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Bactericidal Effects of Natural Tenderizing Enzymes on *Escherichia Coli* and *Listeria monocytogenes*

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Abstract

The objective of this study was to determine the antimicrobial activity of proteolytic, meat-tenderizing enzymes (papain and bromelain) against *E. coli* and *L. monocytogenes* at three different temperatures (5, 25 and 35°C). Two overnight cultures of *E. coli* JM109 and *L. monocytogenes* were separately suspended in 1% peptone water and exposed to a proteolytic enzyme (papain or bromelain) at three different temperatures. Bromelain concentrations (4 mg/ml) and (1 mg/ml) tested at 25°C against *E. coli* and *L. monocytogenes,* respectively, were the most effective concentrations tested reducing populations by 3.37 and 5.7 log CFU/ml after 48 h, respectively. Papain levels of (0.0625 mg/ml) and (0.5 mg/ml) were the most effective concentration tested at 25°C against *E. coli* and *L. monocytogenes,* respectively, reducing populations by 4.94 and 6.58log CFU/ml after 48h, respectively. Interestingly, the lower papain concentration tested (0.0625 mg/ml) was more effective than the higher concentration (0.5 mg/ml) against *E. coli* at all three temperatures. As expected, the temperature was directly related to enzyme efficacy against both *E. coli* and *L. monocytogenes.*

Keywords: proteolytic enzymes, bromelain, papain, meat tenderizing, *Escherichia coli*, *Listeria monocytogenes*

1. Introduction

Consumer acceptance or rejection for cut or processed meat after initial purchase is strongly influenced by tenderness. Meat tenderness is related to structural integrity of myofibrillar and connective tissues proteins (Marsh et al., 1966; Nishimura et al., 1995). Many studies have investigated methods to improve tenderness and overall meat quality using different tenderizing methods including: chemical tenderization of meat with enzymes, salts, or calcium chloride, and physical tenderization by pressure treatments, blade tenderization or electrical stimulation (Ketnawa & Rawdkuen, 2011). Pathogenic bacteria are also a serious concern for consumers in further processed meat products. Gudbjomsdottir et al. (2004) reported the incidence of *Listeria monocytogenes* in meat processing plants was between 0 and 15% and in poultry plants was 20.6 to 24.1%. A majority of food product recalls associated with *L. monocytogenes* contamination involve RTE meat and poultry products (USDA-FSIS, 2005). Lee et al. (2009) reported 9.1% of beef, poultry and pork raw samples contained *E. coli* with 39 pathogenic isolates found among these isolates.

Plant proteolytic enzymes have also received attention in the field of medicine and biotechnology due to their proteolytic properties including papain from papaya (*Carica papaya*), bromelain from pineapple (*Ananas comosus*) and ficin from figs (*Ficus spp.*) (Ketnawa et al., 2010; Bhardwaj et al., 2012). These enzymes have been widely used in the food, medical–pharmaceutical, cosmetic and other industries. In the food industry, the primary application has been for meat tenderization.About 95% of tenderizing enzymes used for meat in the United States are from plant proteases. This marked tenderizing effect is due to the strong proteolytic activity of these enzymes (Amid et al., 2011).

Bromelain is mixture of proteolytic enzymes, many of which are cysteine proteases derived from the pineapple plant (*Ananas comosus*), which is a member of *Bromeliaceae* family (Hale et al., 2005). In the United States, bromelain is sold in health food stores as a nutritional supplement to promote digestive health and as an anti-inflammatory drug (Borrelli et al., 2011). Bromelain also has demonstrated antimicrobial effects including

antihelminthic activity against gastrointestinal nematodes, anti-candida effects, and can resolve infectious skin diseases such as pityriasis lichenoides chronica (Bromelain Monograph, 2010). Corzo et al. (2012) reported that optimum pH and temperature conditions for proteolytic activity of bromelain are in range of pH 6.5-7.5 and 50-60°C, respectively. Lopez-Garcia et al. (2006) reported that bromelain could be used as an alternative to chemical fungicides against Fusarium spp. plant pathogens. Salampessy et al. (2006) isolated antimicrobial peptides produced through bromelain hydrolysis of raw food.

