNTMEHVSSSEESIISQQTTKEE KNMAINPSKENL	1 - 35	35	3961.91	5.10	-2	63%
CSTF	36 - 39	4	457.18	5.52	0	25%
CKEVVRNANEEEYSIGSSSEES AEVATEEVKITVDDKHYQKAL	40 - 82	43	4814.29	4.50	-6	56%
NEINEF	83 - 88	6	765.34	3.79	-2	67%
YQKF	89 - 92	4	585.3	8.59	1	50%
PQYL	93 - 96	4	520.28	5.95	0	25%
QYL	97 - 99	3	423.22	5.52	0	33%
YQGPIVL	100 - 106	7	789.45	5.52	0	14%
NPWDQVKRNAVPITPTL	107 - 123	17	1949.06	8.75	1	35%
NREQL	124 - 153	5	659.35	6.00	0	80%
STSEENSKKTVDMESTEVF	129 - 147	19	2147.96	4.25	-3	63%
TKKTKL	148 - 153	6	718.48	10.30	3	50%
TEEEKNRL	154 - 161	8	1018.52	4.78	-1	76%
NF	162 - 163	2	280.13	5.52	0	50%
L	164	1	132.1	5.52	0	0%
L	165	1	132.1	5.52	0	0%
KISQRTQKF	166 - 174	9	1135.66	11.17	3	67%
AL	175 - 176	2	203.14	5.57	0	0%
PQYL	177 - 180	4	520.28	5.95	0	25%
KTVYQHQKAMKPWIQPKTKVI PYVRYL	181 - 207	27	3343.89	10.22	6	33%

[1]Analyzed at Expasy with Peptide Mass program found at http://us.expasy.org/tools/.
[2]Analyzed at Innovagen with Peptide Property Calculator found at http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp.

TABLE 1.6. Theoretical trypsin *Bos* β-Casein A^2-5P digest properties.

Peptide Sequence[1]	Fragment Position[1]	Number of Amino Acids[1]	Mass[1]	pI[1]	Net Charge (pH 7)[2]	% Hydrophilic Residues[2]
R	1	1	175.12	9.75	1	100%
ELEELNVPGEIVESLSSSEESITR	2 - 25	24	2646.3	3.83	-6	58%
INK	26 - 28	3	374.23	8.75	1	67%
K	29	1	147.11	8.75	1	100%
IEK	30 - 32	3	389.24	6.00	1	67%
FQSEEQQQTEDELQDK	33 - 48	16	1981.86	3.77	-5	81%
IHPFAQTQSLVYPFPFPIPNSLPQ NIPPLTQPPVVVPPFLQPEVMK	49 - 94	46	5159.78	6.75	0	24%
VSK	95 - 97	3	333.21	8.72	1	67%
VK	98 - 99	2	246.18	8.72	1	50%
EAMAPK	100 - 105	6	646.32	6.10	0	33%
HK	106 - 107	2	284.17	8.76	1	50%
EMPFPK	108 - 113	6	748.37	6.10	0	33%
YPVQPFTESQSLTLTDVENLHL PPLLLQSWMHQPHQPLPPTVM FPPQSVLSLSQSK	114 - 169	56	6358.27	5.75	-2	34%
VLPVPEK	170 - 176	7	781.48	5.97	0	29%
AVPYPQR	177 - 183	7	830.45	8.79	1	29%
DMPIQAFLLYQQPVLGPVR	184 - 202	19	2185.18	5.84	0	26%
GPFPIIV	203 - 209	7	742.45	5.52	0	0%

[1]Analyzed at Expasy with Peptide Mass program found at http://us.expasy.org/tools/.
[2]Analyzed at Innovagen with Peptide Property Calculator found at http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp.

TABLE 1.7. Theoretical pepsin *Bos* β-Casein A[2]-5P digest properties.

Peptide Sequence[1]	Fragment Position[1]	Number of Amino Acids[1]	Mass[1]	pI[1]	Net Charge (pH 7)[2]	% Hydrophilic Residues[2]
REL	1 - 3	3	417.25	6.00	0	67%
EEL	4 - 6	3	390.19	3.80	-2	67%
NVPGEIVESL	7 - 16	10	1056.56	3.67	-2	40%
SSSEESITRINKKIEKF	17 - 33	17	1996.07	8.22	1	71%
QSEEQQQTEDEL	34 - 45	12	1463.61	3.42	-5	83%
QDKIHPF	46 - 52	7	884.46	6.74	0	43%
AQTQSL	53 - 58	6	647.34	5.57	0	50%
VYPF	59 - 62	4	525.27	5.49	0	0%
PF	63 - 64	2	263.14	5.96	0	0%
PIPNSL	65 - 70	6	640.37	5.96	0	33%
PQNIPPL	71 - 77	7	778.45	5.96	0	29%
TQPPVVVPPF	78 - 87	10	1080.61	5.19	0	10%
L	88	1	132.1	5.52	0	0%
QPEVMKVSKVKEAMAPKHKE MPF	89 - 111	23	2669.4	9.40	2	43%
PKYPVQPF	112 - 119	8	975.53	9.01	1	25%
TESQSL	120 - 125	6	664.31	4.00	-1	67%
TL	126 - 127	2	233.15	5.19	0	0%
TDVENL	128 - 133	6	690.33	3.67	-2	50%
HL	134 - 135	2	269.16	6.74	0	0%
PPL	136 - 138	3	326.21	5.96	0	0%
L	139	1	132.1	5.52	0	0%
L	140	1	132.1	5.52	0	0%
QSWMHQPHQPL	141 - 151	11	1388.65	6.92	0	36%
PPTVMF	152 - 157	6	691.35	5.96	0	0%
PPQSVL	158 - 163	6	640.37	5.96	0	33%
SL	164 - 165	2	219.13	5.24	0	50%
SQSKVL	166 - 171	6	661.39	8.47	1	67%
PVPEKAVPYPQRDMPIQAF	172 - 190	19	2183.13	6.49	0	32%
L	191	1	132.1	5.52	0	0%
L	192	1	132.1	5.52	0	0%
YQQPVL	193 - 198	6	747.4	5.52	0	33%
GPVRGPF	199 - 205	7	729.4	9.75	1	14%
PIIV	206 - 209	4	441.31	5.96	0	0%

[1]Analyzed at Expasy with Peptide Mass program found at http://us.expasy.org/tools/.
[2]Analyzed at Innovagen with Peptide Property Calculator found at http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp.

TABLE 1.8. Theoretical trypsin *Bos* κ-Casein B-1P digest properties.

Peptide Sequence[1]	Fragment Position[1]	Number of Amino Acids[1]	Mass[1]	pI[1]	Net Charge (pH 7)[2]	% Hydrophilic Residues[2]
EEQNQEQPIR	1 - 10	11	1270.6	4.25	-2	80%
CEK	11 - 13	3	379.16	5.99	0	67%
DER	14 - 16	3	419.19	4.37	-1	100%
FFSDK	17 - 21	5	643.31	5.84	0	60%
IAK	22 - 24	3	331.23	8.75	1	33%
YIPIQYVLSR	25 - 34	10	1251.71	8.59	1	30%
YPSYFLNYYQQKPVALINNQFL PYPYYAKPAAVR	35 - 68	34	4100.11	8.38	3	29%
SPAQILQWQVLSDTVPAK	69 - 86	18	1981.08	5.55	0	39%
SCQAQPTTMAR	87 - 97	11	1193.54	7.96	1	36%
HPHPHLSFMAIPPK	98 - 111	14	1608.85	8.77	1	14%
K	112	1	147.11	8.75	1	100%
NQDK	113 - 116	4	504.24	5.84	0	100%
TEIPTINTIASGEPTSTPTTEAVE STVATLEDSPEVIESPPEINTVQ VTSTAV	117 - 169	53	5453.7	3.28	-9	34%

[1] Analyzed at Expasy with Peptide Mass program found at http://us.expasy.org/tools/.
[2] Analyzed at Innovagen with Peptide Property Calculator found at http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp.

TABLE 1.9. Theoretical pepsin *Bos* κ-Casein B-1P digest properties.

Peptide Sequence[1]	Fragment Position[1]	Number of Amino Acids[1]	Mass[1]	pI[1]	Net Charge (pH 7)[2]	% Hydrophilic Residues[2]
EEQNQEQPIRCEKDERF	1 - 17	17	2177.99	4.42	-3	76%
F	18	1	166.09	5.52	0	0%
SDKIAKYIPIQYVL	19 - 32	14	1650.95	8.15	1	36%
SRYPSYF	33 - 39	7	919.43	8.31	1	43%
L	40	1	132.1	5.52	0	0%
NYYQQKPVAL	41 - 50	10	1223.64	8.5	1	40%
INNQF	51 - 55	5	635.31	5.52	0	60%
L	56	1	132.1	5.52	0	0%
PYPYYAKPAAVRSPAQIL	57 - 74	18	2005.09	9.55	2	22%
QWQVL	75 - 79	5	673.37	5.52	0	40%
SDTVPAKSCQAQPTTMARHPH PHL	80 - 103	24	2610.27	8.01	1	29%
SF	104 - 105	2	253.12	5.24	0	50%
MAIPPKKNQDKTEIPTINTIASG EPTSTPTTEAVESTVATL	106 - 146	41	4282.2	5.58	-2	34%
EDSPEVIESPPEINTVQVTSTAV	147 - 169	23	2441.19	3.45	-5	43%

[1] Analyzed at Expasy with Peptide Mass program found at http://us.expasy.org/tools/.
[2] Analyzed at Innovagen with Peptide Property Calculator found at http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp.

TABLE 1.10. Possible number of peptide fragments and their properties that were generated from theoretical bovine casein digests with pepsin or trypsin.

Properties	Enzyme	
	Pepsin[1]	Trypsin[2]
Fragment Amino Acid Length		
Above 25 Amino Acids	5	6
Below 25 Amino Acids	88	73
Range (Number of Amino Acids)	1 to 52	1 to 56
Fragment Mass		
Above 2700 Daltons	5	6
Below 2700 Daltons	88	73
Range (Daltons)	132.10 to 5880.74	147.11 to 6358.70
Fragment pI		
Acidic	71	39
Neutral (pH: 7 ± 0.25)	2	1
Basic	20	39
pI Range	3.72 to 11.17	3.28 to 11.00
Net Charge (pH 7)		
Acidic (Negative Charge)	20	40
Neutral (No Charge)	51	18
Basic (Positive Charge)	22	21
% Hydrophilic Residues		
Above 50%	22	33
Between 25 – 50%	41	39
Below 25%	30	7

[1] Total number of generated peptides from pepsin digested casein was 93.
[2] Total number of generated peptides from trypsin digested casein was 79.

