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I am submitting herewith a thesis written by Kellie Parks Burris entitled "Antimicrobial Activity of Trypsin and Pepsin Hydrolysates Derived From Acid-Precipitated Bovine Casein." 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.

P. Michael Davidson_____
Major Professor

We have read this thesis
and recommend its acceptance:

Svetlana Zivanovic_____

David Golden_____

Accepted for the council:

_Anne Mayhew_____
Vice Chancellor and
Dean of Graduate Studies

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

Antimicrobial Activity of Trypsin and Pepsin Hydrolysates Derived From Acid-Precipitated Bovine Casein

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Abstract

Foodborne pathogens are a major concern to the food industry and consumers but they may be controlled with antimicrobials. Naturally occurring antimicrobials may be isolated from a variety of plant, animal and microbial sources. Previous studies have demonstrated that peptides isolated from enzyme hydrolyzed milk proteins may have *in vivo* and *in vitro* antimicrobial activity. Such compounds could be of use as inhibitors of foodborne pathogens. The objectives of this study were to determine the antimicrobial effectiveness against *Salmonella* Typhimurium and *Listeria monocytogenes* of digests of bovine acid-precipitated casein with the enzymes pepsin and trypsin and to determine if these peptides were effective in combination with ethylendiaminetetraacetic acid (EDTA) and sodium lactate against these foodborne pathogens.

Whole casein was precipitated from fresh, unpasteurized skimmed cow's milk by addition of 2 N HCl. Precipitated casein was separated by centrifugation, washed and lyophilized. Rehydrated casein was hydrolyzed with either pepsin or trypsin and the reaction mixture was heated to inactivate each enzyme.

For method 1, solutions with hydrolyzed protein were dialyzed against water and freeze-dried. For method 2, 5.0% casein was dissolved in buffer and treated similarly to method 1, however the peptides that were created from enzymatic hydrolysis were separated by centrifugation after inactivation of the enzymes by heat and were not dialyzed against water. For both methods,

hydrolysates created were adjusted to pH 7 and filter sterilized through a 0.45 μm membrane filter

The inhibitory effect of filtered pepsin and trypsin hydrolysates (0.5% and 1.0%) (method 1) and filtered supernate (pepsin and trypsin) (method 2) alone and in combination with EDTA and sodium lactate against four strains each of *L. monocytogenes* and *S.* Typhimurium DT104 was determined. Growth was monitored over 24 hours using a microbroth dilution assay for all hydrolysates. Growth curves were used to relate microtiter data to actual colony counts.

For method 1, pepsin hydrolysates were not very effective in inhibiting the growth of any of the four strains of *S*. Typhimurium or *L. monocytogenes* while trypsin hydrolysates were only slightly effective at extending the lag phase and/or reducing the final growth level of all four strains of *L. monocytogenes*. This ineffectiveness was most likely due to the loss of small molecular weight peptides during the dialysis step. The addition of EDTA had little effect in enhancing the inhibitory effect of pepsin or trypsin hydrolysates against either microorganism.

For method 2, trypsin hydrolysates were effective in extending the lag phase and/or reducing the final growth level of all four strains of *L. monocytogenes* tested; however, they were not effective against any of the strains of *S.* Typhimurium. Pepsin hydrolysate was not effective in extending the lag phase or reducing the final growth level in *S.* Typhimurium. However, pepsin did reduce the final growth level of one strain of *L. monocytogenes,* 101. Trypsin and pepsin hydrolysates derived from bovine milk in combination with EDTA and sodium lactate had antimicrobial activity against both *L. monocytogenes* and *S.*

Typhimurium in tryptic soy broth (TSB). Trypsin hydrolysates also enhanced the antimicrobial activity of sodium lactate against *Listeria monocytogenes*.

The protein concentrations of pepsin and trypsin hydrolysates (before and after membrane filtration) prepared using method 2 was determined with three different protein assays, Bradford dye-binding, modified Lowry and UV 280 nm. For all three methods, non-filtered hydrolysates were higher in protein concentration than those that were filtered. Using the Bradford method, pepsin hydrolysates were higher in protein concentration than trypsin hydrolysates in both filtered and non-filtered samples. The opposite results were observed when using both modified Lowry method and UV 280 nm method to determine protein concentration. By examining the location of the peptide bond hydrolysis of the enzymes, it was possible to determine that small molecular weight peptides were created by the addition of trypsin and pepsin to bovine casein. Variation in number of amino acids as well as types of amino acids of peptides created during hydrolysis likely influenced the antimicrobial effectiveness of each hydrolysate.

Casein-derived peptides could provide an alternative or adjunct to antimicrobials currently used in foods. It is suggested that antimicrobial peptides can be created by enzymatic hydrolysis of casein with trypsin and these peptides have the potential to serve as antimicrobials in food systems. Further research needs to be conducted in enhancing the activity by concentrating the hydrolysates or isolating and characterizing those peptides with the greatest antimicrobial potential.

Table of Contents

1. Introduction 1
2. Literature Review 4
   2.1 *Listeria monocytogenes* 4
      2.1.1 Characteristics of the Organism 4
      2.1.2 Sources of Food Contamination 5
      2.1.3 Epidemiology of Disease 6
   2.2. *Salmonella* Typhimurium 8
      2.2.1 Characteristics of the Organism 8
      2.2.2 Sources 9
      2.2.3 Epidemiology of Disease 10
   2.3 Milk 12
      2.3.1 Introduction 12
      2.3.2. Milk Proteins 13
      2.3.3 Antimicrobial Properties 16
   2.4 Enzymatic Protein Hydrolysis 17
   2.5 Bioactive Peptides from Milk 20
      2.5.1 Introduction 20
      2.5.2 Opioid Activity 21
      2.5.3 Immunomodulatory Peptides 22
      2.5.4 Antihypertensive Peptides 22
      2.5.5 Other Milk Components 23
      2.5.6 Antimicrobial mechanism of action 25
3. Materials and Methods 27
   3.1 Hydrolysate Preparation 27
   3.2 Culture Preparation 28
   3.3 Micro-broth Dilution Assay 28
   3.4 Growth Curve 29
   3.5 Determination of Protein Content 30
4. Results and Discussion 32
   4.1. Method 1 32
   4.2. Method 2 33
      4.2.1 Antimicrobial Activity 33
      4.2.2 Protein Concentration and Peptide Fragment Determination in Method 2 39
5. Conclusions 42

References 43
Appendix 52
Vita 72

List of Figures

Figure 1. Antimicrobial activity of hydrolysates of acid-precipitated casein, digested with pepsin and trypsin, dialyzed and lyophilized (method 1) at 0.5% and 1.0% on *Listeria monocytogenes* (A) 101 (B) 108 (C) 310 and (D) Scott A. 53
Figure 2. Antimicrobial activity of acid-precipitated casein digested with pepsin and trypsin, dialyzed and lyophilized at 0.5% and 1.0% (method 1) on *Salmonella* Typhimurium (A) 2380 (B) 2576 (C) 2582 and (D) 2486. 54
Figure 3. Antimicrobial activity of acid-precipitated casein digested with pepsin, dialyzed and lyophilized at 0.5% and 1.0% (method 1) alone and in combination with EDTA against *Listeria monocytogenes* (A) 101 (B) 108 (C) 310 and (D) Scott A. 55
Figure 4. Antimicrobial activity of acid-precipitated casein, digested with trypsin, dialyzed, lyophilized at 0.5% and 1.0% (method 1) alone and in combination with EDTA against *Listeria monocytogenes* (A) 101 (B) 108 (C) 310 and (D) Scott A. 56
Figure 5. Antimicrobial activity of acid-precipitated casein digested with trypsin, dialyzed and lyophilized at 0.5% and 1.0% (method 1) alone and in combination with EDTA against *Salmonella* Typhimurium (A) 2380 (B) 2576 (C) 2582 and (D) 2486. 57
Figure 6. Antimicrobial activity of acid-precipitated casein, digested with pepsin, dialyzed, lyophilized at 0.5% and 1.0% (method 1) alone and in combination with EDTA against *Salmonella* Typhimurium (A) 2380 (B) 2576 (C) 2582 and (D) 2486. 58
Figure 7. Antimicrobial activity of acid-precipitated casein digested with (A) pepsin (B) trypsin, dialyzed, lyophilized at 5% against *Listeria monocytogenes* 108 and 310. 59
Figure 8. Antimicrobial activity of acid-precipitated casein digested with (A) pepsin (B) trypsin, dialyzed, lyophilized at 5% against *Salmonella* Typhimurium 2582 and 2486. 60
Figure 9. Antimicrobial activity of acid-precipitated casein digested with (A) trypsin (B) pepsin, centrifuged (method 2) on *Listeria monocytogenes* and acid-precipitated casein digested with (C) trypsin and (D) pepsin, centrifuged (method 2) on *Salmonella* Typhimurium. 61
Figure 10. Antimicrobial activity of acid-precipitated casein digested with trypsin, centrifuged (method 2), and combined with EDTA and sodium lactate on *Listeria monocytogenes* (A) 101 (B) 108 (C) 310 and (D) Scott A. 62
Figure 11. Antimicrobial activity of acid-precipitated casein digested with trypsin, centrifuged (method 2), and combined with EDTA and sodium lactate on *Salmonella* Typhimurium (A) 2380 (B) 2576 (C) 2582 and (D) 2486. 63
Figure 12. Antimicrobial activity of acid-precipitated casein digested with pepsin and trypsin, centrifuged (method 2) alone or in combination on *Listeria monoctyogenes* (A) 101 (B) 108 (C) 310 and (D) Scott A. 64

Figure 13. Antimicrobial activity of acid-precipitated casein digested with pepsin and trypsin, centrifuged (method 2) alone or in combination on *Salmonella* Typhimurium (A) 2380 (B) 2576 (C) 2582 and (D) 2486. 65
Figure 14. Antimicrobial activity of acid-precipitated casein digested with trypsin and centrifuged (method 2) on (A) *Listeria monocytogenes* 108 and 310 and (B) *Salmonella* Typhimurium 2582 and 2486. 66
Figure 15. Antimicrobial activity of acid-precipitated casein digested with trypsin and centrifuged (method 2) on (A) *Listeria monocytogenes* 101 and Scott A and (B) *Salmonella* Typhimurium 2582 and 2486. 67
Figure 16. Sequence of amino acids for (A) α_{s1}-casein (B) α_{s2}-casein indicating cleavage at the amino acids, lysine (bold) and arginine (underline), using trypsin. 68
Figure 17. Sequence of amino acids for (A) β-casein (B) κ -casein indicating cleavage at the amino acids, lysine (bold) and arginine (underline), using trypsin. 69
Figure 18. Sequence of amino acids for (A) α_{s1}-casein (B) α_{s2}-casein indicating cleavage at the amino acids, phenylalanine (bold), tyrosine (italics) and trytophan (underline) using pepsin. 70
Figure 19. Sequence of amino acids for (A) β-casein (B) κ-casein indicating cleavage at the amino acids, phenylalanine (bold), tyrosine (italics) and trytophan (underline) using pepsin. 71

1. Introduction

Foodborne pathogens are a major concern in the food industry. Consumers are at risk for the harmful effects caused by many foodborne pathogenic bacteria, including *Salmonella* Typhimirium and *Listeria monocytogenes*. There are numerous methods of inhibiting or inactivating microorganisms in foods including, but not limited to, heating, refrigeration, freezing, dehydration, addition of sugars, salts or acids, fermentation, smoking, and the use of alternative packaging (Potter and Hotchkiss 1998). However, another means of inhibiting the growth of foodborne pathogens is through the use of chemical antimicrobials. One of the major drawbacks to the use of regulatory-approved traditional antimicrobials is their lack of activity in foods with pH less than 5.0. This limits their usefulness to control pathogens in most low acid foods. In addition, food processors are interested in so-called “natural” or bio-based compounds, such as those derived from animal products and plants, as antimicrobials because they perceive that consumers are interested in a reduction in the use of such preservatives (Dufour et al. 2002).

Milk is not only nutritionally beneficial, but is known to possess bioactive compounds that may reduce or prevent bacterial growth. Casein, the main protein found in milk, is a good source of such peptides/protein fragments that have certain physiological functions such as aiding in gastrointestinal function and digestion, hemodynamic modulation (antihypertension and increased blood

flow), probiotic support of gut microflora, non-immune disease protection, passive immunity, immunoregulation, anti-inflammation, growth and development (Tome and Debabbi 1998; Schanbacher et al. 1997). Biologically active peptides have been obtained *in vitro* by proteolysis (Bellamy et al. 1992; Zucht et al. 1995; Recio and Visser 1999) and *in vivo* by gastric digestion of milk proteins (Kuwata et al. 1998; Meisel and Bockelmann 1999). Bioactive peptides derived from milk with specific antimicrobial properties, released from protein hydrolysis, have been found in milk protein hydrolysates, including those with opioid, antihypertensive and antithrombic properties (Schlimme and Meisel 1995; Korhonen et al. 1998; Clare and Swaisgood 2000; Pihlanto-Leppala 2001).

Hydrolysis of the milk components, lactoferrin (Kimura et al. 2000; Kuwata et al. 1998; Nibbering et al. 2001; Groenink et al. 1999; Branen and Davidson 2000; Qian et al. 1995), bovine hemoglobin (Froidevaux et al. 2001), casein (Liepke et al. 2001; Lahov and Regelson 1996; Recio and Visser 1999), α-lactoalbumin (Pellegrini et al. 1999), α-lactoglobulin (Pellegrini et al. 2001) and whey (Pihlanto-Leppala 2001) produce hydrolysates with antimicrobial activity. Known fragments of bovine casein with antimicrobial activity are isracedin (Hill et al. 1974), casocidin-I (Zucht et al. 1995), α-casecidins (Otani and Suzuki 2003), kappacin (Malkoski et al. 2001), and the κ-casein-derived glycomacropeptide (Stromqvist et al. 1995; Aniansson et al. 1990).

The objectives of this study were to: (1) determine the antimicrobial effectiveness against *Salmonella* Typhimurium and *Listeria monocytogenes* of

digests of bovine acid-precipitated casein with the enzymes pepsin and trypsin and (2) if these hydrolysates were effective in combination with ethylenediaminetetraacetic acid (EDTA) and sodium lactate against these foodborne pathogens.

2. Literature Review

2.1 *Listeria monocytogenes*

2.1.1 Characteristics of the Organism

Listeria monocytogenes was first identified in animals in 1911 and in humans in 1929; however, it is only within the last twenty years that the microorganism was recognized as a foodborne pathogen. It has increased importance among the foodborne pathogens because of its ability to grow at refrigeration temperatures (FDA 2004).

L. monocytogenes is a small Gram-positive, non-spore-forming, non-acid-fast rod. *Listeria* species have been found in a wide range of environments, have tolerances to stresses such as low pH and high NaCl concentrations (10-12%) and can grow over large temperature (0-45^{o}C) and pH (4.4 to 9.6) ranges. *Listeria* are injured by heating to 50^{o}C and above (Swaminathan 2001). Tryptose broth with up to 0.1% acetic, citric, or lactic acids has been found to inhibit the growth of *L. monocytogenes* (Ahamad and Marth 1989). Frozen storage and freezing affect the inactivation and injury of *L. monocytogenes* differently depending on the rate of freezing and type of substrate (Swaminathan 2001). *L. monocytogenes* is a facultative anaerobe that is not greatly affected by vacuum packaging (Swaminathan 2001). Resistance of the microorganism to such environments, in combination with its ability to colonize and grow in harsh conditions, makes *L. monocytogenes* a major threat to consumers and the food industry (Fenlon 1999).

2.1.2 Sources of Food Contamination

L. monocytogenes is widely distributed and is commonly found in the soil and water as well as on decaying plant material; however, there are low numbers of organisms present in most environments (Fenlon 1999). *L. monocytogenes* has also been found in the feces of a wide range of healthy animal species including sheep, goats and cattle (Fenlon1999). Humans can be symptomatic and asymptomatic carriers (Fenlon 1999).

Listeria can enter a food processing facility in a number of ways including, on the shoes and clothing of workers, on transportation equipment, through contaminated animal hides, on raw foods or by way of human carriers. Processing environments, such as drains and floors, with high humidity and nutrient sources favor the growth of *Listeria* (Swaminathan 2001; Rocourt and Cossart 1997).

Foods that are at highest risk for causing listeriosis are ready-to-eat (RTE) and stored at refrigeration temperatures for long periods, such as deli meats and hot dogs, and dairy foods using unpasteurized milk, such as some soft cheeses (Swaminathan 2001; Rocourt and Cossart 1997). These foods may be contaminated with high populations of the bacteria (>100 CFU/g or ml). In a survey of retail vacuum-packaged meat samples, 53% tested were contaminated with *L. monocytogenes* (Grau and Vanderlinde 1992). *L. monocytogenes* can survive for long periods on foods and in food processing environments and can be found on both raw and processed foods. Ice cream, raw and cooked chicken, raw vegetables, and raw and smoked fish have all been associated with

infection. An important mode of transmission is post-processing contamination where RTE foods are contaminated after processing (Swaminathan 2001; Rocourt and Cossart 1997).