Papain is another important plant peptidase derived from the latex of unripe papaya fruit (*Carica papaya*, *Caricaceae*) useful as a meat tenderizer due to its powerful proteolytic activity. Papain is characterized by its ability to hydrolyze large proteins into smaller peptides and amino acids. Its ability to break down tough fibers has been used for many years in the US as a natural tenderizing agent and is included as a component in meat tenderizers (Llerena-Suster, 2011).

Anibijuwon and Udeze (2009) concluded that *Carica papaya* maybe used for treatment of gastroenteritis, urethritis and wound infections. They also concluded that antimicrobial activity against both gram-negative and gram-positive bacteria is an indication that the *Carica papaya* is a potential source for production of a broad-spectrum bactericide. Moreover, Emeruwa (1982) supported that *Carica papaya* fruit extract had antibacterial activity against both gram-positive and gram-negative bacteria like *E. coli* and *S. aureus*. He also suggested that the site of action of the antibacterial was at cell wall since the cell morphology appeared to be changed. Raw papain extract was mixed with hydroxy methyl cellulose at a 1:2 ratio and tested against *Enterococcus faecalis* as a debriding gel for dentistry and showed 68% inhibition.

Ming et al. (2002) reported that optimum pH and temperature conditions for proteolytic activity of papain are in range of pH 6.0-7.0 and 65-80°C respectively. While (Anibijuwon & Udeze, 2009) said that the increase in temperature enhances the activity, whereas alkaline pH decreases the activity of papain. Meat consumption is increasing around the world and there are concerns related to the meat quality (tenderness) and meat. Meat tenderness can be addressed in different ways and meat hygiene concerns are mostly of a biological nature and include bacterial pathogens, such as *Escherichia coli* O157:H7, Salmonella and Campylobacter in raw meat and poultry, and *Listeria monocytogenes* in ready-to-eat processed products (Sofos et al., 2010). Since proteolytic enzymes are used in meat marinades and as meat tenderizers and these natural enzymes have displayed antimicrobial activity, they may have use in reducing pathogen risk in meat. Tests against common meat pathogens at temperatures used to hold and store meat seem appropriate. Therefore, the objective of this study was to examine two proteolytic enzymes (bromelain and papain) for antimicrobial activity against *E.coli* and *L.monocytogenes* when held at different temperatures (5, 25 and 35°C).

2. Method

2.1 Inoculum Preparation

Ampicillin-resistant *E.coli JM* 109 was preserved by freezing the culture at -70°C in vials containing tryptic soy broth (Becto™ Tryptic Soy Broth, Becton Dickinson and company Sparks, MD 21152 USA) supplemented with 20% (v/v) glycerol (Sigma, St. Louis, MO). To propagate the culture, a frozen vial was thawed at room temperature, and 0.1 ml of the thawed culture was transferred to 9.9 ml of Enrichment TSB with 0.5% ampicillin (DIFCO) in screw-capped tubes and incubated aerobically for 16-18h at 37°C with shaking (Thermolyne Maxi-Mix III type 65800, Barnstead/ Thermolyne, Dubuque, IA). The inoculum was prepared from a second transfer of this culture (0.1 ml) to another 9.9 ml tube of Enrichment TSB (DIFCO), and incubated aerobically for 16-18h at 37°C with shaking. After overnight incubation, washed cells were harvested by centrifugation at 1107 × g (IEC HN-SII Centrifuge, International Equipment CO., Inc., Needham Heights, MA), the pellet resuspended in sterile peptone water 0.1% (w/v) (Bacto peptone, Becton Dickinson) to obtain a population of approximately 8-9 log CFU/ml. One ml of the suspension was transferred into 99 ml of sterile peptone water 0.1% (w/v) to obtain a final population of approximately 5-6 log CFU/ml. Initial cell populations were verified by enumeration of the cells following pour-plating in TSA with 0.5% ampicillin (DIFCO™ Tryptic Soy Agar, Becton Dickinson and company Sparks, MD 21152 USA) and incubating at 37°C for 24 h. The same procedure was followed with *Listeria monocytogenes* (ATCC 15313) grown in Listeria broth (DIFCO™ Listeria Enrichment broth, Becton Dickinson and Company Sparks, MD 21152 USA).