Chapter 2: Effects of Pepsin and Trypsin-Casein Hydrolysates on *Listeria monocytogenes* and *Escherichia coli* O157:H7

Abstract

Listeriosis has the highest fatality and hospitalization rates of foodborne illnesses. Recently, milk and its proteins have gained recognition as the largest source of biologically active peptides, and it stands reason that several antimicrobial peptides (AMP) can be released from casein as it is the most abundant milk protein. The purpose of this study was to evaluate trypsin and pepsin casein hydrolysates (PCH and TCH, respectively) in minimally processed form as antimicrobials against *Listeria monocytogenes* and *Escherichia coli* O157:H7. Sodium caseinate was hydrolysed with pepsin or trypsin. *L. monocytogenes* (strains Scott A and 310) and *E. coli* O157:H7 (Salami strain) were incubated in 0, 10, 20, and 40% PCH and 0 and 50% TCH concentrations over a 24-h period. PCH were effective against all three test strains at 40% PCH concentration; growth of *L. monocytogenes* Scott A was suppressed by 1.76 log CFU/mL while initial populations of *L. monocytogenes* 310 and *E.* coli O157:H7 Salami were reduced by 0.52 and 0.62 log CFU/ml, respectively. TCH suppressed growth of *L. monocytogenes* Scott A and 310 by fewer than 0.2 log CFU/ml and not at all for *E. coli* O157:H7. Results of this study demonstrate that minimally processed PCH are slightly antimicrobial against *L. monocytogenes* and *E. coli* O157:H7 and that TCH are not antimicrobial against either bacterium. Further research should be conducted to determine the usefulness of PCH as an antimicrobial agent in food matrices.

Introduction

Listeriosis is a foodborne illness caused by *Listeria monocytogenes*. Listeriosis is a sporadic disease but has the highest fatality rate of 20 - 30% and hospitalization requirements of approximately 90% of foodborne illnesses (*11, 14, 19)*. This bacterium causes two types of illness manifestations. One is non-invasive, which is commonly called listerial gastroenteritis, where symptoms range from nothing to fever, vomiting, and diarrhea in healthy adults *(11, 14)*. Invasive listeriosis, the second and most serious form of the disease, affects infants, pregnant women including their fetuses, immunocompromised individuals, such as AIDS and cancer patients, children, and the elderly *(11, 14, 19)*. The illnesses most often experienced by non-pregnant adults are sepsis, meningitis, meningoencephalitis, or bacteremia *(11)*. Symptoms include fever, malaise, ataxia, seizures, and altered mental status that may not show up for several weeks *(11)*. Pregnant women usually present with flu-like symptoms when infected with *L. monocytogenes (11)*. It is believed that fetal listeriosis is acquired transplacentally or from an intrauterine infection that leads to death or spontaneous abortion of the fetus *(11)*. In 1985, the USDA-FSIS announced that an outbreak associated with *L. monocytogenes*-contaminated Mexican-style cheese caused 29 deaths *(13)*. Of these deaths, eight were neonates, 13 were still births, and eight were non-neonatal *(13)*.

Recently, milk and its proteins have gained recognition as the largest source of biologically active peptides and it stands reason that several antimicrobial peptides (AMP) can be released from casein as it is the most abundant milk protein. One casein-derived antimicrobial that has been studied is casocidin-I. Casocidin-I was isolated from acidified milk and determined to be segment 165 – 203 of bovine α_{S2}-casein *(20)*. Zucht et al. (1995) reported that casocidin-I has an isoelectric point (pI) of 8.9, molecular weight of 4870 Daltons and an amphipathic

structure *(20)*. Synthesized casocidin-I was proven to be antibacterial against *Escherichia coli* and *Staphylococcus carnosus (20)*.

The antimicrobial activity of all AMPs results from their interaction with the cell wall or cell membrane of bacteria. AMPs have an affinity for the anionic phospholipids and lipopolysaccharides found in cell walls and membranes of bacteria *(1)*. Lopez-Exposito et al. *(9)* reported that α_{S2}-casein f(183 – 207) peptide initially binds with lipoteichoic acid of Gram-positive bacteria and lipopolysaccharides in Gram-negative bacteria. It is thought that the cationic side chains of arginine, lysine, and histidine mediate binding *(1)*. After the initial binding, many AMPs fold into their final configuration, possibly with α-helixes *(1, 5)*.

Once attached, an AMP inserts its hydrophobic domain into the bacterial membrane forming pores *(5)* by lateral diffusion and forming complexes with other AMPs *(15)*. AMPs are capable of transversing the cell wall and membrane, as has been shown with Lfcin, α_{S2}-casein f(183 – 207) *(9)*, and nisin *(7)*. α_{S2}-casein f(183 – 207) was found to transverse both the inner and outer membranes into the cytoplasm of Gram-negative bacteria and cause leakage of the cytoplasmic contents in Gram-positive bacteria after initial attachment *(9)*. In addition to the anionic cell wall and membrane constituents being targets, AMPs also target DNA, RNA, and proteins and inhibits macromolecular synthesis and enzymes. For example, nisin forms pores and also interferes with cell wall biosynthesis by binding to lipid II, a peptidoglycan precursor *(7)*. The formation of pores allows the leakage of cellular contents and the loss of membrane potential. As an example, when nisin forms pores it disrupts the proton motive force and the pH equilibrium *(7)*. This causes the leakage of ions, hydrolysis of ATP, and finally, cell death *(7)*.

Although research has been conducted to determine the antimicrobial effect of trypsin and pepsin casein hydrolysates *(3)*, it failed to prepare hydrolysates as a minimally processed

product and determine the appropriate concentration that shows antimicrobial effects against *L. monocytogenes*. Over processing of the hydrolysate may cause the loss of potential AMPs. The purpose of this study was to evaluate trypsin and pepsin casein hydrolysates and their concentrations as an antimicrobial against *L. monocytogenes* in minimally processed product form.

Materials and Methods

Pepsin and trypsin casein digestion. A 10% sodium caseinate solution was prepared by slowly adding sodium caseinate (Sigma Product # C8654; St. Louis, MO) to deionized water to avoid large clumps. It was stirred until completely dissolved for about one hour at ambient temperature. For the pepsin digestion, the sodium caseinate solution was adjusted to pH 2.0 ± 0.5 with 6N HCl. When the solution was pH stable for 10 minutes, a 0.2% pepsin (Sigma Product # P6887; St. Louis, MO) solution was added for final solution concentration of 5% casein and 0.1% pepsin. For trypsin digestion, the 10% caseinate solution was adjusted to pH 8.0 with 1N NaOH. When the solution was pH stable for 10 minutes, a 0.2% trypsin (Sigma Product # T1426; St. Louis, MO) solution was added for final solution concentration of 5% casein and 0.1% trypsin. The solutions were allowed to digest on a stir plate for 2 h at ambient temperature. After digestion, solutions was placed into a 95°C water bath until it reached 95°C for 5 minutes to deactivate the enzymes and then cooled to 25°C in an ice water bath. Solutions were centrifuged in a Sorvall RC-B5 Plus (ThermoEletric; Waltham, MA) at 45,000 x *g* for 30 minutes at 10°C. The supernatants were decanted, filter sterilized (0.22 μm), and stored at 4°C.

pH determination of digests. Pepsin-casein hydrolysate (PCH) and trypsin-casein hydrolysate (TCH) were diluted to 100%, 80%, 60%, 40%, 20%, 10%, 4%, and 2%, and pH was

measured with a Denver Instruments 215 pH meter (Denver Instruments; Denver, CO). PCH and TCH were further diluted 1:1 with double strength tryptic soy broth (Difco; Franklin Lakes, NJ) to a final concentration of 50%, 40%, 30%, 20%, 10%, 5%, 2%, and 1% hydrolysate, and pH was measured again.

Culture preparation and maintenance. *L. monocytogenes* (strains Scott A and 310) culture stocks were maintained on tryptose phosphate agar (TPA) slants (Difco; Franklin Lakes, NJ) and re-cultured every two to four weeks. When re-culturing, a loopful of culture was streaked onto a TPA slant and incubated at 32°C for 24 h, and then returned to refrigerated storage. *E. coli* O157:H7 Salami stock culture was treated in the same manner except it was cultured on tryptic soy agar (TSA) slants (Difco; Franklin Lakes, NJ) and incubated at 35°C.

In preparation for use, a loopful of *L. monocytogenes* stock culture was placed into tryptose phosphate broth (TPB) (Difco; Franklin Lakes, NJ) and incubated at 32°C for 24 h, followed by an additional two 24-h transfers before use. *E. coli* O157:H7 was treated in the same way except it was cultured in tryptic soy broth (TSB) (Difco; Franklin Lakes, NJ) and incubated at 35°C.

Microdilution assay: spectrophotometric determination of effective PCH and TCH concentrations. A 24-h culture of *L. monocytogenes* was diluted to $1x10^5$ CFU/ml in double-strength TPB (DS-TBP). Then 125 µl was added to each well of a 96 well microplate. PCH or trypsin-casein hydrolysate (TCH) was diluted to 100%, 80%, 60%, 40%, 20%, 10%, 4%, and 2% in sterile deionized water. Then 125 µl of each dilution was added to the microplate in columns of three. The control plate was set up with TPB in three columns for a negative control and 125 µl DS-TPB plus 125 µl *L. monocytogenes* for the positive control. Plates were incubated at 32°C for 0, 2, 4, 8, 12, and 24 h. At each incubation period, plates were read with an ELx800

Universal Microplate Reader (Bio-Tek Insturments; Winooski, VT). *E. coli* O157:H7 was also treated in the same way except double-strength TSB was used and incubated at 35°C.

The acidified media plates were set up with $1x10^6$ CFU/ml *L. monocytogenes* diluted in 0.1% peptone water (Difco; Franklin Lakes, NJ). The final dilution of $1x10^5$ CFU/ml *L. monocytogenes* was diluted in TPB. The plate was filled with 250 µl and then incubated at 32°C for 0, 2, 4, 8, 12, and 24 h. A negative control was also included in the plates. At each incubation period, the plates were read by ELx800 Universal Microplate Reader. *E. coli* O157:H7 was also treated in the same way except TSB was used and incubated at 35°C.

Bio-broth Assay: plate count confirmation of the antimicrobial activity on bacteria by PCH and TCH. *L. monocytogenes* was diluted to $1x10^7$ CFU/ml in 0.1% peptone water, and then further diluted to $2x10^5$ CFU/ml into DS-TPB. Solutions of 0, 10, 20, and 40% PCH were added to sterile tubes along with an equal volume of a $2x10^5$ CFU/ml bacterial culture. The biobroth tubes were incubated at 32°C for 0, 2, 4, 8, 12, and 24 h. At each incubation period, a 1.0 ml sample was collected, diluted, and spiral plated onto TPA by a WASP2 Spiral Plater (Microbiology International; Frederick, MD). TPA plates are incubated at 32°C for 24 to 48 h. *E. coli* O157:H7 was also handled in the same manner except the final serial dilution was into TSB and the samples were spiral plated on TSA. All plates were read by the ProtoCOL Colony Counter (Microbiology International; Frederick, MD).

The TCH biobroth assay was conducted in the same manner as the PCH biobroth assay except the TCH test concentrations were 0 and 50%.