2.1.3 Epidemiology of Disease

According to the Centers for Disease Control (CDC), *L. monocytogenes* causes 2500 cases and 500 deaths annually (2003*).* The first documented outbreak of foodborne listeriosis occurred in Canada in 1981 and was traced to the consumption of contaminated coleslaw. The coleslaw, which was fertilized with sheep manure, was thought the most possible source of *Listeria* contamination (Rocourt and Cossart 1997). The largest outbreak in the US was in California in 1985 and implicated *L. monocytogenes* 4b in a Mexican style cheese called queso blanco. There were 142 cases and 48 deaths in the outbreak. The cause was theorized to be due to use of raw milk in the cheese and/or general contamination of the processing plant and workers (CDC 2004). The consumption of contaminated turkey meat resulted in 54 illnesses, 8 deaths, and 3 fetal deaths in 9 states in 2002 (CDC 2003). A majority of listeriosis cases are sporadic and difficult to link epidemiologically (FDA 2004) while the annual incidence of listeriosis has decreased by 38% from 1996 to 2002 (CDC 2003)., outbreaks continue to occur.

When ingested, *L. monocytogenes* colonizes the intestinal tract, invades the tissues and enters the blood stream. Listeriolysin O (LLO), a secreted protein of 58-60 kDa belonging to the family of pore-forming, sulfhydryl-activated

cytolysins, has been identified as the substance responsible for beta-hemolysis of erythrocytes and the engulfment and destruction of phagocytic cells (Kuhn and Goebel 1999). LLO is very similar to streptolysin O (SLO), its prototype produced by *Streptococcus pyogenes*. LLO and SLO are only active on membranes containing cholesterol. *Listeria* enters the phagocytes either directly into the phagosomes or from the phagosomes into the phagocytic cytoplasm. *Listeria* are internalized in membrane-bound vacuoles, which are then lysed within 30 min. LLO is the main factor in the lysis of the vacuole. The intracellular bacteria are released into the cytosol and begin to multiply. LLO assists *L. monocytogenes* once inside macrophages by aiding in their escape from the phagolysosomal membranes into the cytosol. Once in the cytosol, ActA, a 610 amino acid surface protein anchored to the bacterial cytoplasmic membrane, facilitates the production of actin tails which provide the organisms the ability to move toward the cytoplasmic membrane (Swaminathan 2001; Rocourt and Cossart 1997). The bacteria become covered with cell actin filaments which rearrange into a “comet tail”, composed of actin microfilaments that are continuously assembled and left behind in the cytosol by moving bacteria. At the plasma membrane, the bacteria form protrusions with a bacterium at the tip. The protrusions are internalized by a neighboring cell, forming a two-membrane-bound vacuole. Lysis of this new vacuole starts a new cycle of replication, movement and spreading of bacteria (Swaminathan 2001; Rocourt and Cossart 1997).

Listeriosis is the disease caused by *L. monocytogenes*. Healthy individuals, that are neither immuno-compromised nor pregnant, are highly

resistant to contracting listeriosis. At risk groups for disease include the elderly, pregnant women and the immuno-compromised. AIDS patients are nearly 300 times more likely to develop the disease than those individuals with a normal immune system. Healthy children and adults occasionally develop listeriosis; however, the illness is not usually severe. The infectious dose is believed to be more than 100 CFU/ml (Swaminathan 2001). Onset of symptoms for serious forms of the infection can be from a few days up to three weeks. The most common symptoms of listeriosis are meningitis and sepsis with mortality rates of 20 to 25% (Swaminathan 2001). Pregnant females typically develop the disease in the third trimester but may not show any signs or very mild signs such as flu-like symptoms. Pregnant females contracting the disease can deliver prematurely; abort, or have stillbirths (Swaminathan 2001; Rocourt and Cossart 1997).

2.2. *Salmonella* Typhimurium

2.2.1 Characteristics of the Organism

Salmonella enterica ssp. *enterica* serotype Typhimurium is a gram-negative, motile, non-spore forming rod, primarily found in the intestinal tract of animals. Besides the peptidoglycan layer, gram-negative bacteria, such as *S.* Typhimurium contain an additional lipolysaccharide (LPS) layer that contains both polysaccharide and protein linked together in the outer layer, further protecting the bacterial cell (D'Aoust et al. 2001; D'Aoust 1997).

Salmonella species are organisms that are highly adaptable to extreme environmental conditions (D'Aoust et al. 2001). Optimal growth for *Salmonella* is at 37^{o}C and pH range 6.5 to 7.5 with the ability to grow at elevated temperatures greater than 54^{o}C and pH values in the range of 4.5 to 9.5 (D'Aoust et al. 2001). Growth is inhibited at $a_w \leq 0.93$ in microbiological media at neutral pH. Salmonellae are unable to grow in 3 to 4% NaCl, however they have the ability to survive in salt concentrations of up to 30% with increasing temperature (D'Aoust et al. 2001). *Salmonella* are generally easily destroyed by heating to 63^{o}C which is the temperature of milk pasteurization. *Salmonella* Typhimurium DT (definitive type) 104 is characterized by its pentaresistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline. Additionally, resistance to gentamicin, trimethoprim and fluoroquinolones has been observed (D'Aoust et al. 2001; D'Aoust 1997).

2.2.2 Sources

S. Typhimurium is found in the intestinal tract of animals such as birds, reptiles, farm animals, humans and some insects. Foods mostly associated with the presence of *Salmonella* spp. are typically of animal origin and include poultry, eggs, milk, and beef. The exposure of livestock to environmental sources of *Salmonella* such as contaminated feeds likely has contributed to the persistence of *Salmonella* in the meat industry (D'Aoust et al. 2001). Fruits and vegetables that have contacted contaminated soil, water or surfaces are also associated with *Salmonella*. Foods may also be contaminated by improper sanitation of food

contact surfaces, i.e., “cross contamination,” as well as through poor hygiene of foodservice workers (D’Aoust et al. 2001; D’Aoust 1997).

2.2.3 Epidemiology of Disease

According to the CDC, it is estimated that the total number of human *Salmonella* infections in the United States ranges from 800,000 to 4,000,000 annually with an estimated range of 59,200 to 296,000 of those being *S.* Typhimurium DT104 (Hogue et al. 1997). Of the Salmonella isolates reported to the CDC in 1996, approximately 24% were *Salmonella* Typhimurium (Hogue et al. 1997). *Salmonella* Typhimurium DT104 is now one of the more prevalent strains isolated from humans. It was first isolated from humans in the United Kingdom in 1984 and became a major cause of salmonellosis in humans in the late 1980s. It was first recognized in the United States and elsewhere in the mid-1990s. (CDC 2004).

Salmonella species have numerous virulence factors. These virulence factors include those necessary for the organism to adhere to intestinal surfaces, to invade the epithelial cells of the host, and the ability to grow and survive in phagocytic cells. *Salmonella* contain greater than 200 virulence factors, and at least 60 genes are required for virulence in *S.* Typhimurium (IFT 2004; D’Aoust et al. 2001). *Salmonella* species use a combination of six adhesins, filamentous, hair-like structures or hair-like fimbriae composed of glycoproteins or glycolipids on their surfaces that aid in the intestinal colonization by their ability to target specific host cell molecules (IFT 2004; D’Aoust et al. 2001). Further, *Salmonella* species have the ability to enter host cells by penetrating the intestinal epithelial

barrier. The Type III secretion system, a complex secretion apparatus that delivers numerous bacterial proteins into the cytosol of host cells is used by *Salmonella* (IFT 2004; D'Aoust et al. 2001). The microorganism invades host cells by affecting normal cellular processes such as those that control the actin cytoskeleton and other signal transduction pathways causing a rearrangement in the actin beneath the adherent bacterium. This causes membrane ruffling, leading to the engulfment of the bacteria into a membrane-bound vacuole (IFT 2004; D'Aoust et al. 2001). It is within the vacuole that the bacteria survive and reproduce. *Salmonella* species have an additional Type III secretion system that encodes the factors needed for their survival in the intracellular compartment formed in the phagocytic cell. Salmonella have virulence plasmids that have additional factors, such as the ability to induce lysis, inflammatory responses and enteritis in animal hosts, aiding in the extended survival of the bacteria within the host cells (IFT 2004; D'Aoust et al. 2001).

Salmonellosis is the disease caused by *Salmonella* species. Healthy individuals, that are neither immuno-compromised nor pregnant, are less susceptible to contracting salmonellosis. At risk groups for disease include the elderly, pregnant women and the immuno-compromised. The ingestion of just a few cells can cause infection. Symptoms of nausea, vomiting, abdominal pain, headache, chills and diarrhea can develop within 12-14 hours of ingestion and last for 2-3 days. Other symptoms that can be observed along with above symptoms are weakness, faintness, moderate fever, drowsiness and restlessness. A mortality rate of 2% to 15% has been observed depending on

age of the affected. Approximately 5% of those infected can become carriers of the disease (D'Aoust et al. 2001; Jay 2000; D'Aoust 1997).

2.3 Milk

2.3.1 Introduction

The secretion of the mammary gland of female mammals is referred to as milk. Milk is typically the only nourishment for young mammals and contains components that provide necessary immunological protection. The major constituent of milk is water, while the remaining components include fat, lactose, and protein (both whey and casein). Smaller quantities of minerals, certain blood proteins, enzymes and intermediates of mammary synthesis are also found in milk. Each of the components found in milk are important to its structural and functional properties. Milk fat is important to flavor and potential off-flavor that develop in milk. Proteins bind calcium and stabilize colloidal particles. Lactose contributes to osmotic pressure, freezing point depression, and boiling point elevation (Singh and Bennett 2002).

Milk fat and protein composition vary with bovine breed, including Friesian, Jersey, Guernsey, Ayrshire, Brown Swiss and Holstein, as well as among individual cows within the same herd. This variation in composition is mainly due to genetic variation, but can be caused by time of lactation as well as other environmental and physiological factors such as type and amount of feed, mastitis, weather changes, stress, exhaustion, time of day milking occurs and milking frequency (Singh and Bennett 2002). Following calving, colostrum (the first secretion of milk) has a large concentration of fat and protein and low

concentration of lactose. Concentrations of these components change to that of normal mature milk gradually within two to four weeks (Singh and Bennett 2002).

2.3.2. Milk Proteins

Bovine milk contains approximately 3.5% protein which can be separated into two main groups—whey and casein (Table 1) (Singh and Bennett 2002). Casein can be subdivided into four different proteins: α_{s1}-, α_{s2}-, β- and κ-casein. Caseins which are derived from the actions of native milk proteases are termed γ-caseins. All caseins are phosphoproteins with their phosphate groups esterified to serine residues within the protein chains. These phosphate groups have the ability to bind large quantities of calcium and aid in development of the structures of the casein micelles. Of the casein found in normal milk, 95% exists as micelles with an average diameter of 150 nm and a range of diameters of 80 to 300 nm. There are approximately 10^{14} casein micelles/ml milk. These micelles are approximately 94% protein and 6% colloidal calcium phosphate, which is calcium, phosphate, magnesium and citrate (Singh and Bennett 2002).

The isoelectric point (pI) of casein is at pH 4.6 (Singh and Bennett 2002; Swaisgood 1996). Caseins are highly soluble in their native state and heat stable at pH > 6. This solubility along with the hydrophobic amino acids covering the surface give casein its amphiphilic structure This amphiphilic structure provides casein with good emulsifying properties. Hydrophobic residues within the casein molecule are not distributed uniformly across the polypeptide chain. The three

Table 1. Milk proteins (casein and whey) and their characteristics (Singh and Bennett 2002; Swaisgood 1996).

	% of total milk protein	Estimated Average MW range (kDa)
Casein	80	
α_{s1}-casein	34	23.6
α_{s2}-casein	8	25.2
β-casein	25	24.0
κ-casein	9	19.0
γ-casein	4	20.5
Whey protein	20	
β-lactoglobulin	9	18.4
α-lactalbumin	4	14.2
Proteose peptone	4	
Blood proteins		
Serum albumin	1	66.3
Immunoglobulin	2	
Total	100	

hydrophobic regions of α_{s1}-casein are located at residues 1-44, 90-113, and 132-199 while the two hydrophobic regions of α_{s2}-casein are at segments 90-120 and 160-207. The C-terminal two-thirds of β-casein is the greatest hydrophobic portion of the caseins. The hydrophobic segments of κ-casein are 5-65 and 105-115. The charged residues are clustered. κ-casein is more polar than α_{s1}-, α_{s2}- and β-caseins (Singh and Bennett 2002).

The remaining soluble proteins found in milk are referred to as whey proteins (Singh and Bennett 2002). Whey proteins can be further subdivided into α-lactalbumin, β-lactoglobulin, bovine serum albumin (BSA), immunoglobulins and proteose peptone (Singh and Bennett 2002; Swaisgood 1996). Whey proteins have a net negative charge at pH 6.8, the physiological pH of milk. The distribution of hydrophobic, polar and charged residues is uniform in whey

proteins. Whey proteins fold intramolecularly, concealing their hydrophobic residues, and therefore, do not interact with other proteins. β -lactoglobulin is the most abundant whey protein (50%) with a β -barrel structure, similar to the structure of a β-sheet but rolled up to form a cylinder, with α-helix on the surface and a molecular weight of around 18.0 kDa. With this structure, β-lactoglobulin binds with many small hydrophobic molecules. β-lactoglobulin has two disulfide bonds in its internal structure and a free single thiol group that account for the changes in milk during heating. Heating causes aggregation due to formation of κ-casein-β-lactoglobulin complexes through a disulfide linkage. The second most abundant whey protein is α-lactalbumin (20%) with a structure that is compact, forming a spherical globular protein and a molecular weight of around 14.0 kDa. α-lactalbumin contains four disulfide bonds within the chain which allows for the strong binding of two calcium atoms. When these calcium atoms are removed, the protein is easily denatured. Immunoglobulins are the third most abundant whey protein (10%) and are antibodies that are created by the presence of foreign macromolecular antigens. The fourth most abundant whey protein is serum albumin (5%) which is made in the liver and enters milk through the secretory cells and appears to function as a carrier of small molecules such as fatty acids, but have no known specific role. Other minor components within whey proteins are β-microglobulin, lactoferrin, transferrin, protease peptones, and acyl glycoproteins (Singh and Bennett 2002; Swaisgood 1996).

2.3.3 Antimicrobial Properties

Milk is not only nutritionally beneficially, but is known to possess compounds that may reduce or prevent bacterial growth. For example, in the form of colostrum, milk is the first natural defense in the protection and development of newborns (Naidu 2000a).

Lactoferrin, an iron-binding protein found in milk, has antimicrobial properties when used at higher concentrations (Law and Reiter 1977; Naidu 2000b). The antimicrobial activity of lactoferrin is related to its ability to bind essential iron which slows the growth of microorganisms (Law and Reiter 1977), to bind directly to the surface of microorganisms (Arnold et al. 1977), or to directly damage the outer membrane of gram-negative bacteria releasing lipopolysaccharide (Ellison et al. 1988; Ellison et al. 1990). Lactoferricin, or pepsin hydrolyzed lactoferrin, provides broad-spectrum activity against various bacteria, viruses, fungi and parasites, making it a potent natural antimicrobial (Bellamy et al. 1992). At 0.5 to 500 mg/ml, lactoferricin has antimicrobial activity against gram-positive and gram-negative bacteria (Bellamy et al. 1992). This activity is reduced or eliminated in complex microbiological media and in foods (Branen and Davidson 2000).

Lactoperoxidase, an oxidoreductase and the most abundant enzyme secreted in bovine milk, protects the mammary gland as well as the intestinal tract of newborns from infection by pathogenic microorganisms (Naidu 2000c). Thiocyanate ions and hydrogen peroxide, together with lactoperoxidase compose the lactoperoxidase system. It is the through the enzymatic reaction of

lactoperoxidase with thiocyanate and hydrogen that provide a broad spectrum of activity against bacteria, viruses and fungi (Naidu 2000c).

Lactoglobulins, or milk-derived immunoglobulins or antibodies, are another component which has been examined for its potential to inactivate microorganisms. Immunoglobulins can recognize and precipitate bacteria, viruses, polysaccharides, nucleotides, peptides and proteins. Colostrum contains high levels of these immunoglobulins that provide the primary source of protection to the newborn from the mother (Bostwick et al. 2000).

Lactolipids are another known form of natural antimicrobials found in milk. The antimicrobial activity of lipids found in milk is due to the presence of long-chain unsaturated fatty acids and the medium-chain saturated fatty acids and their monoglycerides that act by destabilizing the bacterial membrane by forming holes and increasing its porosity (Lampe and Isaacs 2000).

2.4 Enzymatic Protein Hydrolysis

Enzymatic protein hydrolysis is the degradation of proteins into peptides and/or amino acids by using proteolytic enzymes, such as trypsin and pepsin. During protein hydrolysis, peptide bonds are cleaved, and with the addition of water, peptides and free amino acids are released (Adler-Nissen 1993). Proteolysis is dependent upon the protein substrate, type of proteases used and hydrolysis conditions. The sequence of amino acids and the three-dimensional structure of the protein affect the ability of the protein to undergo hydrolysis and the type of peptides formed during hydrolysis. Enzyme-substrate binding is

essential for protein hydrolysis. Globular proteins may be composed of single chains or two or more chains which can interact in different ways and are spherical in shape (Damodaran 1996). Globular proteins, such as whey protein, have most of the peptide bonds within the interior of the protein and, therefore, they are inaccessible to the enzyme (Damodaran 1996). Therefore, for successful hydrolysis of these proteins, reversible unfolding is necessary in order to expose the interior peptide bonds for hydrolysis. Caseins are flexible proteins due to their open structure and can be easily hydrolyzed (Swaisgood 1996). Hydrolysis of proteins with different amounts of hydrophobic and charged groups can result in the formation of peptides varying in their distribution of hydrophobic and hydrophilic side groups.