2.2 Preparation of Enzyme Concentrations

The concentrations of bromelain (B4882-25G, sigma) used with *L.monocytogenes* were 0 mg/ml, 0.25 mg/ml, 0.375 mg/ml and 1.0 mg/ml while for *E. coli*, 0 mg/ml, 1.0 mg/ml, 2.0 mg/ml and 4.0 mg/ml were used based on preliminary experiments. These concentrations were prepared by mixing appropriate amount of the peptone solution, enzyme stock solution and bacterial solution. The same procedure was followed with papain

(P4762-500MG) using different concentrations. For example, concentrations of papain with *E.coli* and *L. monocytogenes* were 0 mg/ml, 0.0625 mg/ml, 0.125 mg/ml, 0.25 mg/ml, and 0.5 mg/ml.

Enzyme and peptone solution of the different concentrations were mixed for 30 sec. until a homogenized solution was achieved. At t = 0 h the bacteria was added to the different mixtures and finally transferred to sterile petri dishes and placed on an orbit shaker at 40 rpm (Model 3520 Orbit shaker, Lab-Line Instruments, Inc) at different temperatures 5, 25, 35°C.

2.3 Sampling Time

At t = 0, 2, 4, 8, 24, and 48 h, 0.1 ml of each enzyme concentration were serially diluted and appropriate serial dilutions were surface plated on enrichment agar, Listeria agar (DIFCO Detroit, MI) for *L. monocytogenes* and TSA (DIFCO, Detroit, MI) for *E. coli*, in duplicate.

The inoculated plates were incubated (Model 2300 CO_2 incubator, VWR Scientific Product) at 37°C for 48 h for *L. monocytogenes* and 24 h for *E. coli* and dilution plates with 25-250 colonies were counted (LEICA, QUEBEC DARK FIELD colony counter, Buffalo, NY 14240 USA model 3325) and populations were reported a CFU/ml and log CFU/ml.

2.4 Statistical Analysis

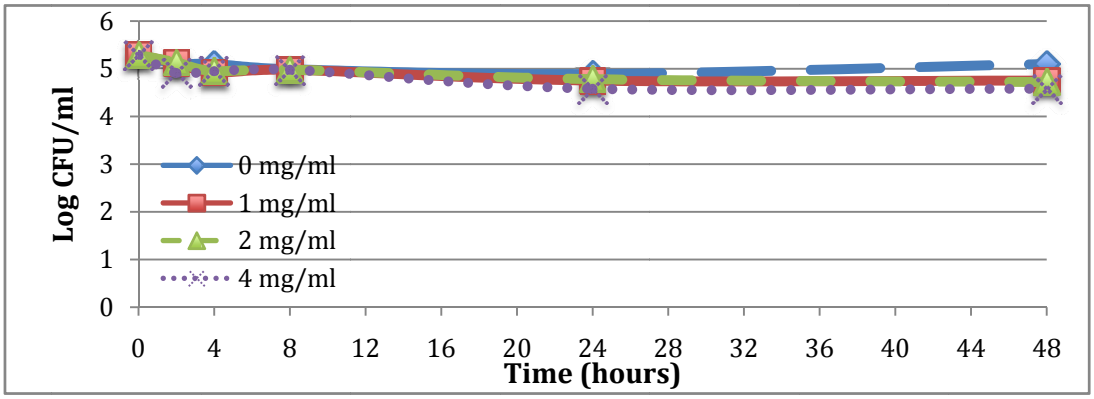
The experiment was conducted as a repeated measures split-plot experimental design. The response variable was logarithmic function of the colony forming units (log CFU) per ml. The whole-plot treatment factor was enzyme concentration and sub-plot treatment factor was temperature. Measurements were repeated over time (0, 2, 4, 8, 24 and 48 h) the covariance matrix was modeled using spatial power law that is a generalization of the first-order autoregressive covariance structure. The PROC MIXED procedure from SAS® was used to analyze the data and the Tukey multiple comparison procedure was for mean separation. All comparisons were made using $\alpha = 0.05$.