For the acidified media assay, TPB was adjusted to pH 5.1 with 1N HCl. *L. monocytogenes* was diluted in 0.1% peptone water to $1x10^7$, and then further diluted to $2x10^5$ CFU/ml in pH 5.1 TPB. The tubes were incubated at 32°C for 0, 2, 4, 8, 12, and 24 h. At each

incubation period, a 1.0 ml sample was taken, diluted, and spiral plated on TPA by a WASP2 Spiral Plater. The plates are then incubated at 32°C for 24 to 48 h. *E. coli* O157:H7 was also handled in the same manner except the final serial dilution was into TSB and the samples were spiral plated on TSA. All plates were read by the ProtoCOL Colony Counter (Microbiology International; Frederick, MD).

Statistical analysis. Statistical analysis was performed by SPSS Statistical software (Version 17, SPSS Inc., Chicago, IL). Analysis of variance was performed to determine statistically significant differences ($P \leq 0.05$) of mean values between treated and control samples and between treatments at each sampling time.

Results

pH determination of digests. pH values of PCH and TCH solutions in deionized water and after mixing with TSB are shown in Table 2.1 (all tables and figures are located in the Appendix). After mixing with deionized water, pH of PCH solutions increased from 2.7 for 100% PCH to 3.81 for 2% PCH. The pH further increased to 5.13 for 50% PCH to 7.04 for 1% PCH. For TCH, pH decreased from 7.10 (100%TCH) to 6.86 for 2% TCH after mixing with deionized water. After the addition of TSB, pH remained constant between 7.04 for 50% TCH and 7.06 for 1% TCH.

Microdilution assay: spectrophotometric determination of effective PCH and TCH concentrations. Figures 2.1 – 2.6 show the results for the microdilution assay. The first three correspond to the effect of PCH on *L. monocytogenes* (Scott A and 310) and *E. coli* O157:H7, respectively. Figures 2.4 – 2.6, refer to the effects of TCH on test organisms. The TSB/TPB (pH 5.1) control and 20% PCH affected *L. monocytogenes* (Figure 2.2) and *E. coli* O157:H7 (Figure

2.3), similarly. Also, 30, 40, and 50% inhibited growth of these organisms. Results for the TSB/TPB (pH 5.1) controls were similar to 20% PCH, but they were significantly different from each other ($p \leq 0.05$). The antimicrobial effect of PCH on *L. monocytogenes* Scott A (Figure 2.1) was less than that for *L. monocytogenes* 310 and *E. coli* O157:H7. The TPB (pH 5.1) control most closely resembled 30% PCH with 40 and 50% PCH suppressing growth. However, there was no significant difference ($p > 0.05$) between the 20% TCH treatment and the TPB (pH 5.1) control, indicating that the two behaved similarly.

The TCH effect on *L. monocytogenes* Scott A (Figure 2.4) was to extend the log phase of growth for 10, 20, 30, 40 and 50% concentrations at 12 h, but *L. monocytogenes* soon recovered and growth was similar to the control at 24 h. For *L. monocytogenes* 310 (Figure 2.5), 40 and 50% TCH extended the log phase at 12 h with 50% having the greatest effect.

The effect of TCH on *E. coli* O157:H7 (Figure 2.6) was the opposite of the expected outcome. The 30, 40, and 50% TCH treatments appeared to increase growth.

Bio-broth assay: plate count confirmation of the antimicrobial activity on bacteria by PCH and TCH. Figures 2.7 – 2.12 lists results for PCH and TCH bio-broth assays. *L. monocytogenes* Scott A (Figure 2.7) was the least sensitive to PCH, with a 1.76 log CFU/ml difference between the 0% PCH control and 40% PCH treatment at 12 h. The pH 5.1 TPB control suppressed growth by 1.29 log CFU/ml at 12 h, indicating that acid alone played a slight role in suppression of *L. monocytogenes*. However, after 24 h, the difference was reduced for both the pH 5.1 TPB (0.53 log CFU/ml difference) and 40% PCH (0.83 log CFU/mL difference).

Growth of *L. monocytogenes* 310 (Figure 2.8) was suppressed by 4.70 log CFU/ml when comparing 40% PCH treatment to the 0% PCH control at 24 h. The initial inoculation for the 40% PCH treatment was on average 5.13 log CFU/ml and at 24 h the population was reduced to

4.61 log CFU/ml, suggesting that growth was inhibited and death may have occurred. The pH 5.1 TPB control suppressed growth by 1.02 log CFU/ml at 24 h, indicating that PCH was the primary antimicrobial agent, with pH having a minor role in inhibition.

At 24 h, *E. coli* O157:H7 (Figure 2.9) was suppressed by 4.33 log CFU/ml when comparing 40% PCH treatment and 0% PCH control. The inhibition of *E. coli* O157:H7 with 40% PCH was much more prominent than that of the pH 5.1 TSB at 0.62 log CFU/ml. This difference indicates that PCH had a major role in suppressing *E. coli* O157:H7. Bacterial populations at all concentrations of PCH for all three test organisms were significantly different ($p \leq 0.05$), except for *E. coli* O157:H7 in 0% and 10% PCH.

The effects of TCH on *L. monocytogenes* Scott A, *L. monocytogenes* 310, and *E. coli* O157:H7 are shown in Figures 2.10 – 2.12. For all three bacteria at 24 h, the difference in growth between 0% TCH control and 50% TCH treatment was only 0.2 log CFU/ml. *L. monocytogenes* 310 (Figure 2.11) was the most sensitive to 50% TCH at 24 h, with a final population difference of 0.52 log CFU/ml between the 0% TCH control and 50% TCH treatment. Although final populations of *E. coli* O157:H7 were slightly higher in 50% TCH than in the 0% TCH control, the difference was not significant ($p > 0.05$). The 0% TCH control and 50% treatment were found to be significantly different ($p \leq 0.05$) for *L. monocytogenes* Scott A and *L. monocytogenes* 310.

Discussion

The pH of the 50% PCH/TSB mixture was pH 5.1 (Table 2.1). That finding gave rise to two issues. The first was the effect of pH change on the potential AMPs. Burris adjusted the pH of the hydrolysates to pH 7.0 and found that PCH was not effective against *Salmonella* Typhimurium and slightly effective at suppressing growth of *L. monocytogenes (3)*. pH plays a

role in the inhibitory effect of AMPs. Both Murdock *(12)* and Wakabayshi et al. *(18)* found this to be true of lactoferricin, which is the pepsin digest of lactoferrin . Wakabayshi et al. *(18)* reported that the effectiveness of lactoferricin was diminished by sodium chloride. In the present study, a consequence of adjusting pH of the PCH solution with base would be the generation of sodium chloride.

The second issue was the effect pH had on bacteria. Both *L. monocytogenes* and *E. coli* O157:H7 survive in broth media at pH 4.5 *(8)*. In light of this information, it was decided to leave the pH unadjusted and to include a pH 5.1 control to all for differentiation between the effects of acid and AMPs on test organisms.

The microdilution assay was used to determine the PCH and TCH concentrations that were effective against *L. monocytogenes* and *E. coli* O157:H7. PCH had an inhibitory effect on all three microorganisms at 30, 40, and 50% concentrations, and the 20% treatment acted similarly to the TPB/TSB (pH 5.1) control. Based on this information, 20% PCH and TPB/TSB (pH 5.1) control were used in the bio-broth assay in order to more closely examine the relationship between them. The 10 and 40% PCH treatments were chosen for low and high inhibitory concentrations.

For all three bacteria, effects of the TCH treatments were very similar at 24 h, with the 50% TCH treatment having the strongest effect. Since there was a small difference, the 50% treatment and a 0% control was used in the bio-broth assay to see if there was a real difference between them.

Results from the bio-broth assay overall show that PCH was effective at either inhibiting or decreasing the final populations of all three bacteria within 24 h. TCH had no noticeable antimicrobial effect on any of the test organisms. Conversely, Burris found that TCH increased

the lag phase and/or reduced the final populations of four strains of *L. monocytogenes (3)*. Also, PCH alone showed no antimicrobial activity against three *L. monocytogenes* strains and *Salmonella* typhimurium *(3)*. This contrast is likely due to the casein hydrolysate preparation. The protocol for this project was different from that of Burris in that a different casein product and enzyme with known activity were used, and the hydrolysates were not pH adjusted or dialyzed. Burris protocol may have resulted in lost of AMPs by dialysis or decreased the AMP activity due to pH adjustments.

E. coli O157:H7 was affected by PCH, with a 4.33 log CFU/ml difference in populations between the control and the 40% concentration, and the final population was reduced by 0.4 log CFU/ml at 24 h. It is well known that ground beef is associated with *E. coli* O157:H7 outbreaks. This treatment may be useful in ground beef or a as a carcass spray much like lactoferrin is used *(10)*.

PCH suppressed growth of *L. monocytogenes* Scott A and resulted in a final population (24 h) reduction of 1.7 log CFU/ml. *L. monocytogenes* 310 was suppressed by 4.7 log CFU/ml. Potentially, a greater suppression of *L. monocytogenes* could be achieved with refrigeration. This would be useful in extending the shelf life of RTE foods such as frankfurters, since they have been associated with *L. monocytogenes* outbreaks *(4)*. PCH also may be useful for food processors as an antibacterial agent, because PCH easily can be made in house. PCH is made from bovine milk casein and an a natural enzyme, which might be appealing to a food processor looking for a natural antimicrobial. Enzymatically hydrolyzed casein has generally considered safe (GRAS) status for use as a flavoring and nutrient in special dietary foods *(16)*. Since GRAS status is use specific *(17)*, PCH would be subject to the current GRAS notification program for use as an antimicrobial compound.

Although eight potential AMPs were identified from previous work, TCH did not suppress growth of bacteria. This solution may not have been at the correct pH, salt content, or temperature to be activated. It is hypothesized that salt content *(2, 12, 18)*, pH *(12, 18)*, and temperature of incubation *(12)* affected the antimicrobial effect of lactofericin. Further research is needed to determine if that is true of TCH.

Conclusion

Results of this study show that minimally processed PCH has some antimicrobial activity against *L. monocytogenes* and *E. coli* O157:H7. The hydrolysates have potential for use in food products for control of pathogenic bacteria, but additional research needs to be conducted. Food matrices are complex, and it is unknown how the hydrolysates will perform in complex systems. Even though TCH was not successful as an antimicrobial agent, continued research should be conducted to determine if changing pH, salt content, or temperature of incubation increases its effect on bacteria.

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Appendix

TABLE 2.1. pH determination of trypsin and pepsin-casein hydrolysate (TCH and PCH) solution at various concentrations when mixed with deionized water and then mixed with tryptic soy broth (TSB).