There are many proteases available for protein hydrolysis and they are classified based on their origins (plant, animal, or microbial), mode of action, or catalytic site (Adler-Nissen 1993). Endoproteases are enzymes which cleave amide bonds within the protein chain. Pepsin and trypsin are examples of endoproteases. Pepsin and trypsin are two of the three proteolytic enzymes found in the digestive system, the other one being chymotrypsin. Pepsin cleaves peptide bonds associated with the aromatic amino acids, phenylalanine, tryptophane and tyrosine and is effective in the pH range of 1 to 4. Trypsin cleaves bonds associated with the amino acids, lysine and arginine and is effective in the pH range of 7 to 9 (Adler-Nissen 1993).

The degree of hydrolysis is dependent upon the conditions, temperature, pH, enzyme to substrate ratio and reaction time. Temperature, pH and enzyme to

substrate ratio determine the rate of the reaction. The reaction time determines the final extent of hydrolysis. At low pH, all amino groups are protonated and only a portion of carboxyl groups are deprotonated, resulting in an uptake of protons for each peptide bond cleaved, increasing the pH. At neutral or alkaline pH, a decrease in pH occurs due to the deprotonation of all the carboxyl groups and partial protonation of amino groups. In order to prevent these changes in pH in a controlled system, a buffer may be used to maintain desired pH (Whitaker 1996).

Protein hydrolysis causes the molecular properties of proteins to change such as a decrease in molecular weight, an increase in charge, the exposure of hydrophobic groups and the exposure of reactive amino acid side chains. As a result of these changes, the functional properties may be affected including nutritional and physiological. Changes in the nutritional properties of hydrolysates include increased digestibility and decreased allergenicity. Smaller peptides are easier to digest than whole proteins, which can be beneficial to those people who suffer from certain digestive disorders. Further, hydrolysis can be used to destroy protein sequences responsible for allergic reactions in those individuals sensitive to certain allergens (Damodaran 1996). Physiological properties changed may include creation of bioactive peptides (Bellamy et al. 1992; Zucht et al. 1995; Recio and Visser 1999), angiotensin converting enzyme (ACE) inhibition (Clare and Swaisgood 2000) and opioid activity (Brantl et al. 1979).

2.5 Bioactive Peptides from Milk

2.5.1 Introduction

Biologically active peptides have been discovered in both animals and plants (Gennaro 1989; Lee 1989; Bevins and Zesloft 1990; Lehrer et al. 1993; Boman 1995; Cowan 1999; Ganz and Lehrer 1999; Meisel 1997, 1997b, 1998; Schanbacher 1997, 1998). Bioactive peptides derived from casein have been found to exhibit a variety of biological, physiological, nutritional and antimicrobial properties/functions. Bioactive peptides from milk protein hydrolysis include those with antimicrobial, opioids, endorphin-like, antihypertensive, decreased blood pressure, and antithrombotic, decreased blood clotting properties (Schlimme and Meisel 1995; Korhonen et al. 1998; Clare and Swaisgood 2000; Pihlanto-Leppala 2001). These biologically active peptides have been obtained *in vitro* by proteolysis (Bellamy et al. 1992; Zucht et al. 1995; Recio and Visser 1999) and *in vivo* by gastric digestion of milk proteins (Kuwata et al. 1998; Meisel and Bockelmann 1999).

Casein is a good source of such peptides/protein fragments that have these certain physiological functions including aiding in gastrointestinal function and digestion, hemodynamic modulation (antihypertension and increased blood flow), probiotic support of gut microflora, nonimmune disesase protection, passive immunity, immunoregulation, anti- inflammation, growth and development (Tome and Debabbi 1998; Schanbacher et al. 1997). Thus, it is

important to study the potential for creating peptides through proteolytic hydrolysis *in vitro* which could have antimicrobial activity.

2.5.2 Opioid Activity

Peptides derived from the enzymatic digests of casein with opioid activity were first reported by Brantl et al. (1979). Peptides with similar activity were found by Ziodrou et al. (1979) in the pepsin hydrolysates of α-casein. Bitri (2004) found that through a mild acidic hydrolysis using pepsin, imitating gastric-like digestion, the release of bioactive material occurred over time. The release of material was correlated with acidity rather than enzymatic activity indicating a chemical proteolysis produced the protein fragments rather than an enzymatic hydrolysis. Biziulevicius et al. (2002) reported on the antimicrobial activity of tryptic casein hydrolysate, its mode of antimicrobial action, and its efficacy as a treatment and prophylaxis of colibacillus in newborn calves by its stimulation to the microbial autolytic system.

Opioid peptides are short peptides, 5-10 amino acids, and have the ability to bind opioid receptors on intestinal epithelial as well as other cells (Teschemacher and Koch 1991; Schlimme and Meisel 1995). Opioid peptides are derived from β-casein, α_{s1}-casein, β-lactoglobulin, and α-lactalbumin (Teschemacher and Koch 1991; Schlimme and Meisel 1995). The major opioid peptides are β-casomorphins, which are the 60 to 70 amino acid residues of bovine β-casein (Clare and Swaisgood 2000). Opioid peptides are responsible

for altering the emptying of gastric contents and decreased intestinal motility (Schanbacher et al. 1997; Clare and Swaisgood 2000).

2.5.3 Immunomodulatory Peptides

Immunomodulatory peptides modulate lymphocyte function, lymphocyte differentiation, enhance killer cell activity and affect both the immune system and cell multiplication responses (Clare and Swaisgood 2000; Schanbacher et al. 1997). Casein hydrolysates were shown to increase the phagocytic activity of human macrophages against red blood cells (Fiat et al. 1989; Jolles et al. 1981; Milgliore-Samour et al. 1989). Small peptides at the N-terminal end of bovine α-lactalbumin and κ-casein increased the multiplication of human peripheral blood lymphocytes (Kayser and Meisel 1996).

2.5.4 Antihypertensive Peptides

The angiotensin converting enzyme (ACE) is a peptidyldipeptidase that cleaves dipeptides from the carboxy terminal end of a protein, and converts angiotensin I to angiotensin II, causing an increase in blood pressure (Clare and Swaisgood 2000). Peptides can act as ACE inhibitors. True inhibitors are those which bind to the active site of ACE without being hydrolyzed by the enzyme. Inhibitor peptides can also be substrates of ACE, which can be cleaved by ACE releasing new peptides. Those new peptides that are created that yield more effective inhibitors are called “pro-drug type inhibitors” while those peptides that create less effective inhibitors are called “substrate type inhibitors.” There have been numerous peptides and hydrolysates derived from food proteins reported to work as ACE inhibitors (Ariyoshi 1993; Meisel and Schlimme 1996; Yamamoto

1997; Shah 2000). Hydrolysis with trypsin results in hydrolysates with good ACE-inhibiting activity (Mullally et al. 1997). ACE inhibition by hydrolysates is mainly due to low molecular weight peptides (Meisel et al. 1997; Pihlanto-Leppala et al. 2000). Casokinins are ACE inhibitors that are derived from the tryptic digestion of bovine casein and provide antihypertensive properties (Clare and Swaisgood 2000). ACE inhibitory peptides have also been created through the hydrolysis of α_{s1}-casein and β-casein with *Lactobacillus helveticus* CP790 (Clare and Swaisgood 2000).

2.5.5 Other Milk Components

Hydrolysis of the milk components, lactoferrin (Kimura et al. 2000; Kuwata et al. 1998; Nibbering et al. 2001; Groenink et al. 1999; Branen and Davidson 2000; Qian et al. 1995), bovine hemoglobin (Froidevaux et al. 2001), casein (Liepke et al. 2001; Lahov and Regelson 1996; Recio and Visser 1999;), α-lactoalbumin (Pellegrini et al. 1999) and α-lactoglobulin (Pellegrini et al. 2001) and whey (Pihlanto-Leppala 2001) produce hydrolysates with antimicrobial activity. Antimicrobial activity of hydrolyzed lactoferrin was observed in peptone glucose yeast extract media, but not tryptic soy broth (Branen and Davidson 2000). When cation and amphipathic peptides were derived from bovine and human lactoferrins, those peptides containing the largest number of positively charged amino acids showed the greatest antimicrobial activity against both gram-positive and gram-negative bacteria (Groenink et al. 1999). Sheep and human lactoferrins as well as their pepsin hydrolysates inhibited thrombin-induced platelet aggregation in a dose dependent manner (Qian et al. 1995). The

1-23 fragment of the peptic digest of bovine hemoglobin had low antimicrobial activity against *Micrococcus luteus* A270 (Froidevaux et al. 2001). Pepsin hydrolysis of κ-casein (residues 63-117) was found to inhibit growth of gram-positive bacteria, gram-negative bacteria and yeasts (Liepke et al. 2001). Using a heat treatment in combination with the enzyme chymosin, casecidines or polycationic low molecular mass peptides with antimicrobial activity were formed (Lahov and Regelson 1996). Three bactericidal domains were isolated and identified using reversed phase chromatography from bovine α-lactalbumin after partial digestion with trypsin and chymotrypsin (Pellegrini et al. 1999) and four bactericidal domains were isolated and identified using reversed phase chromatography from bovine α-lactoglobulin after digestion with trypsin (Pellegrini et al. 2001). Domains derived from both α-lactalbumin and α-lactoglobulin digesion with trypsin were effective against gram-positive bacteria (Pellegrini et al. 2001). Digestion with pepsin created fragments with no antimicrobial activity (Pellegrini et al. 1999). However, Recio and Visser (1999), using peptic hydrolysis, found two distinct antibacterial domains (183-207 and 164-179) within the sequence of bovine α_{s2}-casein.

Known fragments of bovine casein with antimicrobial activity are isracidin (Hill et al. 1974), casocidin-I (Zucht et al. 1995), α-casecidins (Otani and Suzuki 2003), kappacin (Malkoski et al. 2001), and the casein-κ-derived glycomacropeptide (Stromqvist et al. 1995; Aniansson et al. 1990). Isracidin is a non-immunological polypeptide (with a molecular weight of 2770 Da) obtained

from the chymosin digestion of α_{s1}-casein B and consists of the N-terminal segment (1-23) of α_{s1}-casein B (Lahov and Regelson 1996). Isracidin was found to be significantly effective *in vivo* at concentrations comparable to antibiotics against *Staphylococcus aureus* strain Smith as well as produced long-term immune resistance (Lahov and Regelson 1996).

2.5.6 Antimicrobial mechanism of action

The mode of action/mechanism of peptide hydrolysates is not fully understood. Hydrolysates may affect transmembrane pore-forming mechanisms as described for several antimicrobial peptides. Cationic peptides have been shown to kill bacteria by disrupting or permeabilizing the bacterial membrane by inducing the uptake of extracellular K+ ions. This uptake of ions leads to the depolarization of the outer membrane or, in the case of Gram-negative bacteria, permeabilization of the outer membrane. Antimicrobial peptides may accumulate at the target membrane surfaces causing displacement of phospholipids leading to changes in membrane fluidity and membrane disruption (Yeaman and Yount 2003).

It is proposed that the positive charges affect the anionic lipids of the bacterial membrane, causing destabilization and destruction of the membrane by changing their lipid structure (Kragol et al. 2001). A positive net charge and a potentially amphipathic α-helix have been identified as the major structural components that interact with the lipid bilayer to enhance the permeability of membranes (Dathe et al. 1996). Dathe et al. (1996) found that amphipathic

peptide bound the lipid bilayer at the membrane interface by electrostatic interactions between cationic peptide charges and anionic lipid head groups of the membrane and through hydrophobic interactions. Peptides with cationic amphipathic structures show a high affinity for negatively charged bacterial membranes with lipopolysaccharides or anionic phospholipids (Matsuzaki 2001). These cationic peptides target the cell surface of the anionic lipids unique to the microorganisms (Matsuzaki 2001; Hancock and Lehrer 1998).

Peptides that are cysteine-rich are thought to form pores or ion-permeable channels within the lipid bilayer (Marshall and Arenas 2003). Membrane-active peptides that are cationic due to the presence of multiple lysine and arginine residues form amphipathic secondary structures which can enter the membrane (Matsuzaki 1999).

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3. Materials and Methods

3.1 Hydrolysate Preparation

Whole casein was precipitated at room temperature from 10.8 L of fresh, unpasteurized skimmed cow's milk (Broad Acre Farms, Powell, TN) by the slow addition of 300 mL of 2 N HCl to pH 4.6. Precipitated casein was separated by centrifugation at 1370 g for 10 min and was subsequently washed by suspending in 2.25 L of deionized water followed by recentrifugation. The washing was repeated five times. The resulting precipitate was freeze-dried.

In Method 1, a solution of 1.7 % whole dry casein at pH 2 was incubated with pepsin (1:100 w/w) at 37 ^{o}C, and a solution of 1.7 % whole dry casein at pH 8 was incubated with trypsin (1:100 w/w) at 37 ^{o}C. After 5 hr, the reaction solutions were heated for 5 min at 95 ^{o}C to inactivate each enzyme, and rapidly cooled to room temperature. The solutions were dialyzed using Spectra/Por® 2 dialysis membrane (MW cutoff = 12000-14000 Da) (Spectrum Laboratories, Inc., Rancho Dominquez, CA) against water for 40 hr with 5 water changes and freeze-dried. Solutions containing 0.5% and 1% of each hydrolysate were prepared, pH adjusted to ca. 7.0 $\pm$ 0.2 and filter sterilized through a cellulose acetate 0.45 μm membrane filter (Corning Incorporated, Corning, NY).

In Method 2, a solution of 5.0 % casein was prepared by dissolving whole dry casein in 0.1 M pH 7.2 phosphate buffer. The 5.0 % casein solution treated similarly to Method 1, however, the hydrolysates created were separated by centrifugation at 4000 g for 10 min after inactivation of the enzymes and were not

dialyzed against water. The resulting supernatant from centrifugation was pH adjusted to ca. 7.0 ± 0.2 and filtered sterilized as described in Method 1.

3.2 Culture Preparation

Listeria monocytogenes, strains 101, 108, 310 and Scott A and *Salmonella* Typhimurium strains 2380, 2576, 2582 and 2486 were stock cultures obtained from microbiology laboratory at the University of Tennessee. All cultures were grown in tryptic soy broth (TSB; Difco, Sparks, MD) and transferred every 4 wk to maintain viability. Working cultures were obtained by inoculating a loopful of culture into TSB and incubating for 24 hr at 32°C. Working cultures were subsequently transferred every day for 3 days prior to use. After incubation, the cultures were diluted to ca. 5.0 log CFU/mL.

3.3 Micro-broth Dilution Assay

Sterile 96-well microtiter plates with a well capacity of 300 μL were used for all methods. In Method 1, for testing hydrolysate alone, a total volume of 250 μL was used consisting of 125 μL of double strength TSB, 100 μL of filtered hydrolysate and 25 μL of inoculum (ca. 5.0 log CFU/mL). For testing hydrolysate in combination with EDTA, a total volume of 250 μL was used consisting of 125 μL of double strength TSB, 50 μL of filtered hydrolysate (pepsin or trypsin at 0.5 % and 1 %), 50 μL of EDTA (250 μg/mL EDTA for *L. monocytogenes* and 1250 μg/mL for *S*. Typhimurium DT104) and 25 μL of inoculum (ca. 5.0 log CFU/mL).

In Method 2, for testing hydrolysate alone, the same method was used as in Method 1. However, hydrolysates were tested in combination with either EDTA

or sodium lactate and with both EDTA and sodium lactate. For testing hydrolysate in combination with either EDTA or sodium lactate, a total volume of 250 μL was used consisting of 125 μL of double strength TSB, 50 μL of filtered hydrolysate (pepsin or trypsin), 50 μL of EDTA (250 μg/mL EDTA for *L. monocytogenes* and 1250 μg/mL for *S.* Typhimurium DT104) or 50 μL of sodium lactate (1 %) and 25 μL of inoculum (ca. 5.0 log CFU/mL). For testing hydrolysate in combination with both EDTA and sodium lactate, a total volume of 250 μL was used consisting of 125 μL of double strength TSB, 33 μL of filtered hydrolysate (pepsin or trypsin), 33 μL of EDTA (250 μg/mL EDTA for *L. monocytogenes* and 1250 μg/mL for *S.* Typhimurium DT104), 33 μL of sodium lactate (1 %) and 25 μL of inoculum (ca. 5.0 log CFU/mL).

For both methods 1 and 2, microtiter plates were covered with a sterile lid and incubated 24 hr at 32 ^{o}C and the absorbance (630 nm) of each well was read at 0, 3, 6, 12, and 24 hr with a microtiter plate spectrophotometer (EI_x800 Universal Microplate reader, BioTek Instruments, Winooski, VT). The micro-broth dilution assays for all methods were performed in triplicate.

3.4 Growth Curve

In Method 1, pepsin and trypsin hydrolysates (5 %) were mixed with bacteria harvested at late logarithmic phase and diluted to ca. 5.0 log CFU/mL. The bacteria and hydrolysates were incubated in TSB at 32 ^{o}C for 24 hr. Bacterial suspensions were enumerated on tryptic soy agar (TSA; Difco) at 0, 3, 6, 12 and 24 hr. Plates, in duplicate, were incubated for 24 hr at 32 ^{o}C.

In Method 2, trypsin hydrolysates were mixed with bacteria harvested at late logarithmic phase and diluted to ca. 5.0 log CFU/mL. The bacteria and hydrolysates were incubated in TSB at 32 ^{o}C for 24 hr. The bacterial suspensions were enumerated on TSA at 0, 3, 6, 12 and 24 hr. Plates, in duplicate, were incubated for 24 hr at 32 ^{o}C.