Average pH of solution when mixed with DI water								
	% Concentration							
	100%	80%	60%	40%	20%	10%	4%	2%
TCH	7.10	7.12	7.14	7.11	7.05	7.05	6.96	6.86
PCH	2.74	2.77	2.80	2.90	2.99	3.15	3.37	3.81
Average pH of solution from above mixed with TSB								
	% Concentration							
	50%	40%	30%	20%	10%	5%	2%	1%
TCH	7.04	7.04	7.04	7.04	7.05	7.05	7.05	7.06
PCH	5.13	5.50	5.88	6.28	6.67	6.85	6.98	7.04

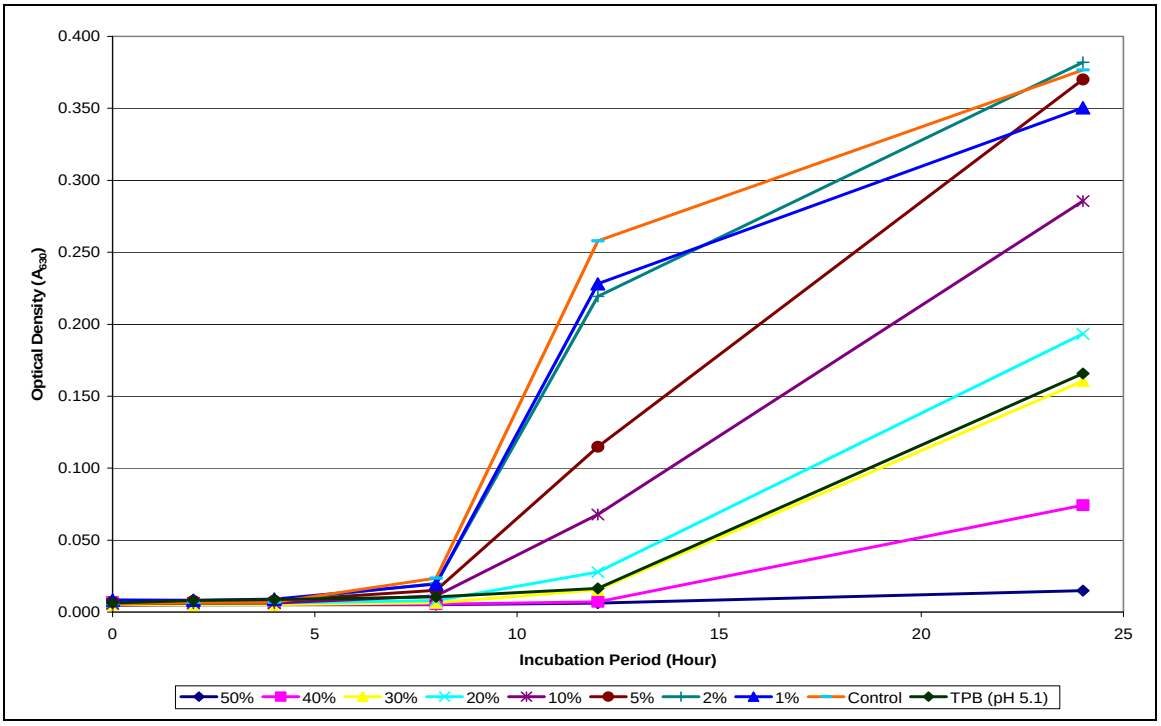

FIGURE 2.1. Microdilution assay: Pepsin-casein hydrolysate (PCH) antimicrobial effect on *L. monocytogenes* Scott A over a 24-h period at 1, 2, 5, 10, 20, 30, 40, 50% concentrations including controls of 0% PCH and tryptose phosphate broth (TPB) at pH 5.1.

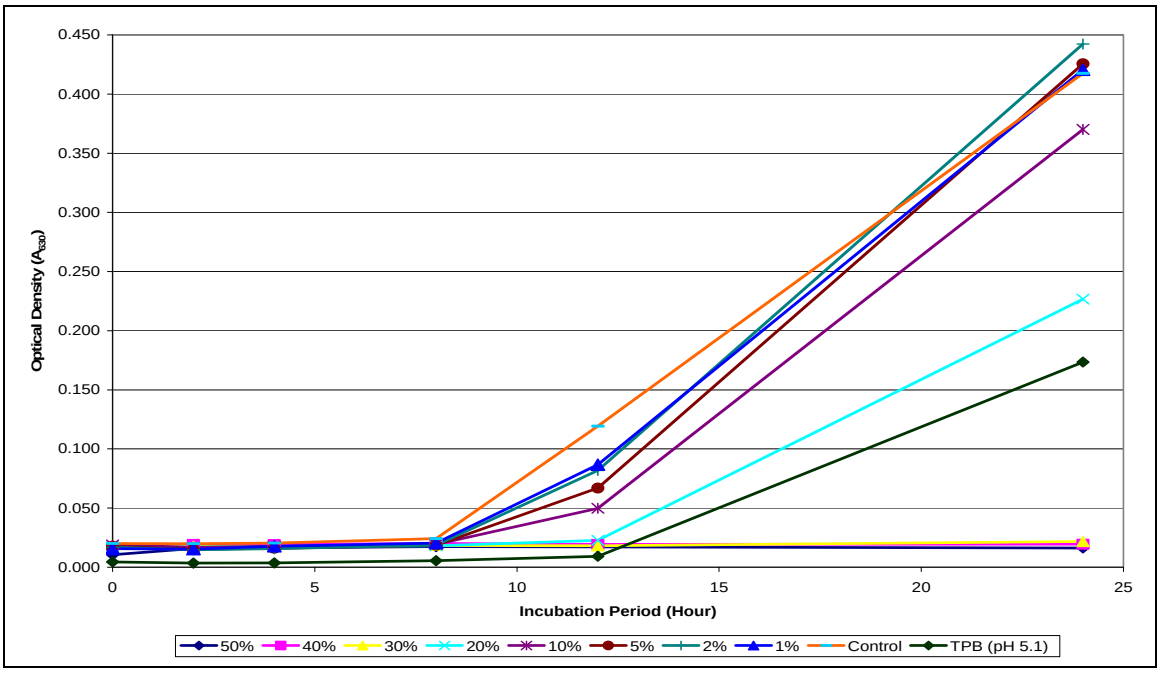

FIGURE 2.2. Microdilution assay: Pepsin-casein hydrolysate (PCH) antimicrobial effect on *L. monocytogenes* 310 over a 24-h period at 1, 2, 5, 10, 20, 30, 40, 50% concentrations including controls of 0% PCH and tryptose phosphate broth (TPB) at pH 5.1.

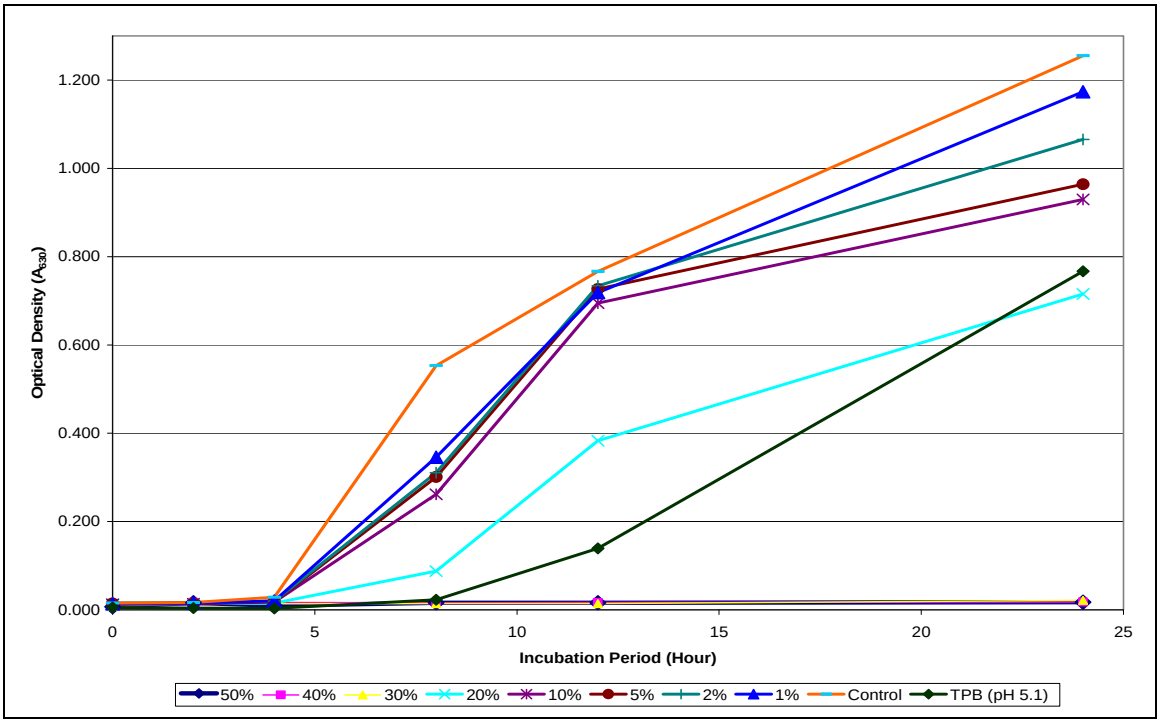

FIGURE 2.3. Microdilution assay: Pepsin-casein hydrolysate (PCH) antimicrobial effect on *E. coli* Salami over a 24-h period at 1, 2, 5, 10, 20, 30, 40, 50% concentrations including controls of 0% PCH and tryptic soy broth (TSB) at pH 5.1.

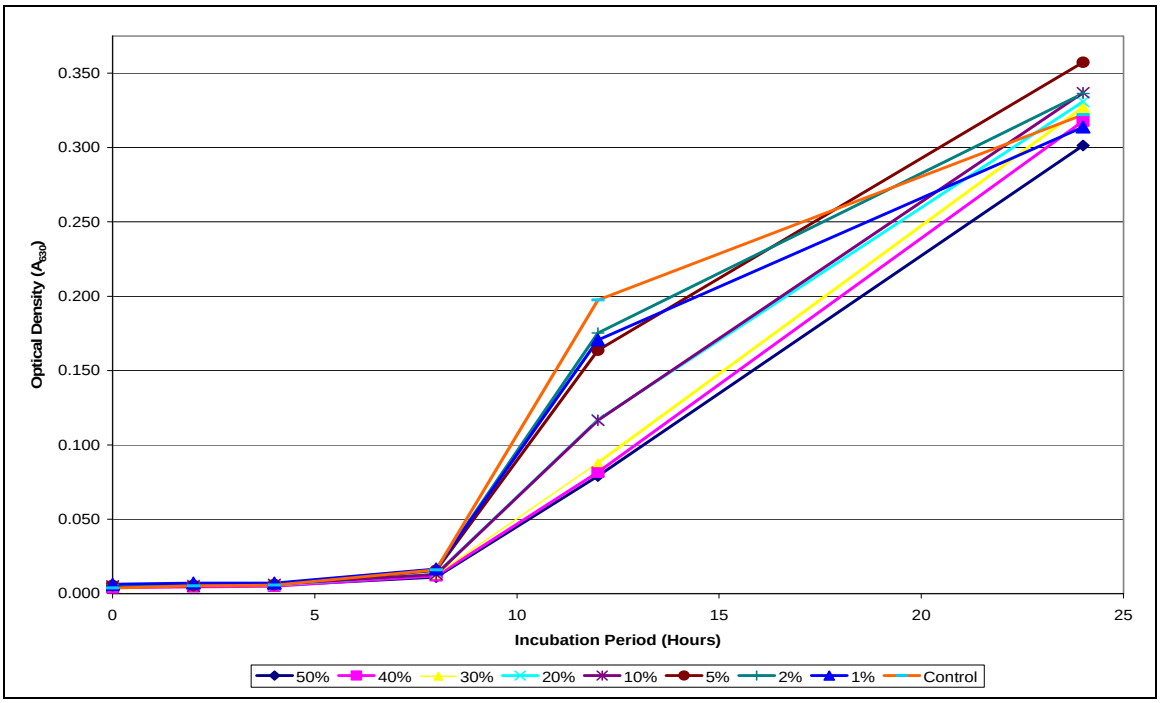

FIGURE 2.4. Microdilution assay: Trypsin-casein hydrolysate (TCH) antimicrobial effect on *L. monocytogenes* Scott A over a 24-h period at 1, 2, 5, 10, 20, 30, 40, 50% concentrations including controls of 0% TCH.

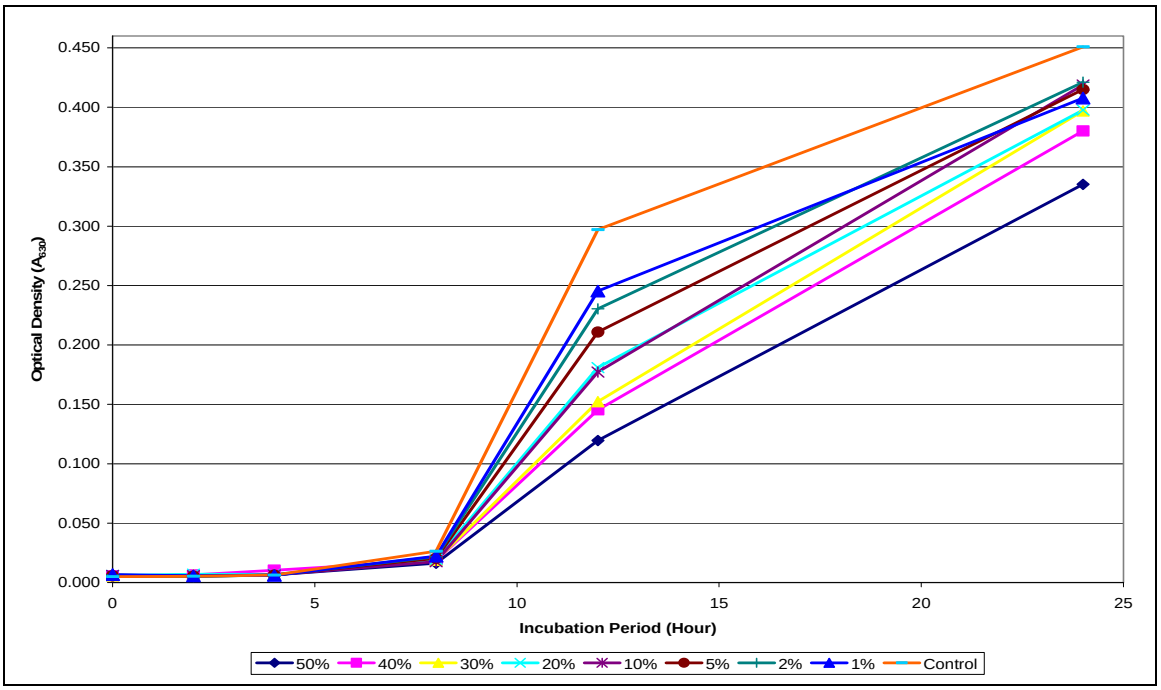

FIGURE 2.5. Microdilution assay: Trypsin-casein hydrolysate (TCH) antimicrobial effect on *L. monocytogenes* 310 over a 24-h period at 1, 2, 5, 10, 20, 30, 40, 50% concentrations including controls of 0% TCH.

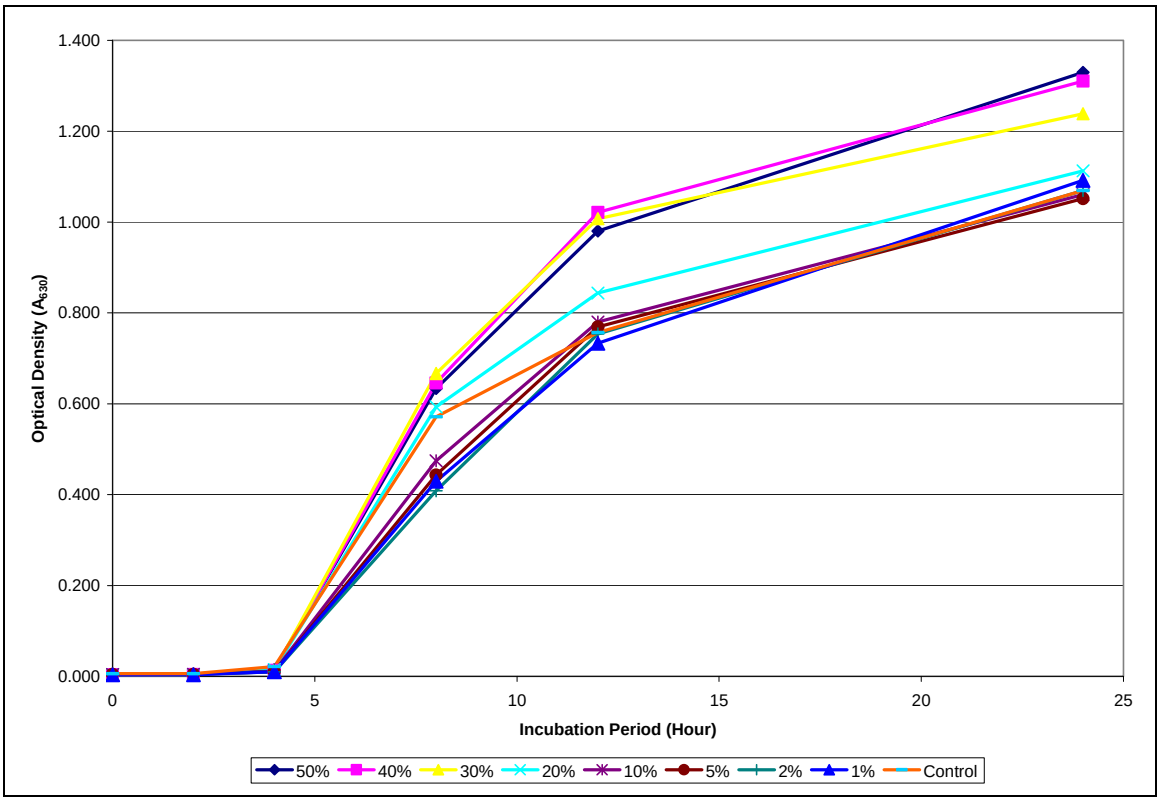

FIGURE 2.6. Microdilution assay: Trypsin-casein hydrolysate (TCH) antimicrobial effect on *E. coli* Salami over a 24-h period at 1, 2, 5, 10, 20, 30, 40, 50% concentrations including controls of 0% TCH.

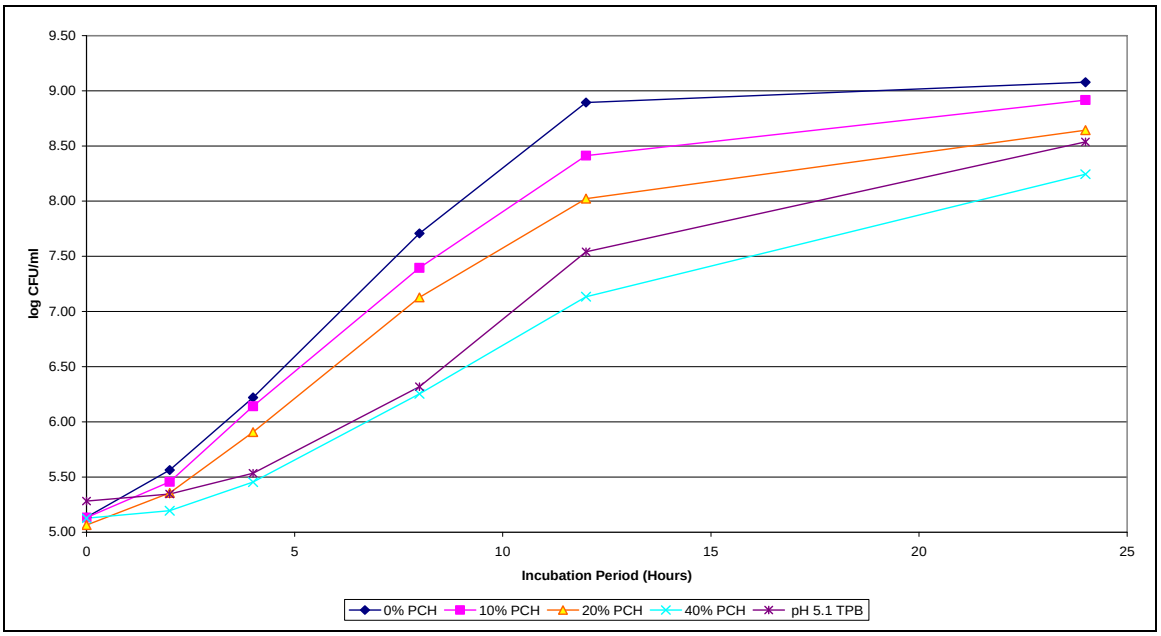

FIGURE 2.7. Biobroth assay: Pepsin-casein hydrolysate (PCH) antimicrobial effect on *L. monocytogenes* Scott A over a 24-h period at 0, 10, 20, 40% concentrations including controls of 0% PCH and tryptose phosphate broth (TPB) at pH 5.1.

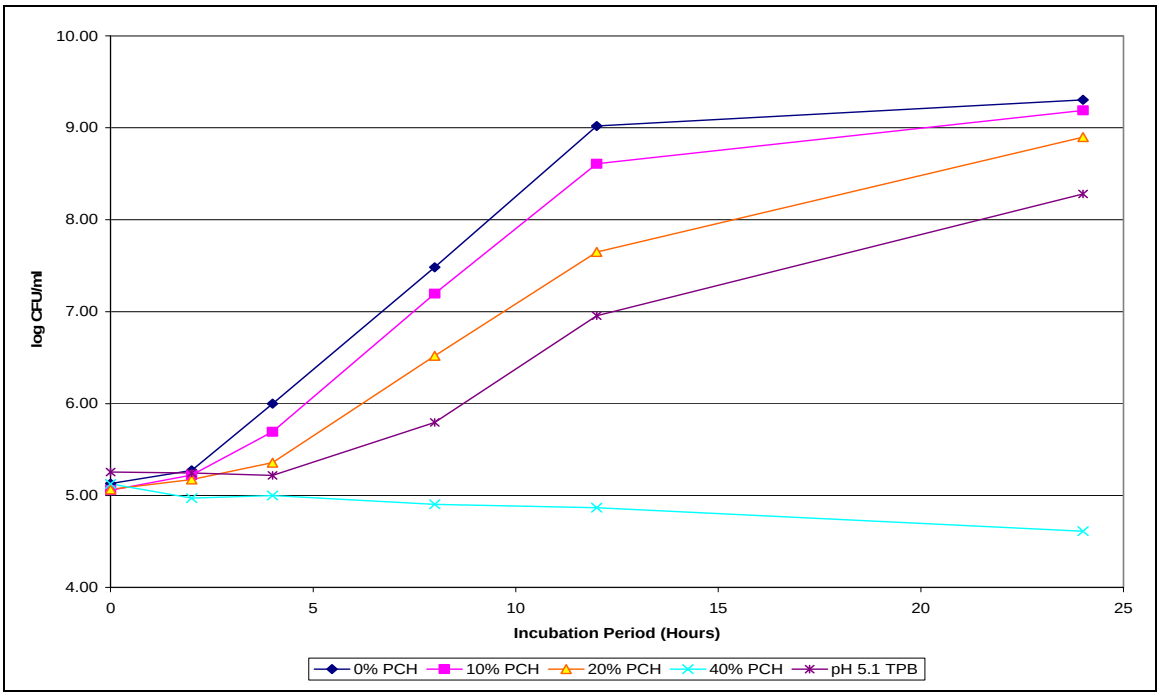

FIGURE 2.8. Biobroth assay: Pepsin-casein hydrolysate (PCH) antimicrobial effect on *L. monocytogenes* 310 over a 24-h period at 10, 20, 40% concentrations including controls of 0% PCH and tryptose phosphate broth (TPB) pH at 5.1.