3.5 Determination of Protein Content

The protein concentrations of pepsin and trypsin hydrolysates (before and after filtering) in methods was determined using three different protein assays, Bradford dye-binding method (Bio-Rad Laboratories, Hercules, CA), modified Lowry method (Pierce, Rockford, IL) and UV 280 nm (Chang 1998) . Protein concentration determined using the Bradford dye-binding method utilized a 5-point standard curve that was prepared using bovine serum albumin (BSA), ranging from 0.2 to 1 mg protein/mL or a 10-point standard curve also prepared using BSA, ranging from 0 to 2 mg protein/mL. To a 50 μL aliquot of unknown or standard, 2.5 mL of diluted (1 volume of Dye Reagent Concentrate with 4 volumes deionized water) and filtered (using Whatman No. 1 paper) Bio-Rad Dye Reagent Concentrate was added and the samples were mixed using a vortex. The samples were allowed to sit at room temperature for at least 10 min to allow for color development. The absorbance was stable for about 1 hr. Each sample (unknowns and standards) was transferred to a disposable polystyrene cuvette and the absorbance was measured at 595 nm using a UV-VIS scanning

spectrophotometer (UV-2101PC, Shimadzu, Japan). Protein concentrations of the unknown samples were determined using the standard curves.

In the modified Lowry Protein Assay Kit (Pierce, Rockford, IL), a 10-point standard curve was developed using BSA ranging from 0 to 1.5 mg protein/ml. A 1N Folin-Ciocalteu Reagent was prepared by diluting the 2N reagent 1:1 with deionized water. To a 0.2 mL aliquot of unknown or standard, 1.0 mL Modified Lowry Reagent (containing cupric sulfate, potassium iodide and sodium tartrate in an alkaline solution buffer) was added, mixed well by vortexing and incubated for exactly 10 min at room temperature. At the end of the incubation period, 100 μL of prepared 1N Folin-Ciocalteu Reagent was added, mixed well by vortexing and incubated for exactly 30 min at room temperature. Samples (unknowns and standards) were transferred to disposable polystyrene cuvettes and the absorbance measured at 750 nm. Protein concentrations of the unknown samples were determined using the standard curves.

For the UV 280 nm method, a 10-point standard curve that was prepared with BSA ranging from 0 to 2 mg protein/mL. Each standard and sample were transferred to a quartz cuvette and the absorbance at 280 nm measured using a UV-VIS spectrophotometer (UNICAM, Cambridge, UK)..The protein concentrations of the unknown samples were determined using the standard curve.

4. Results and Discussion

4.1. Method 1

Pepsin and trypsin hydrolysates produced with Method 1 showed little antimicrobial activity. Trypsin hydrolysates were slightly effective in extending the lag phase and/or reducing the final growth level of three of four strains of *Listeria monocytogenes* (101, 108 and Scott A) (Fig. 1A-D) (all figures located within appendix) at 1.0 % (w/v). Trypsin hydrolysates alone or in combination with EDTA had no activity against any strain of *S.* Typhimurium (Fig. 2A-D, 5A-D) at up to 1.0. Pepsin hydrolysates were effective in slightly extending the lag phase of two of the four strains of *L. monocytogenes* (101 and 108 at 0.5 %) (Fig. 1B) without and with EDTA (3A, 3B) and one of the four strains of *S*. Typhimurium (2486 at 1.0 %) without EDTA (Fig. 2D).

EDTA alone was effective against one of the four strains of *L. monocytogenes* (101) (Fig. 3A, 4A) and all four strains of *S*. Typhimurium (2380, 2576, 2582 and 2486) (Fig. 5A-D, 6A-D) by decreasing the final growth level and/or extending the lag phase; however, EDTA had little effect on enhancing the inhibitory effect of pepsin or trypsin hydrolysates against either microorganism.

To more precisely determine the influence of the compounds on growth of the test microorganisms, numbers of each were monitored over time (Fig. 7, 8). Neither pepsin nor trypsin hydrolysates at 5 % prepared using Method 1 were effective in inhibiting the growth of any of the strains of *L. monocytogenes* (Fig. 7A-B) or *S*. Typhimurium (Fig. 8A-B). *S.* Typhimurium 2486 had decreased

growth from 6 to 12 hr; however, there was no decrease in the final level of growth as compared to the control (Fig 8B).

In conclusion, hydrolysate preparation using Method 1 was shown to produce solutions that demonstrated little or no inhibition of the test microorganisms. The probable reason for this was that low molecular weight peptides were lost during dialysis due to the large pore size (MW cutoff = 12000-14000 Da) in the dialysis tubing. It was theorized that low molecular weight peptides are necessary to have antimicrobial activity. This was supported by research that showed that peptides from bovine casein and hydrolyzed with pepsin and trypsin demonstrated some antimicrobial activity (Miclo et al.. 2001; Recio and Visser 1999). Lack of antimicrobial effectiveness may have also been due to potential enhancement of bacterial growth due to larger peptides being utilized as nutrient sources.

4.2. Method 2

4.2.1 Antimicrobial Activity

Method 1 was modified to capture the low molecular weight peptides proposed to possess the primary antimicrobial activity. By using centrifugation instead of dialysis, the smaller molecular weight peptides were captured in the supernatant while the larger molecular weight peptides were removed in the form of a precipitate.

Using Method 2, trypsin hydrolysates were effective in extending the lag phase and/or reducing the final growth level of all four strains of *L. monocytogenes* tested (Fig. 9A). However, they were not effective against any of

the strain of *S.* Typhimurium (Fig. 9B). It is suggested that proteolytic digestion of bovine casein with trypsin led to the production of several heat stable peptide fragments possessing antimicrobial properties. The peptides produced from the tryptic digestion of casein displayed greater antimicrobial activity against Gram-positive bacteria. This would suggest that they may be similar to other cationic antimicrobial peptides, such as nisin, which act as surface active compounds (Rurh and Sahl 1985). The lack of activity against *S.* Typhimurium, a Gram-negative bacterium, was possibly because of the protection afforded to the microorganism by the outer membrane which is often effective in screening amphiphilic compounds (Matsuzaki 1999). In a similar study, trypsin digestion of rabbit casein also did not produce antimicrobial activity against Gram-negative organisms (Barayni et al. 2003).

Pepsin hydrolysates were not effective in extending the lag phase and/or reducing the final growth level of any of the four strains of *S.* Typhimurium (Fig. 9D). However, they were effective in reducing the final growth level in *L. monocytogenes* 101 (Fig. 9B). This is contrary to the observations of Baranyi et al. (2003) who found that casein-derived peptides isolated from rabbit (*Oryctolagus cuniculus*) milk and digested with chymotrypsin, pepsin and clostipain produced several peptide fragments with antimicrobial activity. Additionally, Liepke et al. (2001) found that hydrolyzing human milk proteins with pepsin produced low-molecular-mass peptide fragments similar to κ-casein that increased the antimicrobial activity against Gram-positive, Gram-negative bacteria and yeasts. The differences in antimicrobial activity between what we

found and those results from Baranyi et al. (2003) and Liepke et al. (2001) most probably are due to their increased purification by the previous researchers of those compounds responsible for activity.

The mechanism of bioactive peptides is thought to be related to their ability to increase permeability of the bacterial membrane or other type of membrane destabilization (Yeaman and Yount 2003). Therefore, combinations of the peptides with traditional, regulatory-approved antimicrobials were tested to observe whether or not the inhibition caused by the peptides could enhance antimicrobial activity. Method 2 results confirm that trypsin hydrolysates derived from bovine milk were effective in extending the lag phase and/or reducing the final growth level *L. monocytogenes* (Fig. 10A-D) and slightly effective in reducing the final growth level of *S.* Typhimurium (Fig. 11A-D). Trypsin hydrolysates were found to enhance the antimicrobial activity of sodium lactate against *L. monocytogenes* (Fig. 10A-D) and only slightly enhance the antimicrobial activity of sodium lactate against *S.* Typhimurium (Fig. 11A-D). Trypsin hydrolysates derived from bovine milk in combination with EDTA have antimicrobial activity against *L. monocytogenes* and *S.* Typhimurium in tryptic soy broth (TSB) (Fig. 10A-D, 11A-D). However, in this study, the activity was primarily due to the presence of EDTA.

Pepsin hydrolysates alone showed no antimicrobial activity against either *L. monocytogenes* or *S.* Typhimurium and did not enhance the antimicrobial activity of either sodium lactate or EDTA (data not shown). Branen and Davidson (2000) found that pepsin hydrolyzed lactoferrin (HLF) was effective against *L.*

monocytogenes, enterohemorrhagic *E. coli* and *Salmonella* Enteritidis in peptone yeast extract broth (PYE) but not in TSB. Addition of EDTA enhanced the activity of HLF in TSB (Branen and Davidson 2000). In a separate study, Branen and Davidson (2004) examined the effect of combining the antimicrobials nisin, lysozyme and monolaurin with EDTA and lactoferrin and found that antimicrobial activity of certain antimicrobials can be enhanced in combination. For example, EDTA enhanced the activity of nisin, monolaurin and lysozyme in TSB against two enterohemorrhagic *E. coli* (Branen and Davidson 2004). Further, while none of the antimicrobials alone were bactericidal, in combination with EDTA, nisin, lysozyme and monolaurin were bactericidal against some Gram-negative bacteria (Branen and Davidson 2004). However, in the present study, addition of pepsin or trypsin hydrolysates did not enhance the antimicrobial activity of EDTA and lactate against Gram-negative bacteria (Fig. 11A-D).

Another test was run to determine whether antimicrobial activity could be increased by combining both pepsin and trypsin hydrolysates. It was found that by adding pepsin hydrolysates, the antimicrobial activity of trypsin hydrolysates actually decreased in activity against *L. monocytogenes* (Fig. 12A-D) and there was no effect on the activity against *S.* Typhimurium (13A-D).

The Method 2 hydrolysates were evaluated against *L. monocytogenes* and *S.* Typhimurium by enumerating the microorganisms over time (Fig. 14 and 15). Compared to the extent of antimicrobial activity of trypsin hydrolysates demonstrated in the spectrophotometric analysis, the activity was reduced using the count method (Fig 14A-B, 15A-B). A possible reason for this may be the

detection limits of the analysis. The limit of detection of cells using the spectrophotometric method is on the order of 5-6 log CFU/mL. In contrast, the cell count method used followed growth from 4 log CFU/mL. The lack of increase in OD seen in the spectrophotometric assay could be due to the reduced final growth level as seen in the count assay.

It is suggested that activity of the peptide solutions may be enhanced if further isolation techniques for the antimicrobial peptides were used. For example, it has been found that isolating specific peptide sequences from casein that possess antimicrobial activity is important to maximal activity (Malkoski et al. 2001; Helinck et al. 2003). Malkoski et al. (2001) isolated kappacin, a novel antimicrobial peptide from bovine milk and the active form of caseinomacropeptide (CMP) consisting of non-glycosylated, phosphorylated κ-casein (residues 106-169). Malkoski et al. (2001) prepared CMP by chymosin digestion of casein and gel-filtration. CMP was effective in inhibiting growth of both gram-positive and gram-negative microorganisms. Further, hydrolyzation of CMP with endoproteinase Glu-C generated the nonglycosylated peptides Ser(P)^{149} κ-casein-A(138-158) that displayed inhibitory activity in the growth of *Streptococcus mutans* (Malkoski et al. 2001). Recio and Visser (1999) isolated and identified two distinct antimicrobial domains from a peptic hydrolysate of bovine α_{s2}-casein and determined the C-terminal part of the α_{s2}-casein interacted with the cation-exchange membrane used to obtain the antimicrobial fraction. Further, in isolating the antibacterial peptides, fragments were found to have antimicrobial activity against both Gram-positive and Gram-negative bacteria

(Recio and Visser 1999). Otani and Suzuki (2003) isolated cytotoxic peptides, α-casecidins, from tryptic digestion of bovine α_{s1}-casein and ion-exchange chromatography. These peptides were found to be cytotoxic toward all lymphocytes examined. The α-casecidins showed little cytotoxic activity towards bovine milk cells. Otani and Suzuki (2003) examined the effects of strength of charge or cationic peptide on cytotoxic activity and determined no correlation. Zucht et al. (1995) found that peptides derived from acid hydrolyzed α_{s2}-casein, casocidin-I, demonstrate activity against both gram-positive and Gram-negative organisms and further demonstrated that gram-positive microorganisms were more sensitive than gram-negative microorganisms. Lahov and Regelson (1996) showed that chymosin-hydrolyzed casein and α_{s1}-casein were antimicrobial against gram-positive microorganisms *in vitro* and against *Staphylococcus aureus in vivo* in mice.

While it is known that further purification steps to isolate antimicrobial peptides from casein are possible, the most useful form of antimicrobials for the food industry are crude extracts or preparation (Davidson and Zivanovic, 2003). Since casein-derived peptides could provide an alternative or adjunct to antimicrobials currently used in foods, further research needs to be conducted to find simple methods for enhancing activity by concentrating the hydrolysates or isolating and characterizing those peptides with the greatest antimicrobial potential.

4.2.2 Protein Concentration and Peptide Fragment Determination in Method 2

A 5 % (w/v) casein (50 mg/mL) starting solution was used for creating each type of hydrolysate. Using the Bradford method, non-membrane filtered hydrolysates were higher in protein than those that were filtered (84.5 μg/mL vs. 37.0 μg/mL respectively). Pepsin hydrolysates were higher in protein than trypsin hydrolysates in both filtered and non-filtered samples. Average protein concentrations for filtered pepsin, non-filtered pepsin, filtered trypsin and non-filtered trypsin were 62.3 μg/mL, 125.9 μg/mL, 11.7μg/mL and 42.9 μg/mL, respectively. The Coomassie brilliant blue dye used in the Bradford method binds to primarily basic (arginine groups) and aromatic amino acids (Compton and Jones 1985).

Using the Modified Lowry method, non-filtered hydrolysates were slightly higher in protein concentration than those that were filtered (30.4 mg/mL vs. 27.8 mg/mL respectively). Pepsin hydrolysates were lower in protein concentration than trypsin hydrolysates in both filtered and non-filtered samples. Average protein concentrations for filtered pepsin, non-filtered pepsin, filtered trypsin and non-filtered trypsin were 11.2 mg/mL, 16.0 mg/mL, 44.4 mg/mL and 44.9 mg/mL, respectively. The Lowry method applies two reagents, one with Cu^{2+} which reacts with peptide bonds giving a blue color reaction and a second folin cupric reagent that reacts with –OH also resulting in a blue color (Lowry et al. 1951). The resulting color indicates the amount of small molecular weight peptides.

Using the UV 280 nm method, non-filtered hydrolysates were higher in protein concentration than those that were filtered (1,680 μg/mL versus 1,500 μg/mL respectively). Trypsin hydrolysates were higher in protein concentration than pepsin hydrolysates in both filtered and non-filtered samples. Average protein concentrations for filtered pepsin, non-filtered pepsin, filtered trypsin and non-filtered trypsin were 480 μg/mL, 920 μg/mL, 2,180 μg/mL, and 2,450 μg/mL, respectively. Just as the Lowry Method, theUV 280 nm determination measures smaller sized proteins.

The actual identity of the peptides isolated from bovine milk casein and digested with pepsin and trypsin cannot be discerned from the methods used. However, by examining the protein sequence and understanding the method of enzymatic cleavage, one can speculate on the peptides formed Fig. (16-19). Trypsin cleaves proteins at the amino acids, lysine and arginine. Trypsin cleavage of α_{s1}-casein yields 40 different peptide sequences varying in length (from one to 41 amino acids) with 28 sequences with 5 amino acids or less (Fig. 16A). Trypsin cleavage of α_{s2}-casein yields 51 different peptide sequences varying in length (from one to 24 amino acids) with 37 sequences with 5 amino acids or less (Fig. 17B). Trypsin cleavage of β-casein yields 31 different peptide sequences varying in length (from one to 55 amino acids) with 23 sequences with 5 amino acids or less (Fig. 17A). Trypsin cleavage of κ-casein yields 27 different peptide sequences varying in length (from one to 53 amino acids) with 18 sequences with 5 amino acids or less (Fig 17B).

Pepsin cleaves proteins at the amino acids, phenylalanine, tyrosine and tryptophan. Pepsin cleavage of of α_{s1}-casein yields 32 different peptide sequences varying in length (from one to 58 amino acids) with 24 sequences with 5 amino acids or less (Fig. 18A). Pepsin cleavage of α_{s2}-casein yields 33 different peptide sequences varying in length (from one to 38 amino acids) with 22 sequences with 5 amino acids or less (Fig. 18B). Pepsin cleavage of β-casein yields 29 different peptide sequences varying in length (from one to 32 amino acids) with 16 sequences with 5 amino acids or less (Fig. 19A). Pepsin cleavage of κ-casein yields 22 different peptide sequences varying in length (from one to 66 amino acids) with 17 sequences with 5 amino acids or less (Fig 19B).

Therefore, it was possible that small molecular weight peptides were created by the addition of trypsin and pepsin to bovine casein. The possible variation in lengths of amino acid sequences created could indicate the varying antimicrobial affects from one method to another as well as the variation in replications. It appears that trypsin hydrolysates contained a greater quantity of small molecular weight peptides which may have contributed to greater antimicrobial activity.

5. Conclusions

Bovine milk contains an assortment of biologically active peptides that can be released during enzymatic proteolysis. The primary objective of the food industry is to protect consumers from the harmful effects of foodborne pathogens. Exploring the potential for use of antimicrobials derived from natural sources or bio-based compounds is important in finding alternatives or adjuncts to regulatory-approved traditional antimicrobials. The antimicrobial activity of traditional antimicrobials may be enhanced by combining them with trypsin or pepsin hydrolysates against both Gram-positive and Gram-negative microorganisms. Further research will need to be done to characterize and identify these “natural” antimicrobial peptides as well as examine their effectiveness in food systems.

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Appendix

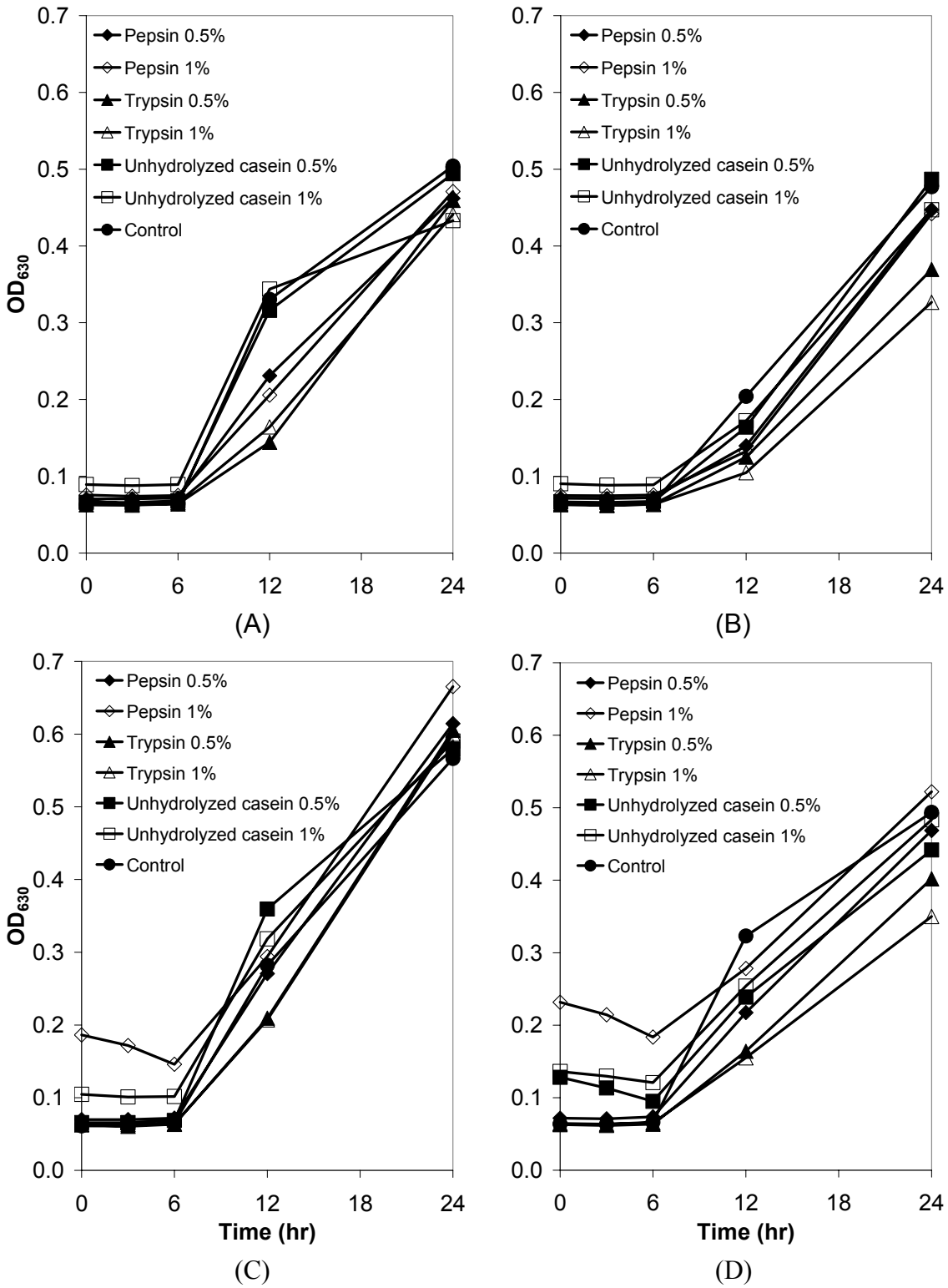


Figure 1. Antimicrobial activity of hydrolysates of acid-precipitated casein, digested with pepsin and trypsin, dialyzed and lyophilized (method 1) at 0.5% and 1.0% on *Listeria monocytogenes* (A) 101 (B) 108 (C) 310 and (D) Scott A.

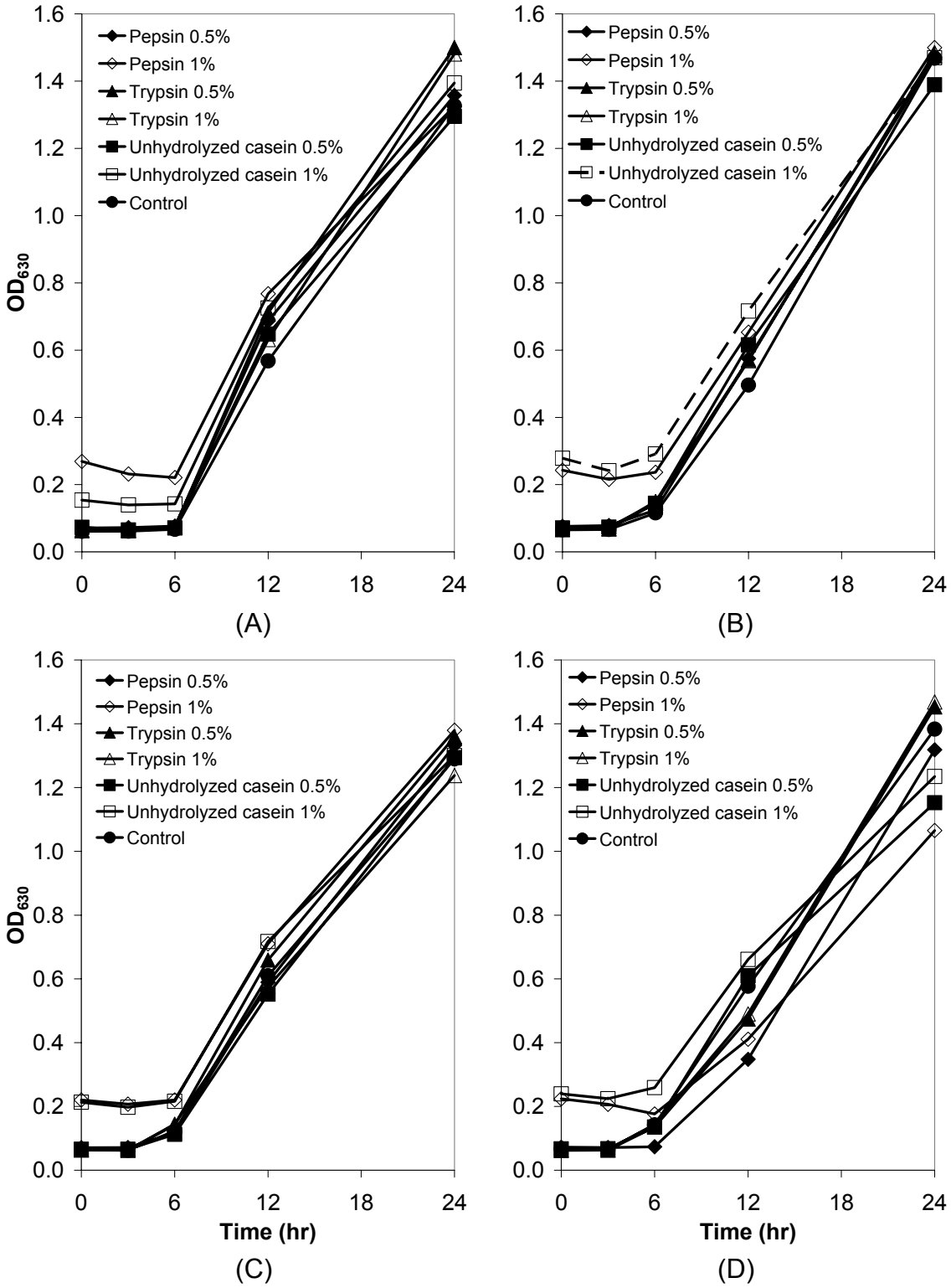


Figure 2. Antimicrobial activity of acid-precipitated casein digested with pepsin and trypsin, dialyzed and lyophilized at 0.5% and 1.0% (method 1) on *Salmonella* Typhimurium (A) 2380 (B) 2576 (C) 2582 and (D) 2486.

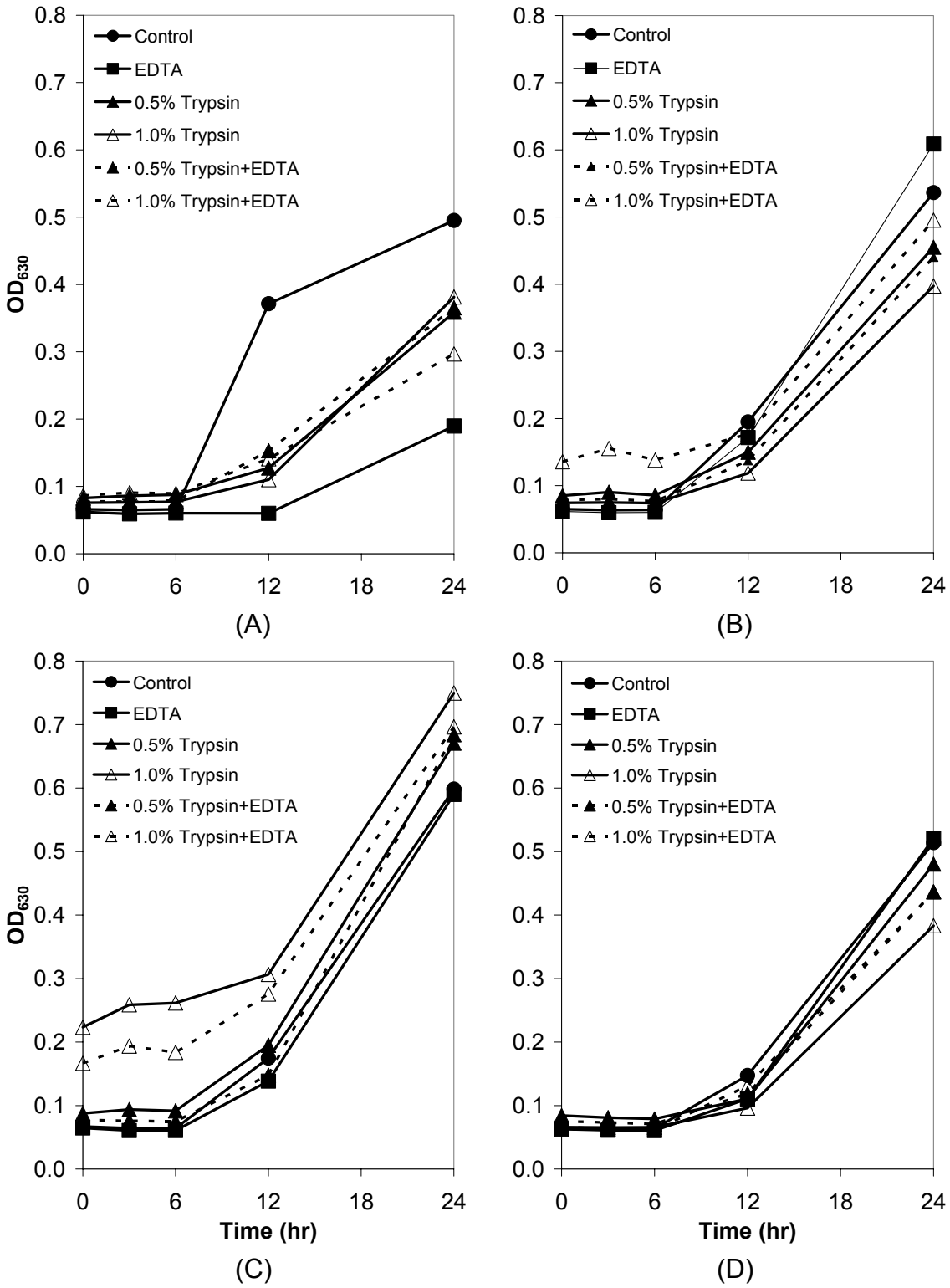


Figure 3. Antimicrobial activity of acid-precipitated casein digested with pepsin, dialyzed and lyophilized at 0.5% and 1.0% (method 1) alone and in combination with EDTA against *Listeria monocytogenes* (A) 101 (B) 108 (C) 310 and (D) Scott A.

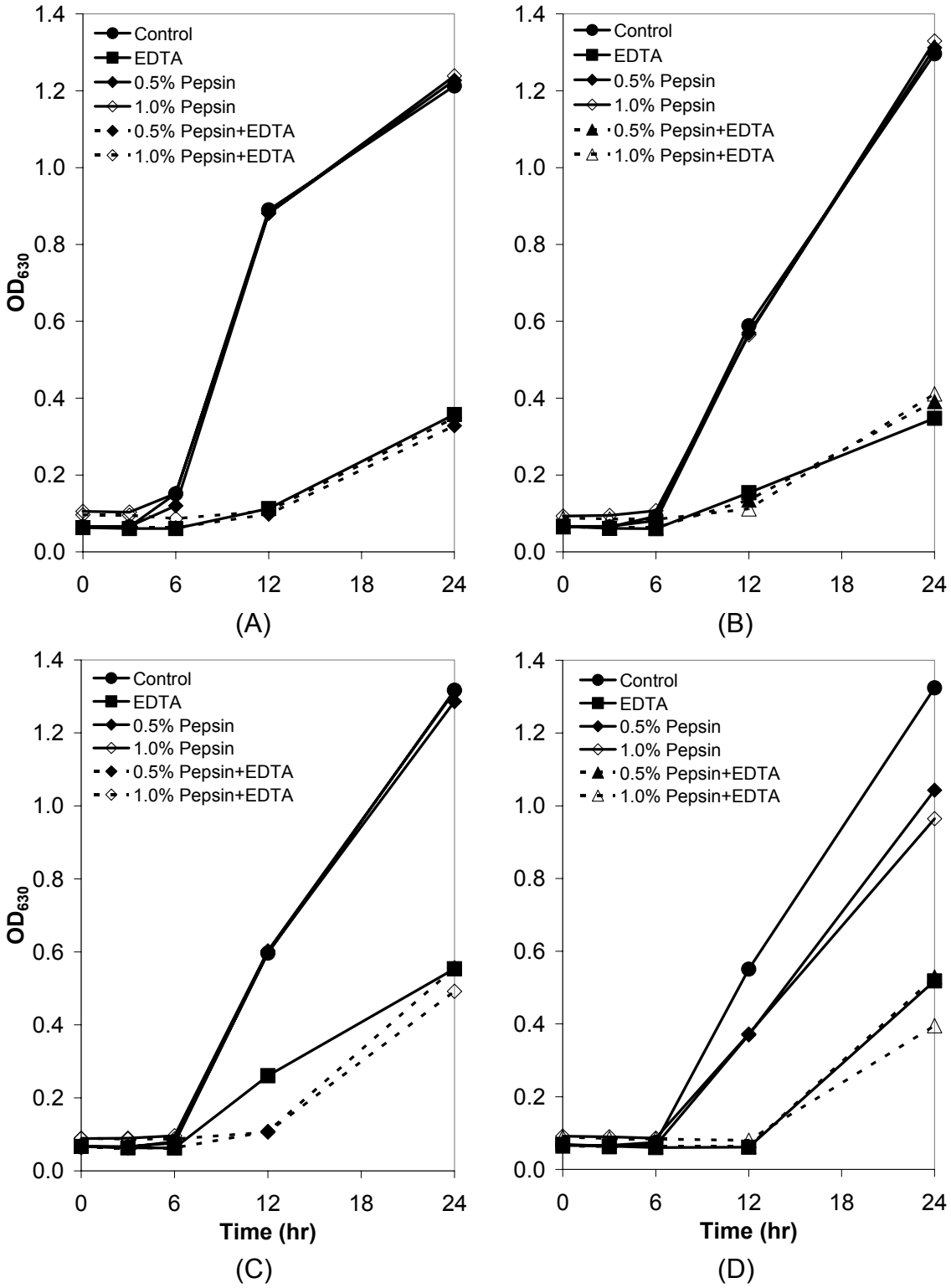


Figure 4. Antimicrobial activity of acid-precipitated casein, digested with trypsin, dialyzed, lyophilized at 0.5% and 1.0% (method 1) alone and in combination with EDTA against *Listeria monocytogenes* (A) 101 (B) 108 (C) 310 and (D) Scott A.