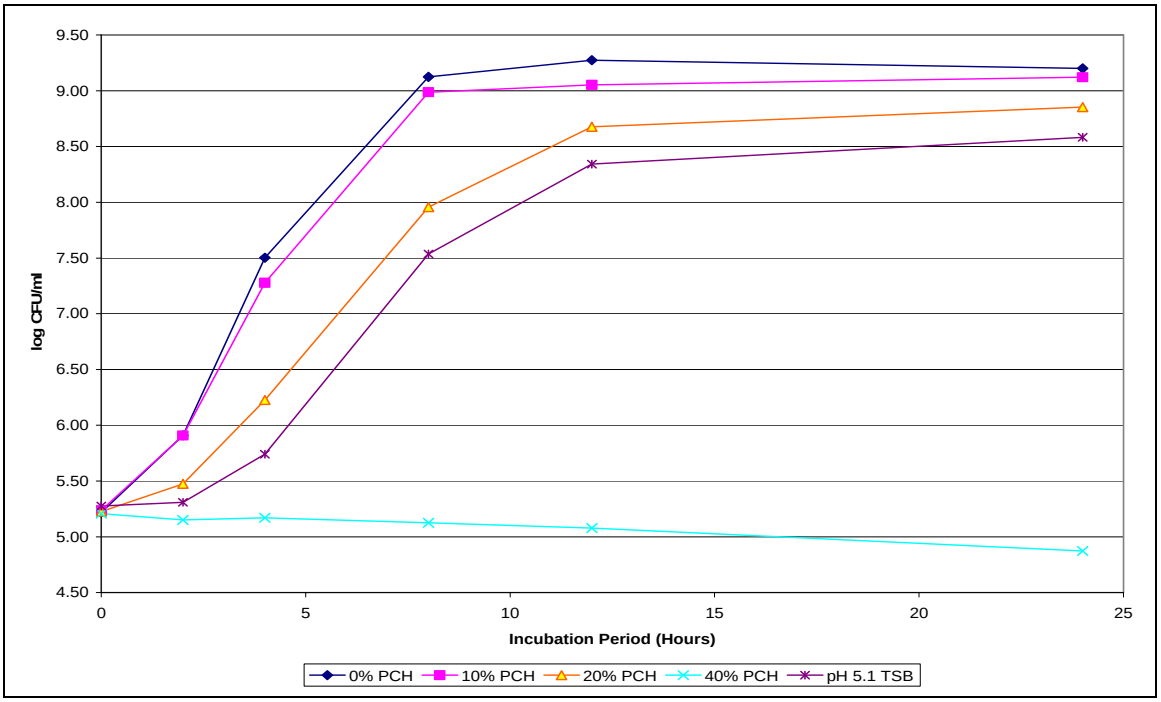

FIGURE 2.9. Biobroth assay: Pepsin-casein hydrolysate (PCH) antimicrobial effect on *E. coli* Salami over a 24-h period at 10, 20, 40% concentrations including control of 0% PCH and tryptic soy broth (TSB) at pH 5.1.

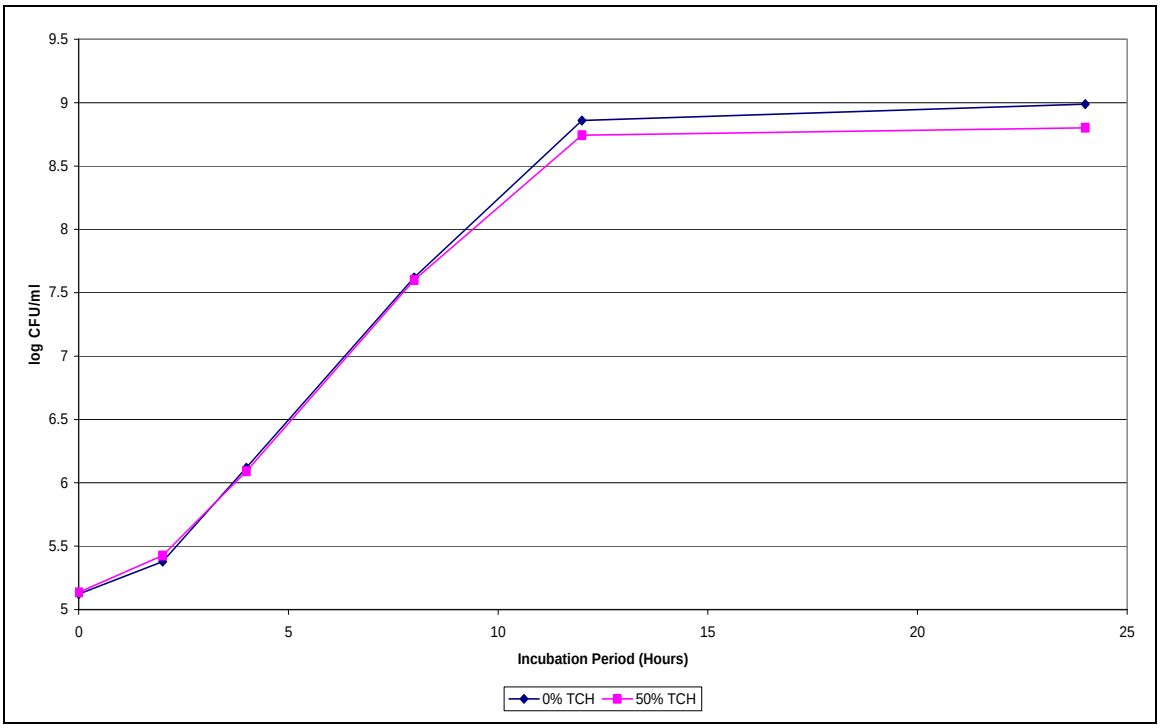

FIGURE 2.10. Biobroth assay: Trypsin-casein hydrolysate (TCH) antimicrobial effect on *L. monocytogenes* Scott A over a 24-h period at 0 and 50% concentrations.

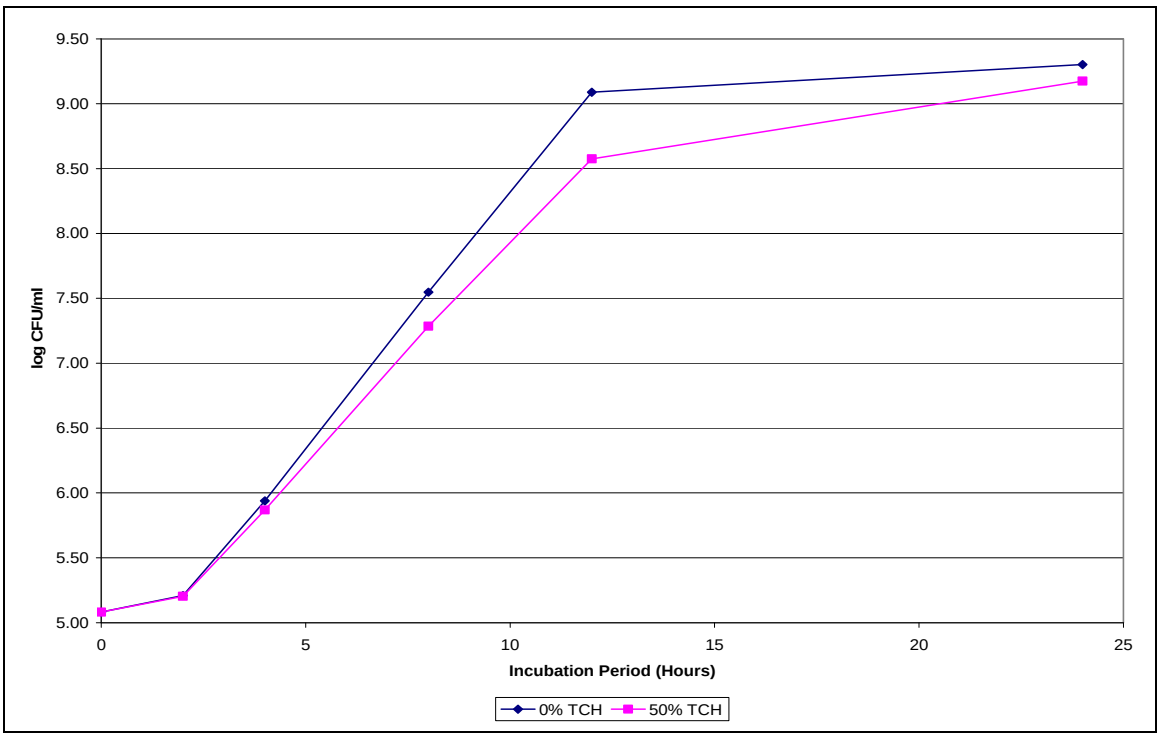

FIGURE 2.11. Biobroth assay: Trypsin-casein hydrolysate (TCH) antimicrobial effect on *L. monocytogenes* 310 over a 24-h period at 0 and 50% concentrations.