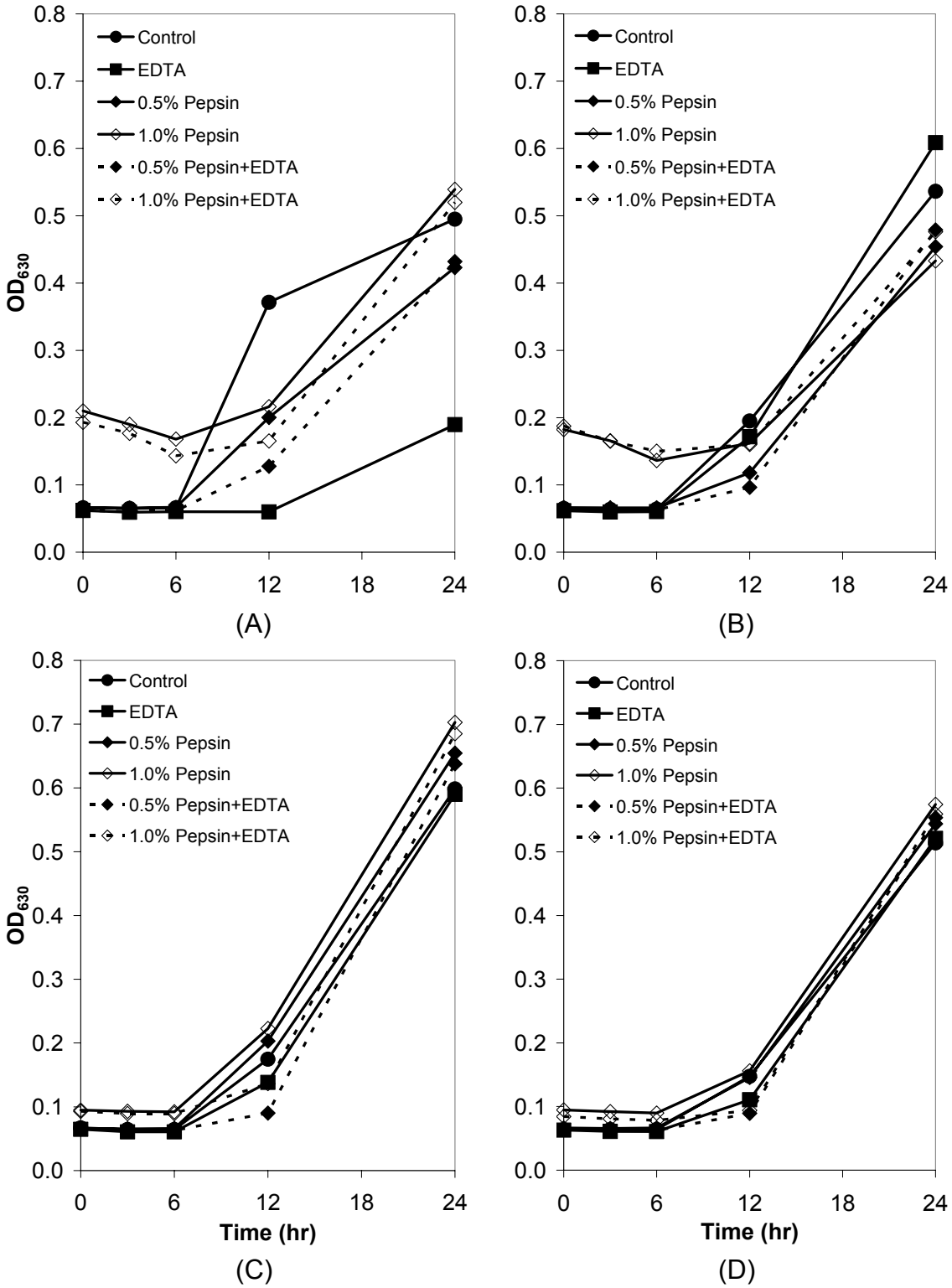


Figure 5. Antimicrobial activity of acid-precipitated casein digested with trypsin, dialyzed and lyophilized at 0.5% and 1.0% (method 1) alone and in combination with EDTA against *Salmonella* Typhimurium (A) 2380 (B) 2576 (C) 2582 and (D) 2486.

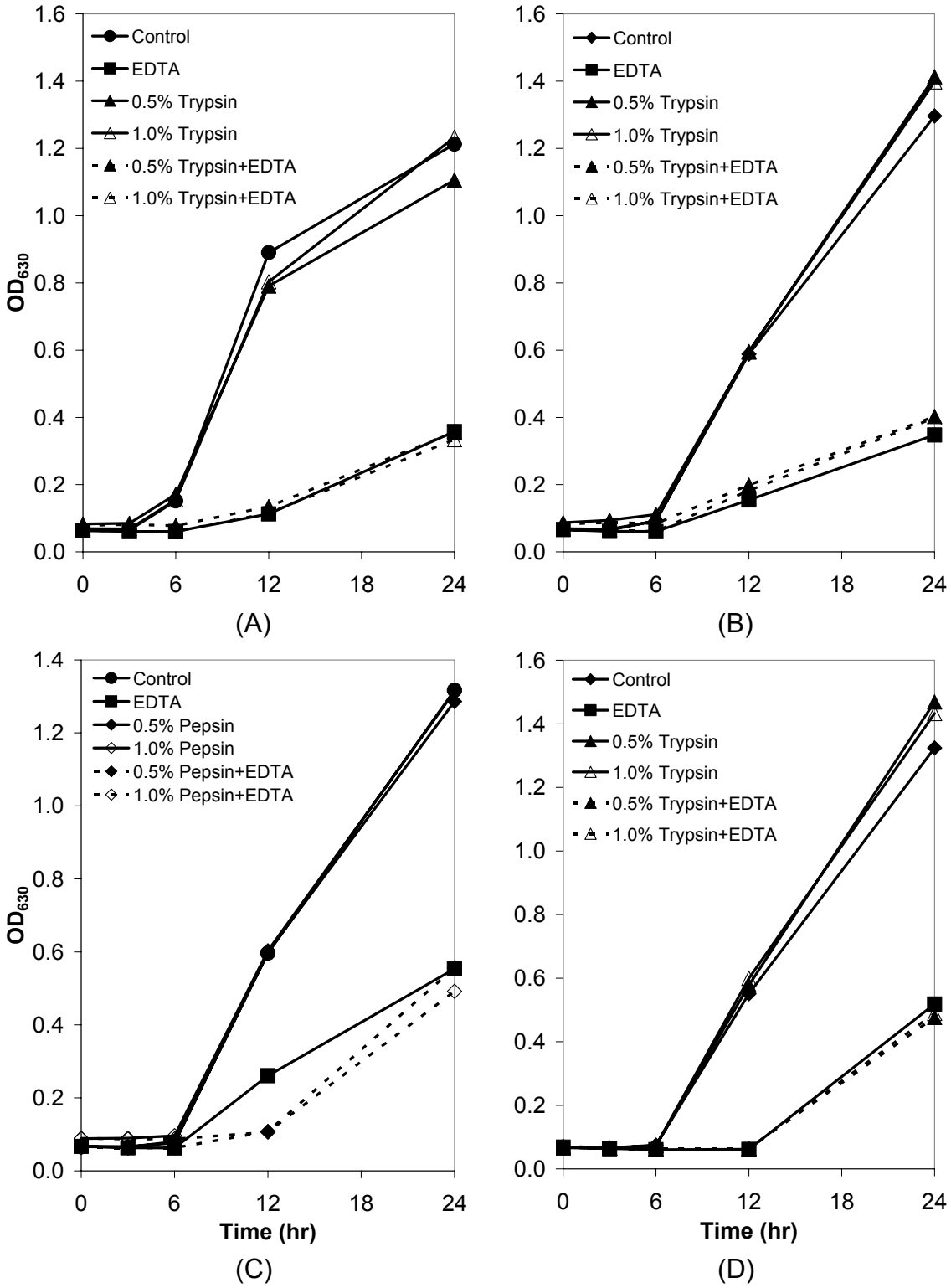
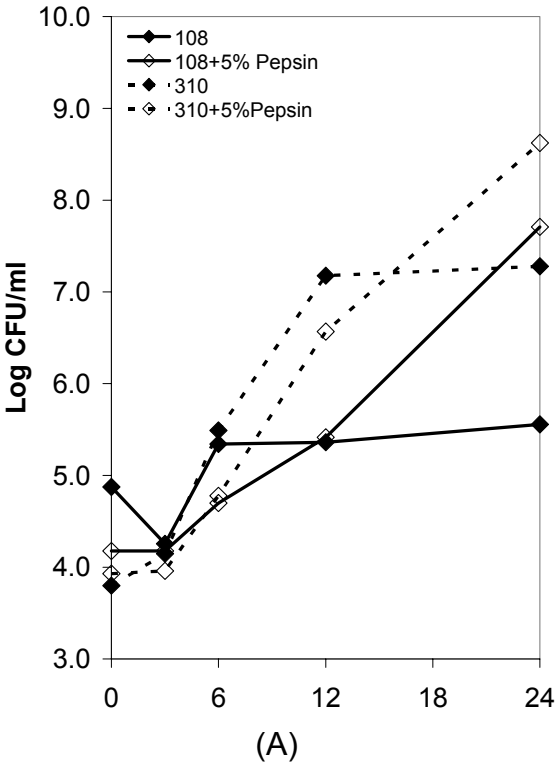
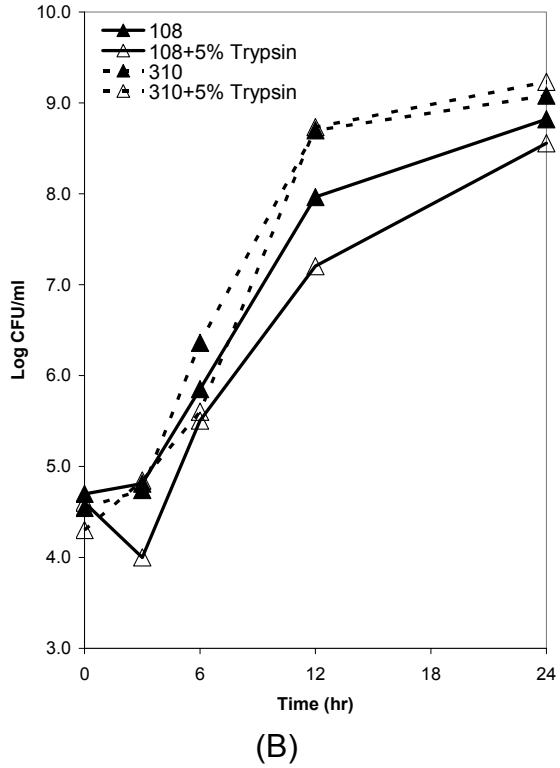


Figure 6. Antimicrobial activity of acid-precipitated casein, digested with pepsin, dialyzed, lyophilized at 0.5% and 1.0% (method 1) alone and in combination with EDTA against *Salmonella* Typhimurium (A) 2380 (B) 2576 (C) 2582 and (D) 2486.

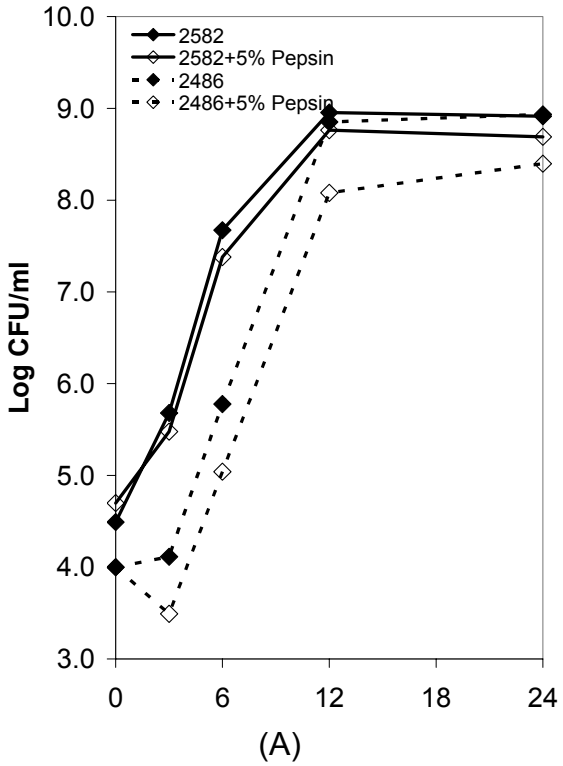


(A)

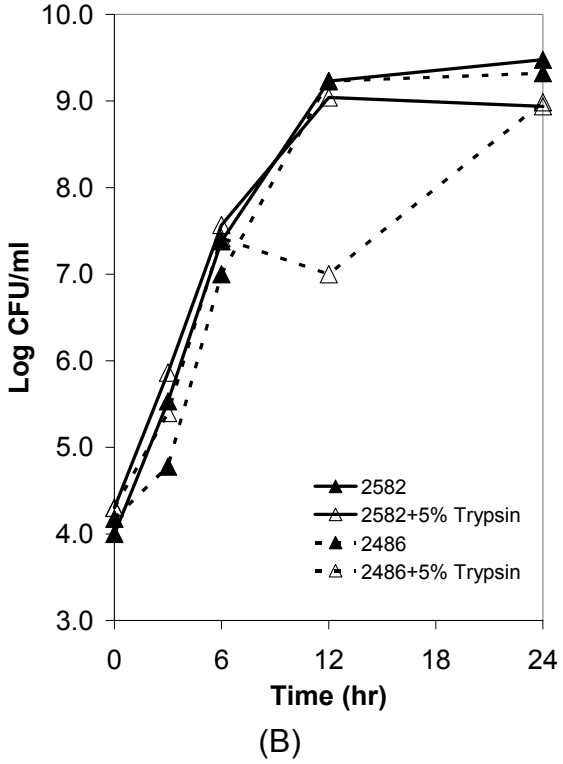


(B)

Figure 7. Antimicrobial activity of acid-precipitated casein digested with (A) pepsin (B) trypsin, dialyzed, lyophilized at 5% against *Listeria monocytogenes* 108 and 310.



(A)



(B)

Figure 8. Antimicrobial activity of acid-precipitated casein digested with (A) pepsin (B) trypsin, dialyzed, lyophilized at 5% against *Salmonella* Typhimurium 2582 and 2486.

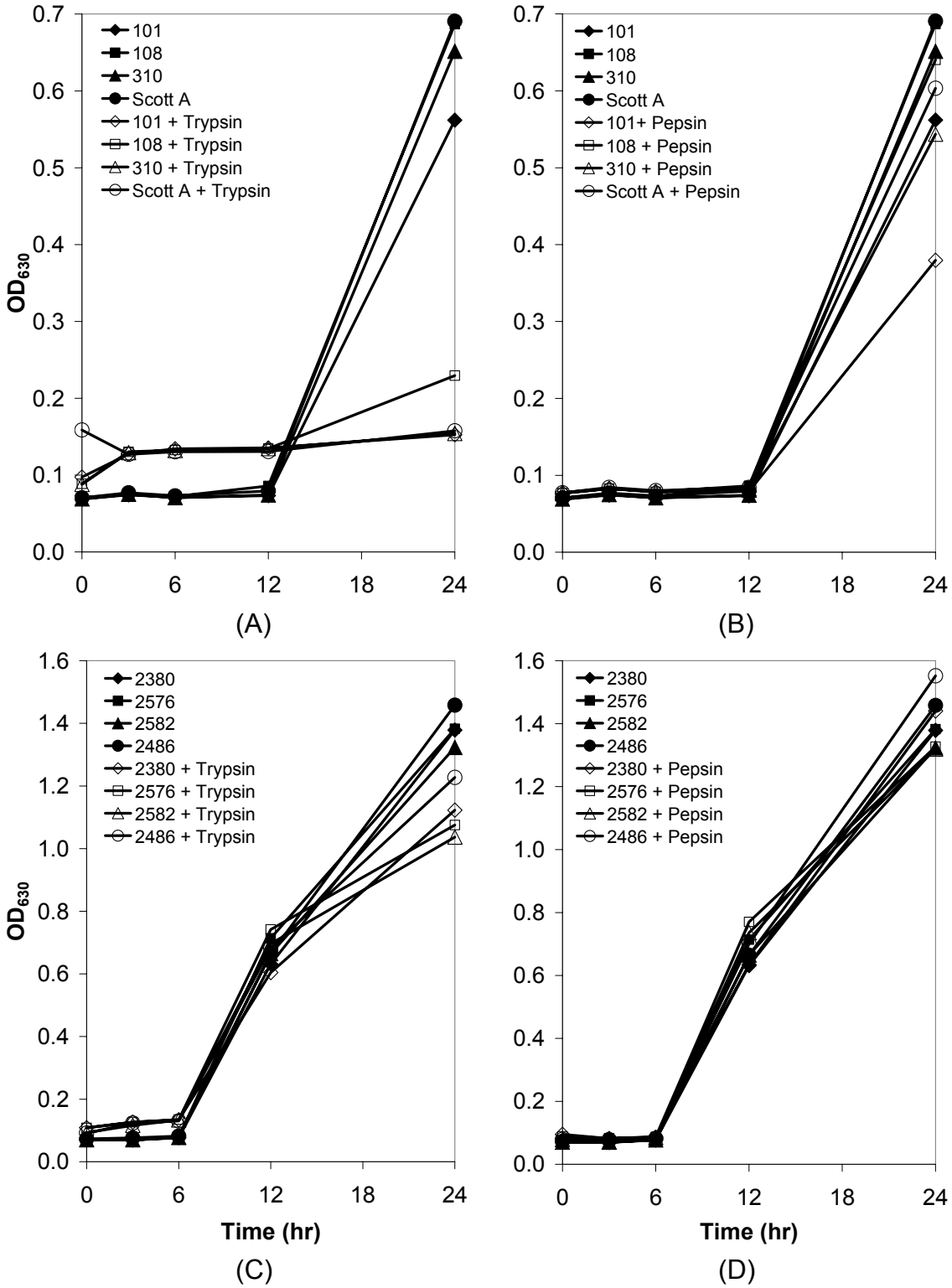


Figure 9. Antimicrobial activity of acid-precipitated casein digested with (A) trypsin (B) pepsin, centrifuged (method 2) on *Listeria monocytogenes* and acid-precipitated casein digested with (C) trypsin and (D) pepsin, centrifuged (method 2) on *Salmonella* Typhimurium.

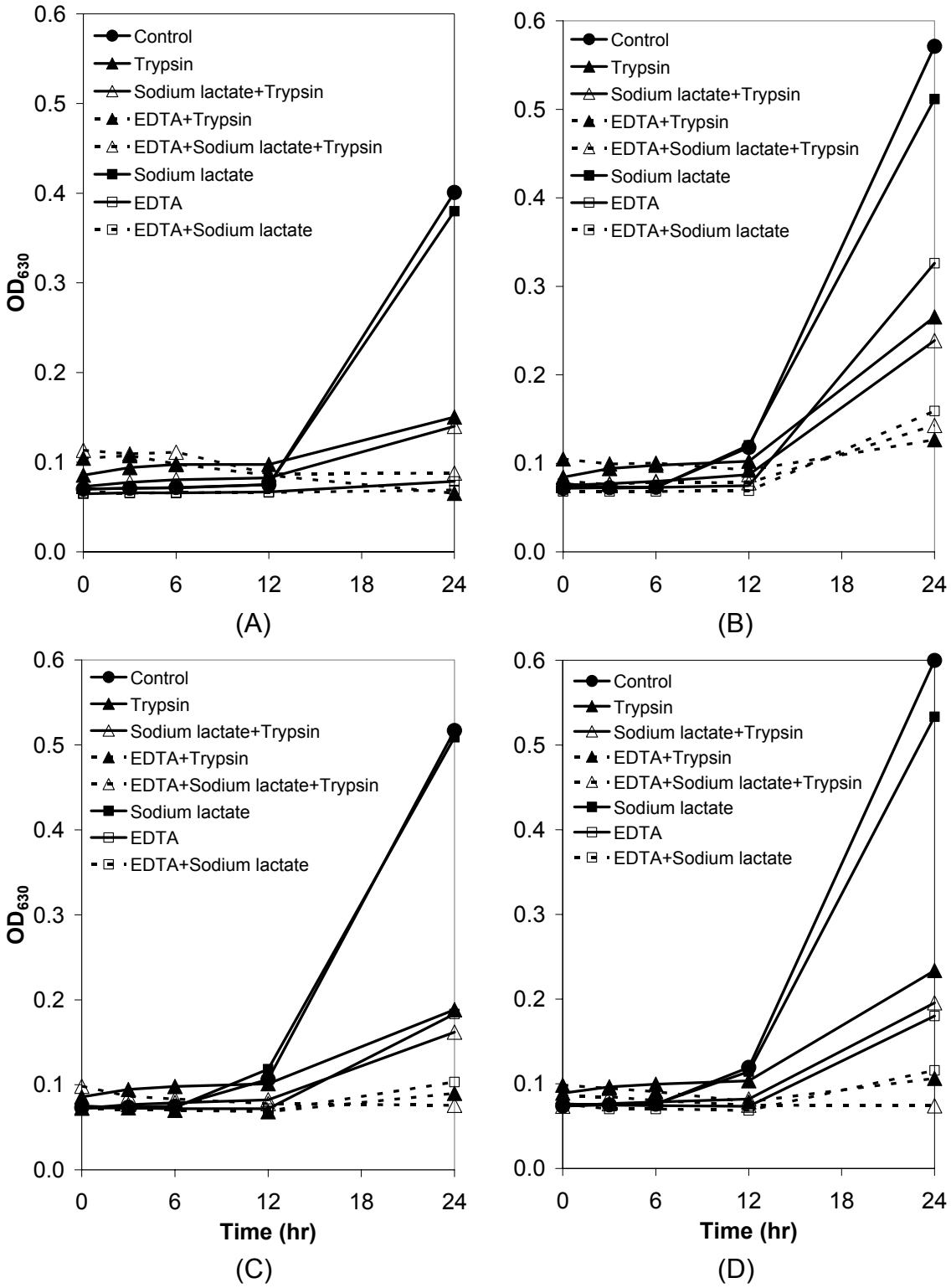


Figure 10. Antimicrobial activity of acid-precipitated casein digested with trypsin, centrifuged (method 2), and combined with EDTA and sodium lactate on *Listeria monocytogenes* (A) 101 (B) 108 (C) 310 and (D) Scott A.

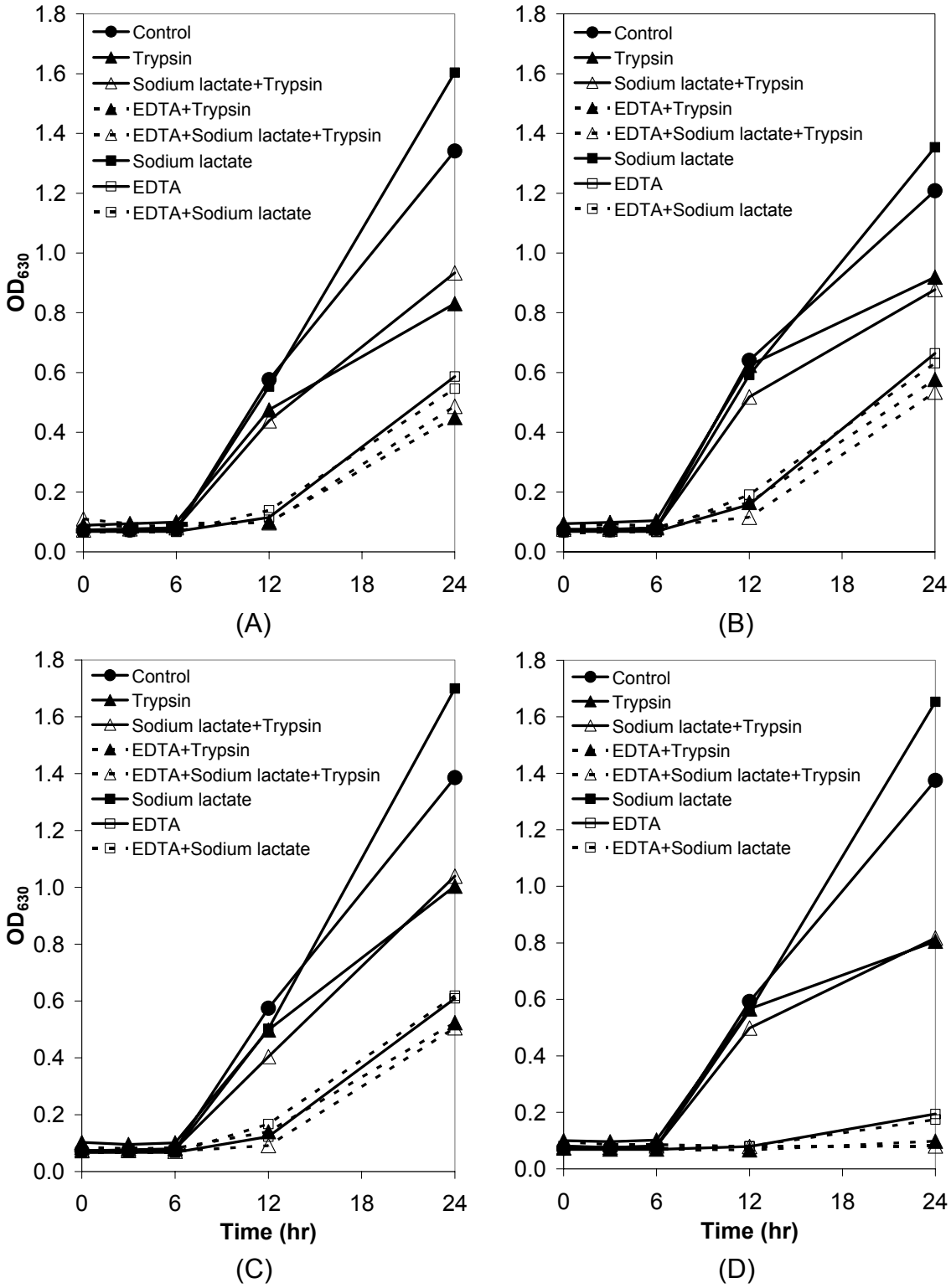


Figure 11. Antimicrobial activity of acid-precipitated casein digested with trypsin, centrifuged (method 2), and combined with EDTA and sodium lactate on *Salmonella* Typhimurium (A) 2380 (B) 2576 (C) 2582 and (D) 2486.

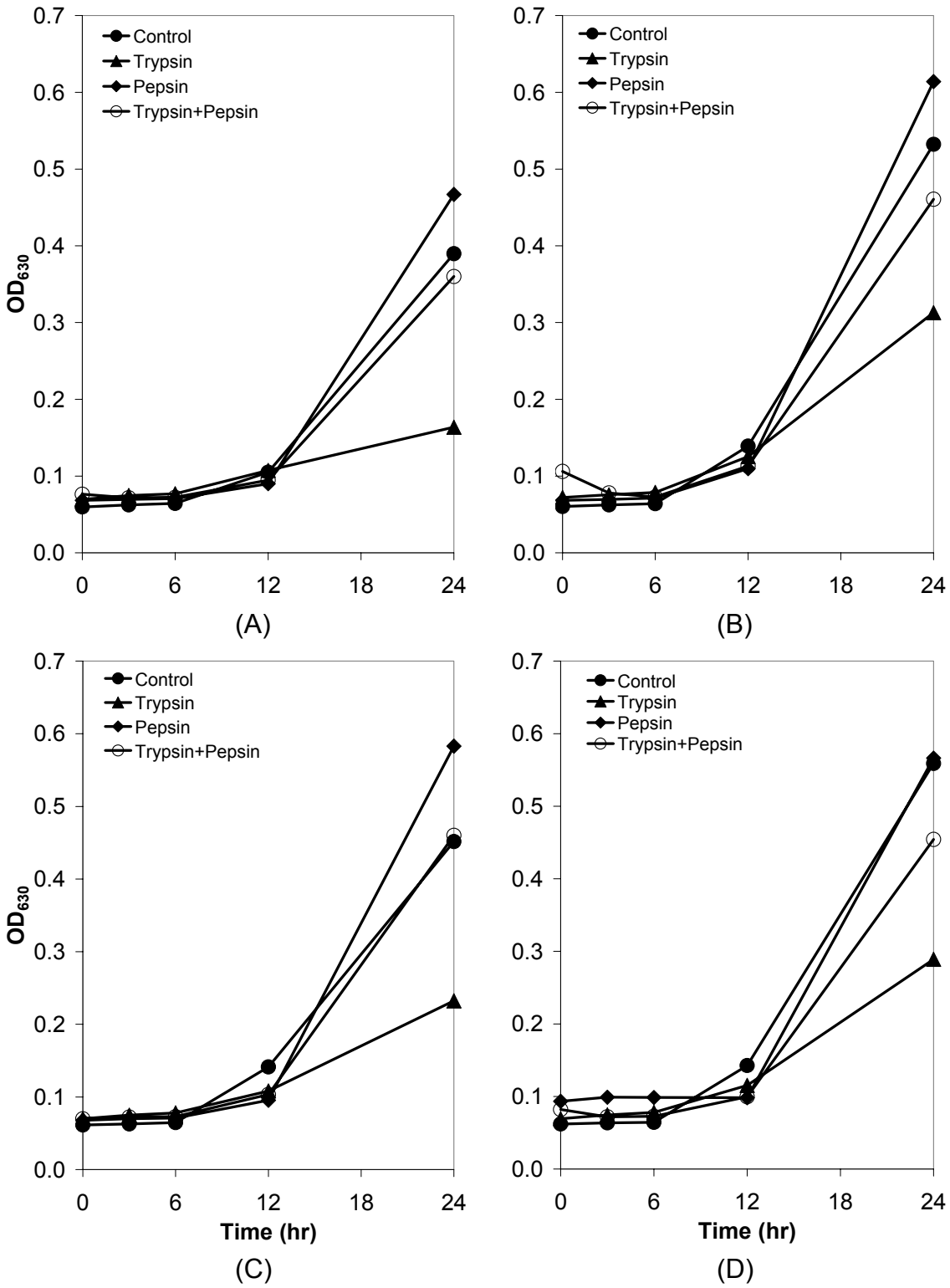


Figure 12. Antimicrobial activity of acid-precipitated casein digested with pepsin and trypsin, centrifuged (method 2) alone or in combination on *Listeria monoctyogenes* (A) 101 (B) 108 (C) 310 and (D) Scott A.

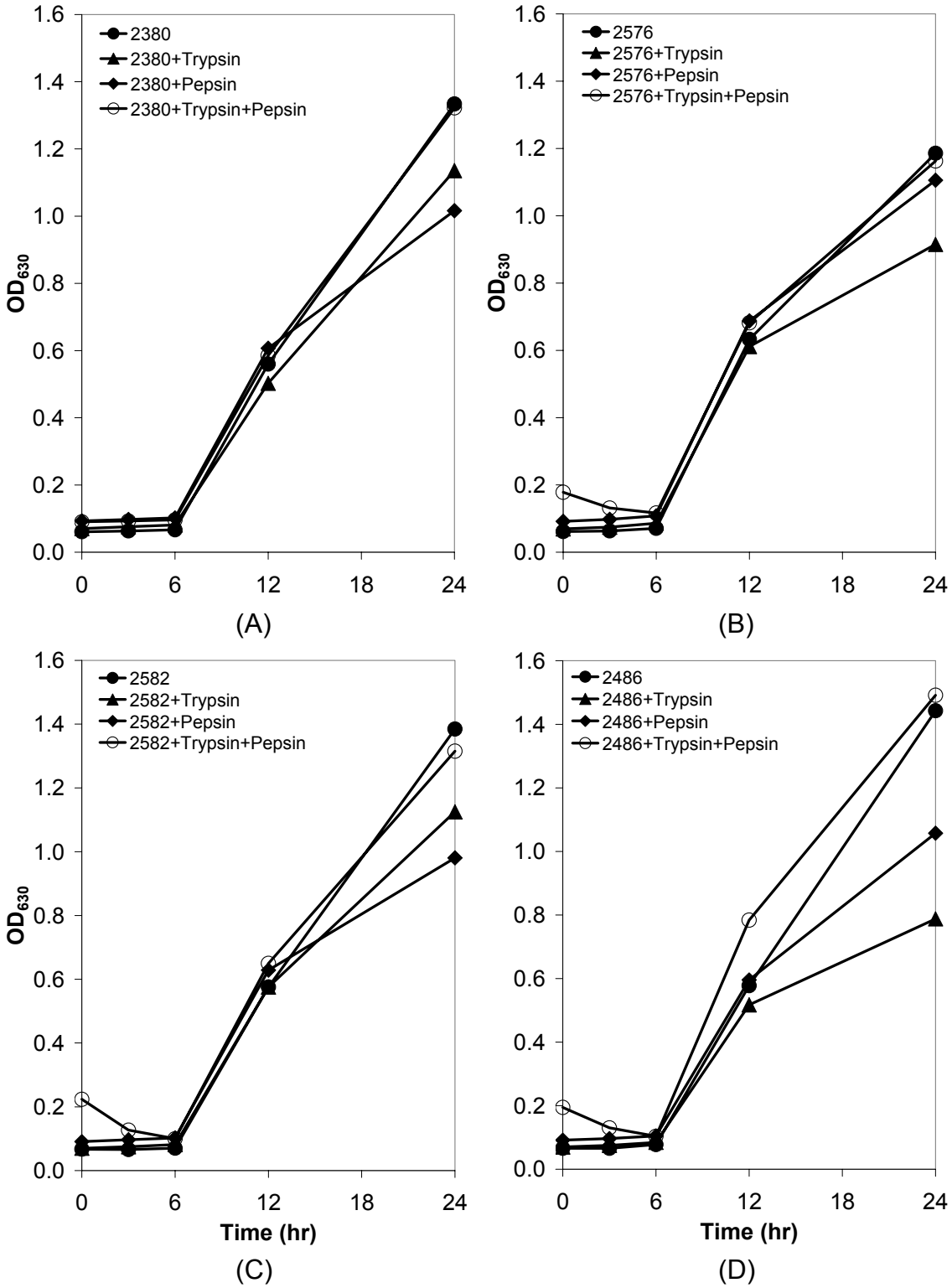


Figure 13. Antimicrobial activity of acid-precipitated casein digested with pepsin and trypsin, centrifuged (method 2) alone or in combination on *Salmonella* Typhimurium (A) 2380 (B) 2576 (C) 2582 and (D) 2486.