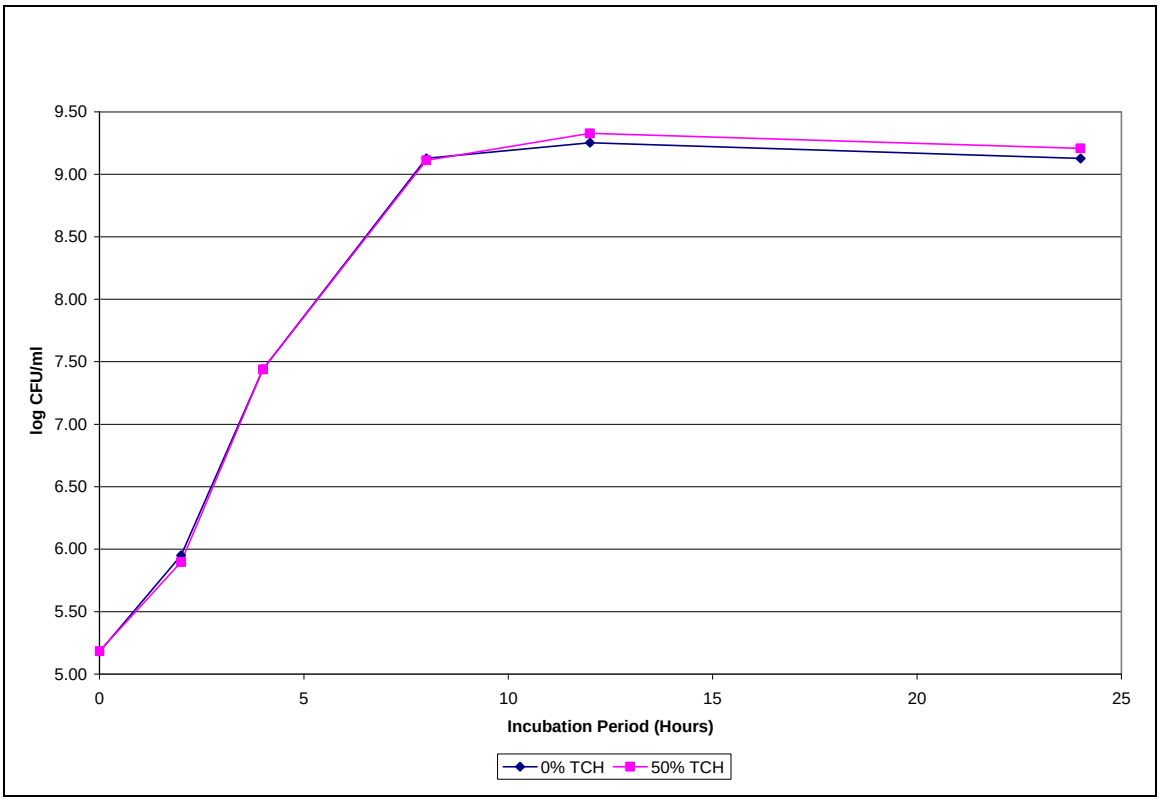

FIGURE 2.12. Biobroth assay: Trypsin-casein hydrolysate (TCH) antimicrobial effect on *E. coli* Salami over a 24-h period at 0 and 50% concentrations.

Chapter 3: Antimicrobial Effect of Pepsin-casein Hydrolysates on *Listeria monocytogenes* Scott A on Frankfurters

Abstract

The objective of this study was to evaluate pepsin-casein hydrolysates (PCH) as post-processing anti-listerial control agents on frankfurters. Frankfurters were spot inoculated with *Listeria monocytogenes* Scott A and dipped into one of five treatments (deionized water, pH 2.7 buffer, pH 5.1 buffer, pH 2.7 PCH, and pH 5.1 PCH) or not at all for 30 seconds. Dipped frankfurters were incubated at 32°C for seven days. Results revealed that there was no significant differences ($p>0.05$) between any of the treatments and controls. Further research should be conducted on dip time, concentration, and salt content to determine the effect these parameters have on antimicrobial activity of PCH on frankfurters.

Introduction

Listeria monocytogenes is a difficult bacterium for food processors, especially the RTE sector to eliminate. Frankfurters are considered a RTE product defined by 9 CFR Part 430.1 as a meat or poultry product that is in a form edible without additional preparation to achieve food safety *(27)*. *L. monocytogenes* is a Gram-positive, non-sporeforming, bacilli-shaped, and highly motile bacteria *(29, 16)*. *L. monocytogenes* is a facultative anaerobe and psychrophilic, which allows it to grow in no oxygen and in refrigeration *(29)*. Also, *L. monocytogenes* is able to grow in curing salt solution up to 13%, nitrate solutions, and pHs between approximately 4.5 and 9.6 at refrigerator temperatures *(29)*. The combination of *L. monocytogenes*'s ability to grow in extreme environments as mentioned above and that franks are often consumed with minimal reheating can lead to *L. monocytogenes* infections.

One of the chief problems with *L. monocytogenes* is its ability to incorporate into biofilms on food processing equipment and other surfaces *(13)*. Biofilms are particularly

problematic, because they produce a polysaccharide matrix that traps and protects *L. monocytogenes* from removal by scrubbing and sanitizers. *L. monocytogenes*, reportedly, is able to persist in food processing environments for months and up to 10 years *(7)*. Since biofilms containing *L. monocytogenes* can form on stainless steel tables, processing equipment, walls, floors, and air handling systems they must be washed and scrubbed frequently. However, biofilms are almost impossible to see on surfaces making it difficult to identify contaminated areas for sanitization.

Frankfurters are considered nearly *L. monocytogenes* free before peeling, but once it is exposed to the environment and surfaces it can become contaminated. According to the FSIS, post-cooking steps such as peeling, sorting, slicing, and packaging are the greatest potential sources of *L. monocytogenes* contamination (*7, 17, 29)*. Once a *L. monocytogenes* bacterium comes in contact with the surface of the frankfurter, it can irreversibly attach itself within five minutes *(6)*. One example of frankfurter associated *L. monocytogenes* outbreak was in 1998. Thirty-eight patients contracted listeriosis from Bil Mar Food's hot dogs and other meat products *(3)*. Of the thirty-eight patients four died: one was an infant and the other three were elderly *(3)*. Interestingly, over half of the patients were women and six were newborns *(3)*. This shows the importance of sanitation as a control for *L. monocytogenes* on frankfurters, but if it fails as it did at Bil Mar Foods, there must be other controls built into the frankfurters or packaging.

There are some anti-listerial agents being used to treat frankfurters. Some anti-listerial agents being used are organic salts and acids, such as sodium chloride *(25)*, sodium lactate, sodium diacetate *(7, 29)*, sodium nitrate, sodium nitrite *(5)*, citric acid, lactic acid *(25)*, and benezoic acid *(25)*. Other antibacterial additives include bateriocins such as nisin *(9, 22, 24, 25)* and pediocin *(18)*. Nisin is the most widely used and approved to be used in meat products *(29)*.

Essential oils such as rosemary *(25, 29)*, garlic, and grapeseed *(24)* have been used as anti-listerial agents. All of these agents are usually incorporated into the meat batter of frankfurters to provide inhibition or bactericidal effects long after being distributed. However, health conscience consumers see chemical additives as unnatural and detrimental to their health and the essential oils impart a flavor profile that may not be pleasing to the palate.

In the desire to answer the demands of a health conscience public for minimally processed foods, researchers are searching food sources that have bioactive peptides (BAP) for use as biopreservatives. Bioactive peptides are antimicrobial peptides (AMPs) that become active when enzymatically cleaved from a parent protein using gastrointestinal enzymes *(10, 21)*. BAPs have been commonly found in bovine and human milk, fish, eggs, and cereals *(10)*. As of recent, milk has provided the largest amount of BAPs *(12, 21)* and it stands reason that several of them may be released from casein as it is the most abundant milk protein. An example of an antimicrobial casein peptide is isracidin. It is bovine α_{S1}-casein segment #1 – 23 that has been cut off by chymosin and has a molecular weight of 2770 daltons *(11)*. Isracidin has antibacterial activity against Lactobacilli, Gram-positive, and Gram-negative bacteria *(11)*. Various Gram-positive bacteria were affected by a concentration between 0.1 and 1 mg/mL *(11)*. Furthermore, Birkemo et al. (2008) showed that isracidin was effective against *Escherichia coli* DPC6053 with a minimum inhibitory concentration (MIC) of 0.2 mg/mL *(2)*. However, this AMP has mostly been tested *in vivo* as immunoprotectants for animal bacterial infection against such bacteria as *L. monocytogenes* and *Staphylococcus aureus (11)*. Isracidin and other milk-derived peptides have been unable to gain FDA approval as clinically useful compounds, because they are typically isomeric mixtures that vary from batch to batch and the FDA requires homogeneity *(11)*.

Although the aforementioned and other casein-derived peptides were found to be antimicrobial, the control of *L. monocytogenes* on frankfurters with antimicrobial casein peptides has not been reported. The studied literature were limited to lactic acid bacteria bacteriocins being added as a single peptide *(13)*, in combination with chemical antimicrobials *(26)*, or bacteriocin producing bacteria itself as a hurdle step *(13)*. Antimicrobial casein peptides may prove to be a post-cooking listerial control for frankfurters.

Materials and Methods

Pepsin-casein hydrolysate (PCH) preparation. A 10% sodium caseinate solution was prepared by slowly adding sodium caseinate (Sigma Product # C8654; St. Louis, MO) to deionized water to avoid large clumps. It was stirred until completely dissolved for about one hour at ambient temperature. The dissolved sodium caseinate solution was adjusted to pH 2.0 ± 0.5 with 6N HCl. When the solution was pH stable for 10 minutes, a 0.2% pepsin (Sigma Product # P6887; St. Louis, MO) solution was added for final solution concentration of 5% casein and 0.1% pepsin. The solution was allowed to digest on a stir plate for 2 h at ambient temperature. After digestion, the solution was placed into a 95°C water bath until it reached 95°C for 5 minutes to deactivate the enzyme and then cooled to 25°C in an ice water bath. The solution was centrifuged in a Sorvall RC-B5 Plus (ThermoEletric; Waltham, MA) at 45,000 x *g* for 30 minutes at 10°C. The supernatant were decanted, filter sterilized (0.22 μm), and stored at 4°C until used.

Culture preparation and maintenance. *L. monocytogenes* (strains Scott A and 310) culture stocks were maintained on tryptose phosphate agar (TPA) slants (Difco; Franklin Lakes, NJ) and re-cultured every two to four weeks. When re-culturing, a loopful of culture was

streaked onto a TPA slant and incubated at 32°C for 24 h, and then returned to refrigerated storage. *E. coli* O157:H7 Salami stock culture was treated in the same manner except it was cultured on tryptic soy agar (TSA) slants (Difco; Franklin Lakes, NJ) and incubated at 35°C.

In preparation for use, a loopful of *L. monocytogenes* stock culture was placed into tryptose phosphate broth (TPB) (Difco; Franklin Lakes, NJ) and incubated at 32°C for 24 h, followed by an additional two 24-h transfers before use. *E. coli* O157:H7 was treated in the same way except it was cultured in tryptic soy broth (TSB) (Difco; Franklin Lakes, NJ) and incubated at 35°C.

Dip solution preparation. Stock solutions of 100 mM potassium hydrogen phthalate buffer (KHPB) were prepared at pH 2.5 and 5.0. The pH 2.5 KHPB was made by mixing 280.2 ml of 0.1 M potassium hydrogen phthalate (KHP) and 0.1 M hydrochloric acid (HCl). The pH 5.0 KHPB is made similarly by mixing 346.8 ml of 0.1 M KHP solution and 153.2 ml of 0.1 M sodium hydroxide (NaOH) solution. KHPB stock solutions were measured for pH accuracy and adjusted with HCl or NaOH as necessary. The solutions were sterilized in an autoclave and stored at room temperature. For use, the solutions were diluted ten-fold with sterile deionized water (1 part KHPB:9 parts water).