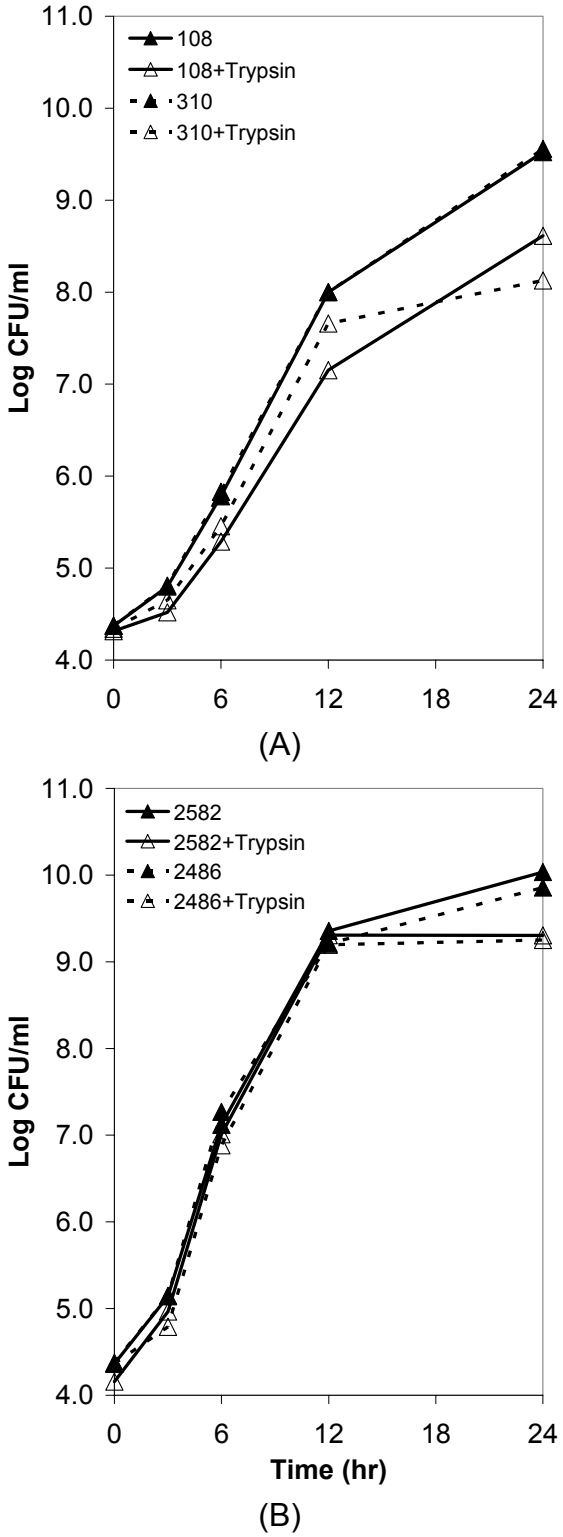
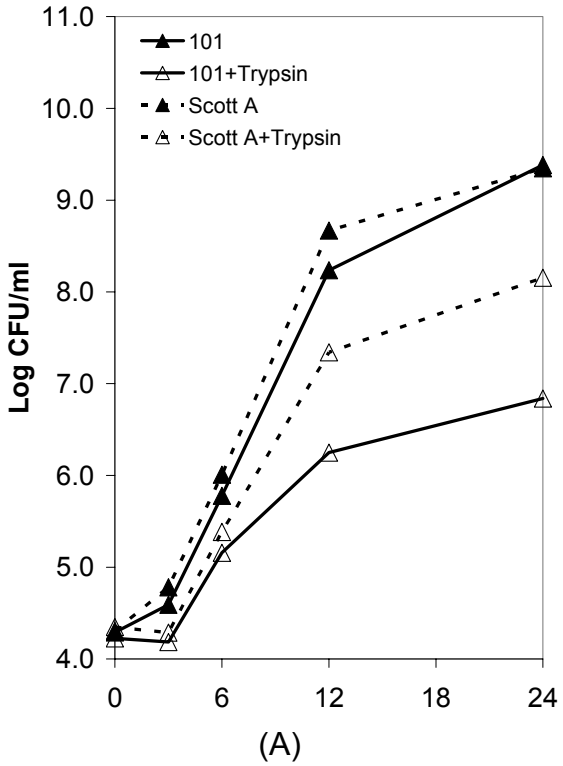
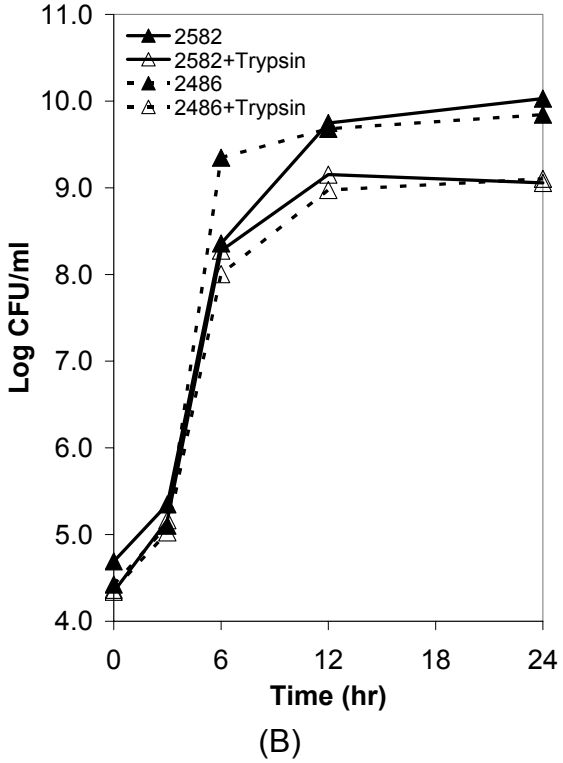


Figure 14. Antimicrobial activity of acid-precipitated casein digested with trypsin and centrifuged (method 2) on (A) *Listeria monocytogenes* 108 and 310 and (B) *Salmonella* Typhimurium 2582 and 2486.



(A)



(B)

Figure 15. Antimicrobial activity of acid-precipitated casein digested with trypsin and centrifuged (method 2) on (A) *Listeria monocytogenes* 101 and Scott A and (B) *Salmonella* Typhimurium 2582 and 2486.

Arg pro **Lys** his pro ile **Lys** his gln gly leu pro gln (glu val leu asn glu asn leu
(Absent in Varient A) 30 40
leu Arg phe phe val ala) pro phe pro gln val phe gly **Lys** glu **Lys** val asn glu leu
P P 50 ThrP in varient D 60
ser **Lys** asp ile gly ser glu ser thr glu asp gln ala met glu asp ile **Lys** glu met
P P P 70 P 80
glu ala glu ser ile ser ser ser glu glu ile val pro asn ser val glu gln **Lys** his
90 100
ile gln **Lys** glu asp val pro ser glu Arg tyr leu gly tyr leu glu gln leu leu Arg
110 P 120
leu **Lys** **Lys** tyr **Lys** val pro gln leu glu ile val pro asn ser ala glu glu Arg leu
130 140
his ser met **Lys** gln gly ile his ala gln gln **Lys** glu pro met gly val asn asn gln
150 160
glu leu ala typ phe tyr pro glu leu phe arg gln phe tyr gln leu asp ala tyr pro
170 180
ser gly ala trp tyr tyr val pro leu gly thr gln tyr thr asp ala pro ser phe ser
190 gly in varient C 199
asp ile pro asn pro ile gly ser glu asn ser glu **Lys** thr thre met pro leu trp OH

(A)

1 P P P 11 P
Lys Asn Thr Met Glu His Val Ser Ser Ser Glu Glu Ser Ile Ile Ser Gln Gln Thr Thr
21 31
Lys Glu Glu **Lys** Asn Met Ala Ile Asn Pro Ser **Lys** Glu Asn Leu Cys Ser Thr Phe Cys
41 51 P P P
Lys Glu Val Val Arg Asn Ala Asn Glu Glu Glu Tyr Ser Ile Gly Ser Ser Ser Glu Glu
P 62 71
Ser Ala Glu Val Ala Thr Glu Glu Val **Lys** Ile Thr Val Asp Asp **Lys** His Tyr Gln **Lys**
81 91
Ala Leu Asn Glu Ile Asn Gli Phr Typ Gln **Lys** Phe Pro Gln Tyr Leu Gln Tyr Lue Tyr
101 111
Gln Gly Pro Ile Val Leu Asn Pro Trp Asp Gln Val **Lys** Arg Asn Ala Val Pro Ile Thr
121 P P
Pro Thr Leu Asn Agr Glu Gln Lue Ser Thr Ser Glu Glu Asn Ser **Lys** **Lys** Thr Val Asp
141 P 151
Met Glu Ser Thr Glu Val Phe Thr **Lys** **Lys** Thr **Lys** Leu Thr Glu Glu Glu **Lys** Asn Arg
161 171
Leu Asn Phe Leu Lsu Lsy Ile Ser Gln Agr Thr Gln **Lys** Phe Ala Leu Pro Gln Tyr Leu
181 191
Lsy Thr Val Tyr Gln His Gln **Lys** Ala Met **Lys** Pro Trp Ile Gln Pro **Lys** Thr **Lys** Val
201 207
Ile Pro Tyr Val Arg Ttr Leu OH

(B)

Figure 16. Sequence of amino acids for (A) α_{s1}-casein (B) α_{s2}-casein indicating cleavage at the amino acids, lysine (bold) and arginine (underline), using trypsin.

Arg glu leu glu glu leu asn val pro gly glu ile val glu ser leu ser ser ser glu
In G 1 Casein, split here 30 P lys in varient E 40
glu ser ile thr Arg ile asn **Lys** **Lys** ile glu **Lys** phe gln ser glu glu gln gln gln
50 In varient C, lys 60
thr glu asp glu leu gln asp **Lys** ile his pro phe ala gln thr gln ser leu val tyr
In varients B, A1 & C his 70 80
pro phe pro gly pro ile pro asn ser leu pro gln asn ile pro pro leu thr gln pro
90 100
pro val val val pro pro phe leu gln pro glu val met **Lys** val ser **Lys** val **Lys** glu
In G 3 Casein, split here Split here in G 2 Casein 120
ala met ala pro **Lys** his **Lys** glu met pro phe pro **Lys** tyr pro val gln pro phe thr
arg in varient B 130 140
glu ser gln ser leu thr leu thr asp val glu asn leu his leu pro pro leu leu leu
150 160
gln ser trp met his gln pro his gln pro leu pro pro thr val met phe pro pro gln
170 180
ser val leu ser leu ser gln ser **Lys** val leu pro val pro glu **Lys** ala val pro tyr
190 200
pro gln Arg asp met pro ile gln ala phe leu leu tyr gln gln pro va; leu gly pro
209
val Arg gly asp met phe pro ile ile val OH
190 200
pro gln Arg asp met pro ile gln ala phe leu leu tyr gln gln pro va; leu gly pro

(A)

Glu Glu Gln Asn Gln Glu Gln Pro Ile Arg Cys Glu **Lys** Asp Glu Arg Phe Phe Ser Asp
21 31
Lys Ile Ala **Lys** Tyr Ile Pro Ile Gln Tyr Val Leu Ser Arg Tyr Pro Ser Tyr Gly Leu
41 1 51 11
Asn Tyr Tyr Gln Gln **Lys** Pro Val Ala Leu Ile Asn Asn Gln Phe Lue Pro Tyr Pro Tyr
61 61
Tyr Ala **Lys** Pro Ala Ala Val Arg Ser Pro Ala Gln Ile Leu Gln Trp Gln Val Leu Ser
81 81
Asp Thr Val Pro Ala **Lys** Ser Cys Gln Ala Gln Pro Thr Thr Met Ala Arg His Pro His
101 105 106 111
Pro His Leu Ser Phe Met Ala Ile Pro Pro **Lys** **Lys** Asn Gln Asp **Lys** Thr Glu Ile Pro
121 131 Ile Varient B
Thr Ile Asn Thr Ile Ala Ser Gly Glu Pro Thr Ser Thr Pro Thr Thr Glu Ala Val Glu
141 Varient B has Ala **P** 151
Ser Thr Val Ala Thr Leu Glu Asp Ser Pro Glu Val Ile Glu Ser Pro Pro Glu Ile Asn
161 169
Thr Val Gln Val Thr Ser Thr Ala Val

(B)

Figure 17. Sequence of amino acids for (A) β-casein (B) κ -casein indicating cleavage at the amino acids, lysine (bold) and arginine (underline), using trypsin.

arg pro lys his pro ile lys his gln gly leu pro gln (glu val leu asn glu asn leu
(Absent in Varient A) 30 40
leu arg **Phe** **Phe** val ala) pro **Phe** pro gln val **Phe** gly lys glu lys val asn glu leu
P P 50 ThrP in varient D 60
ser lys asp ile gly ser glu ser thr glu asp gln ala met glu asp ile lys glu met
P P P P 70 P 80
glu ala glu ser ile ser ser ser glu glu ile val pro asn ser val glu gln lys his
90 100
ile gln lys glu asp val pro ser glu arg *Tyr* leu gly *Tyr* leu glu gln leu leu arg
110 P 120
leu lys lys *Tyr* lys val pro gln leu glu ile val pro asn ser ala glu glu arg leu
130 140
his ser met lys gln gly ile his ala gln gln lys glu pro met gly val asn asn gln
150 160
glu leu ala typ **Phe** *Tyr* pro glu leu **Phe** arg gln **Phe** *Tyr* gln leu asp ala *Tyr* pro
170 180
ser gly ala Trp *Tyr* *Tyr* val pro leu gly thr gln *Tyr* thr asp ala pro ser **Phe** ser
190 gly in varient C 199
asp ile pro asn pro ile gly ser glu asn ser glu lys thr thre met pro leu Trp OH

(A)

1 P P P 11 P
Lys Asn Thr Met Glu His Val Ser Ser Ser Glu Glu Ser Ile Ile Ser Gln Gln Thr Thr
21 31
Lys Glu Glu Lys Asn Met Ala Ile Asn Pro Ser Lys Glu Asn Leu Cys Ser Thr **Phe** Cys
41 51 P P P
Lys Glu Val Val Arg Asn Ala Asn Glu Glu Glu *Tyr* Ser Ile Gly Ser Ser Ser Glu Glu
P 62 71
Ser Ala Glu Val Ala Thr Glu Glu Val Lys Ile Thr Val Asp Asp Lys His *Tyr* Gln Lys
81 91
Ala Leu Asn Glu Ile Asn Gli Phr Typ Gln Lys **Phe** Pro Gln *Tyr* Leu Gln *Tyr* Lue *Tyr*
101 111
Gln Gly Pro Ile Val Leu Asn Pro Trp Asp Gln Val Lys Arg Asn Ala Val Pro Ile Thr
121 P P
Pro Thr Leu Asn Agr Glu Gln Lue Ser Thr Ser Glu Glu Asn Ser Lys Lys Thr Val Asp
141 P 151
Met Glu Ser Thr Glu Val **Phe** Thr Lys Lys Thr Lys Leu Thr Glu Glu Glu Lys Asn Arg
161 171
Leu Asn **Phe** Leu Lsu Lsy Ile Ser Gln Agr Thr Gln Lys **Phe** Ala Leu Pro Gln *Tyr* Leu
181 191
Lsy Thr Val *Tyr* Gln His Gln Lys Ala Met Lys Pro Trp Ile Gln Pro Lys Thr Lys Val
201 207
Ile Pro *Tyr* Val Arg Ttr Leu OH

(B)

Figure 18. Sequence of amino acids for (A) α_{s1}-casein (B) α_{s2}-casein indicating cleavage at the amino acids, phenylalanine (bold), tyrosine (italics) and trytophan (underline) using pepsin.

arg glu leu glu glu leu asn val pro gly glu ile val glu ser leu ser ser ser glu

In G 1 Casein, split here 30 P lys in varient E 40

glu ser ile thr arg ile asn lys lys ile glu lys **Phe** gln ser glu glu gln gln gln

50 In varient C, lys 60

thr glu asp glu leu gln asp lys ile his pro **Phe** ala gln thr gln ser leu val *Tyr*

In varients B, A1 & C his 70 80

pro **Phe** pro gly pro ile pro asn ser leu pro gln asn ile pro pro leu thr gln pro

90 100

pro val val val pro pro **Phe** leu gln pro glu val met lys val ser lys val lys glu

In G 3 Casein, split here Split here in G 2 Casein 120

ala met ala pro lys his lys glu met pro **Phe** pro lys *Tyr* pro val gln pro **Phe** thr

arg in varient B 130 140

glu ser gln ser leu thr leu thr asp val glu asn leu his leu pro pro leu leu leu

150 160

gln ser Trp met his gln pro his gln pro leu pro pro thr val met **Phe** pro pro gln

170 180

ser val leu ser leu ser gln ser lys val leu pro val pro glu lys ala val pro *Tyr*

190 200

pro gln arg asp met pro ile gln ala **Phe** leu leu *Tyr* gln gln pro va; leu gly pro

209

val arg gly pro **Phe** pro ile ile val OH

(A)

1 11

Glu Glu Gln Asn Gln Glu Gln Pro Ile Arg Cys Glu Lys Asp Glu Arg **Phe** **Phe** Ser Asp

21 31

Lys Ile Ala Lys *Tyr* Ile Pro Ile Gln *Tyr* Val Leu Ser Arg *Tyr* Pro Ser *Tyr* Gly Leu

41 51

Asn *Tyr* *Tyr* Gln Gln Lys Pro Val Ala Leu Ile Asn Asn Gln **Phe** Lue Pro *Tyr* Pro *Tyr*

61 71

Tyr Ala Lys Pro Ala Ala Val Arg Ser Pro Ala Gln Ile Leu Gln Trp Gln Val Leu Ser

81 91

Asp Thr Val Pro Ala Lys Ser Cys Gln Ala Gln Pro Thr Thr Met Ala Arg His Pro His

101 105 106 111

Pro His Leu Ser **Phe** Met Ala Ile Pro Pro Lys Lys Asn Gln Asp Lys Thr Glu Ile Pro

121 131 Ile Varient B

Thr Ile Asn Thr Ile Ala Ser Gly Glu Pro Thr Ser Thr Pro Thr Thr Glu Ala Val Glu

141 Varient B has Ala **P** 151

Ser Thr Val Ala Thr Leu Glu Asp Ser Pro Glu Val Ile Glu Ser Pro Pro Glu Ile Asn

161 169

Thr Val Gln Val Thr Ser Thr Ala Val

(B)

Figure 19. Sequence of amino acids for (A) β-casein (B) κ-casein indicating cleavage at the amino acids, phenylalanine (bold), tyrosine (italics) and trytophan (underline) using pepsin.

Vita

Kellie Parks Burris was born on September 5, 1978 in Morganton, NC to Tom and Gail Parks. She attended Burke County Public Schools and graduated with honors from Freedom High School in 1996. Kellie attended North Carolina State University and graduated *Cum Laude* with two Bachelors' of Science Degrees in Animal Science and Poultry Science in May 2000 and *Magna Cum Laude* with a Bachelor's of Science Degree in Food Science with a minor in nutrition in May 2001. In August 2002, 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. P.M. Davidson. Kellie graduated with her Masters of Science degree in December of 2004. She plans to continue her education by pursuing another Master's degree at the University of Tennessee in the field of Plant Sciences.