Two 50% PCH solutions were made, one at pH 2.7 and another at pH 5.1. Both solutions were mixed 1:1 with deionized water, and pH was adjusted with HCl or NaOH. Solutions were filter-sterilized (0.22 μm filter) and stored at 4°C until used.

Frankfurter assay. A 24-h culture of *L. monocytogenes* Scott A was diluted to $1x10^8$ CFU/ml, and 100 μl was spot-inoculated with 10-μl aliquotes along the surface of a commercial brand beef frankfurter. Inoculated frankfurters were allowed to dry for 45 minutes in a level 2 biosafety cabinet. After drying, frankfurters were dipped into one of five dip solutions for 30

seconds: deionized water, pH 2.7 PCH, pH 5.1 PCH, pH 2.7 KHPB, or pH 5.1 KHPB; inoculated frankfurters that were not dipped served as controls. Frankfurters are then placed into filter stomacher bags directly out of the dip, bags were rolled and taped, and incubated at 10°C. Samples were taken at 0, 1, 4, and 7 days from each dip type. The frank samples were diluted with 100 mL of 0.1% peptone/0.2% w/v Tween 80 solution and stomached with a Seward Stomacher 400 (Seward; Bohemia, NY) at 230 rpm for 2 minutes. Samples were diluted in 0.1% peptone water and spiral plated onto TPA plates using a WASP2 Spiral Plater (Microbiology International; Frederick, MD). TPA plates were incubated at 32°C for 48 h and counts were determined using a ProtoCOL Colony Counter (Microbiology International; Frederick, MD).

Statistical analysis. Statistical analysis was performed by SPSS Statistical software (Version 17, SPSS Inc., Chicago, IL). Analysis of variance was performed to determine statistically significant differences ($P \leq 0.05$) of mean values between treated and control samples and between treatments at each sampling time.

Results and Discussion

The effects of treatments on survival of *L. monocytogenes* on frankfurters dipped in either deionized water, pH 2.7 KHPB, pH pH 5.1 KHPB, pH 2.7 PCH, pH 5.1 PCH, or not dipped are shown in Figure 3.1. All five treatments and the control resulted in similar growth of *L. monocytogenes*. Initial inoculum populations on frankfurters were between 6.1 and 6.6 log CFU/frankfurter, and after seven days, populations of *L. monocytogenes* ranged from 8.1 to 8.5 log CFU/frankfurter.

There are several factors that may have contributed to the inability of PCH to control *L. monocytogenes* on frankfurters. Salt concentration and content can have an effect on the

antimicrobial activity of AMPs. Wakabayashi et al. *(28)* reported that the antimicrobial effectiveness of lactoferricin was diminished by sodium chloride, potassium chloride, and ammonium chloride at concentrations up to 100 mM *(28)*. A main ingredient of frankfurters is salt. The major component of salt is sodium chloride (30%), with magnesium chloride, calcium chloride, and potassium following under four percent *(15)*. It is estimated that a standard sized frankfurter has a volume of 128.7 ml, and the frankfurters used in this study contained 420 mg of sodium chloride per frank. From this information, it was estimated that the franks had a sodium chloride molarity of 56 mM, which falls into the range reported by Wakabayashi et al. *(28)*.

Another reason PCH may have not performed as expected was related to PCH concentration. The franks were dipped in 50% PCH solution for 30 seconds and placed into stomacher bags. During incubation, the hydrolysates and bacteria have a chance to move into the microscopic crevices on the frank surface. Huang *(8)* reported that a vacuum-steam-vacuum process did not completely eliminate *Listeria innocua* from the surface of hot dogs because of the inability of the steam treatment to adequately penetrate the porous surface of the hot dogs. In other studies, antimicrobial agents were successfully incorporated into hot dog formulations before processing *(1, 7, 19, 23)*. Thus, it may be prudent to add PCH to frankfurter formulations in future research projects. Additionally, pH may have an adverse effect on AMP activity in the PCH solution. Wakabayashi et al. *(28)* reported that lactoferricin was active between pH 5.5 and 7.5 against *L. monocytogenes* (28). However, each AMP has its own tolerance to pH due to its amino acid composition. The AMPs in the PCH solution responsible for the effects against *L. monocytogenes* may be more sensitive to pH than lactoferricin. Further studies should be conducted to test pH range of antimicrobial activity of PCH.

PCH also may have been ineffective as an antimicrobial dip against *L. monocytogenes* due to its short dip time (30 seconds). In other studies that utilize a dipping method for delivery of antimicrobial solutions to frankfurters, dip times were five minutes *(20, 26)*, two minutes *(1, 26)*, and only one used 30 seconds *(26)*. By allowing a longer dip time, it may allow the frank more liquid exchanges in the pores and PCH may be more effective.

Conclusion

PCH was ineffective at controlling *L. monocytogenes* on frankfurters. There are many factors that need to be evaluated before a final determination of the usefulness of PCH for controlling *L. monocytogenes* in foods. Further research should be conducted on dip time, concentration, and salt content to determine the effect that these parameters have on antimicrobial activity of PCH dipped frankfurters.

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Appendix

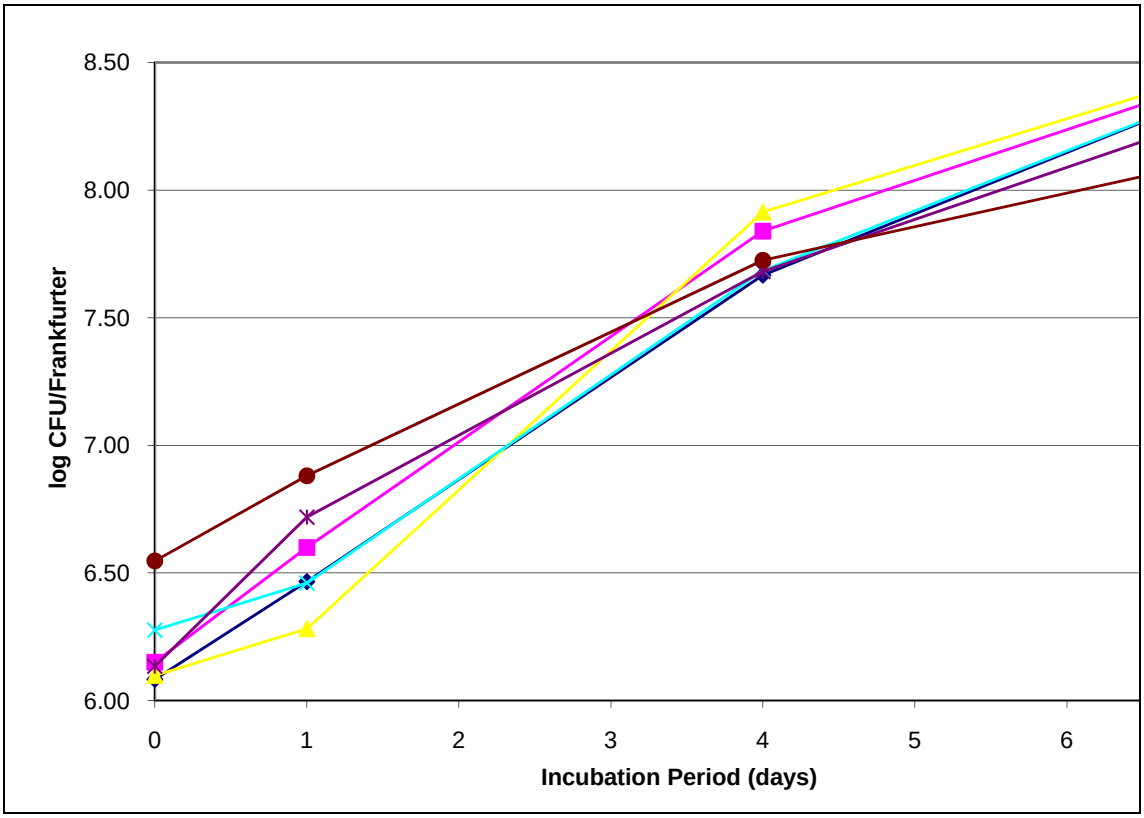

FIGURE 3.1. Antimicrobial effect of pepsin-casein hydrolysate (PCH) on *L. monocytogenes* Scott A inoculated frankfurters dipped into either DI water, 2.7 KHPB, 5.1 KHPB, 2.7 PCH, 5.1 PCH, or not dipped over a seven day period.

Conclusion

The results from the AMP prediction study showed that it is possible to screen for AMPs using known enzyme specificities, primary protein structures, and AMP commonalities. It was also expected that minimally processed hydrolysates would retain the most peptide fragments. Eight potential AMPs were identified for both TCH and PCH in this study. However, results from the microdilution and biobroth assays revealed that TCH did not inhibit bacterial growth, whereas PCH did. There were several factors that might result in TCH being ineffective as an antimicrobial agent, including incorrect pH, salt content, and temperature of incubation. These factors need to be explored before determining that TCH is not an effective antimicrobial solution, since other research has shown the opposite to be true.

The microdilution and biobroth assays both confirmed that minimally processed PCH inhibited growth and/or slightly reduced populations of *L. monocytogenes* and *E. coli* O157:H7 over a 24-h period. When a 50% PCH dip was challenged against *L. monocytogenes* inoculated frankfurters, the solution was unsuccessful in inhibiting bacterial growth under temperature abuse conditions. The reasons for its failure are likely from salt concentration, salt ion content, and dip time. Further research should focus on determining the activity range of PCH as affected by these factors. By knowing those parameters, it would be easier to match PCH with appropriate food systems. Also, incorporating PCH into frankfurters could be researched.

Minimally processed PCH solution could be useful in a applications to inhibit growth of *L. monocytogenes* and *E. coli* O157:H7. Due to the processing conditions used in preparing the hydrolysates, the solutions are likely to be stable under extremes of temperature and pH. As such, PCH could be applicable to pasteurized, hot filled, and acidic products. The solutions can be made on site in a processing facility for use in fluid systems such as an antimicrobial spray on

beef carcasses and in milk, juice, sports drinks, soda, soups, and yogurt. It also could be used in solid systems such as frankfurters, cheese, ground beef, and processed or RTE foods.

Vita

Jessica Marie Christman was born on September 30, 1972 in Denver, CO to John and Jamie Ennis. She attended Ventura County Public Schools and graduated from Simi Valley High School in 1991. Jessica attended Eastern Kentucky University and graduated with a Bachelor of Arts degree in Chemistry in December 2003 and a Bachelor of Science degree in Environmental Health Science in May 2004. In August 2004, she decided to pursue her master's degree in the Department of Food Science and Technology at the University of Tennessee. Her research was focused mainly in the area of food microbiology and antimicrobials under the direction of Dr. David Golden, Dr. P.M. Davidson, and Dr. Svetlana Zivanovic. Jessica graduated with her Master of Science degree in December of 2010. She plans to acquire employment to further her education and utilize training learned from her master's education.