3. Results

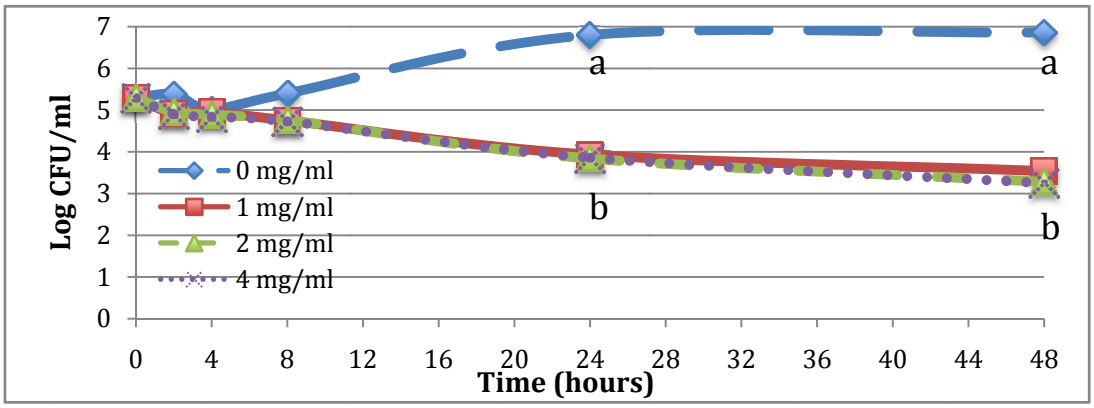
3.1 Bromelain

3.1.1 Effect of Bromelain on *E. coli*

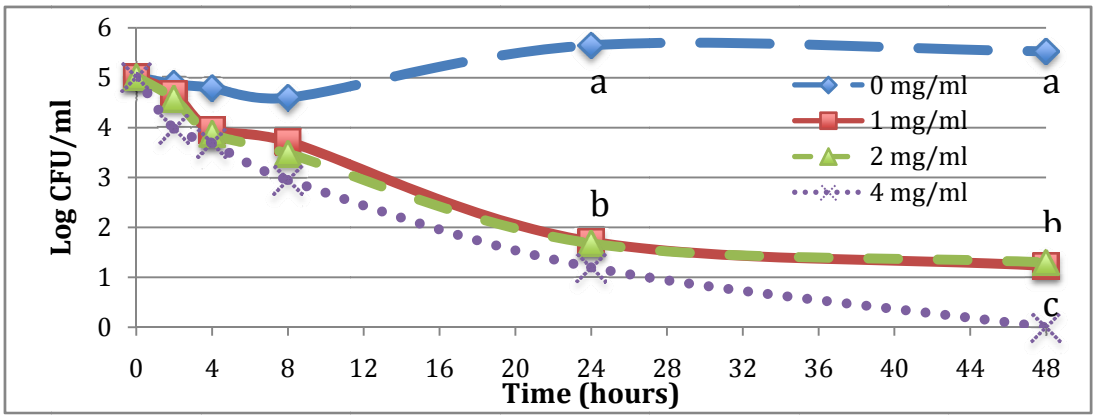
Bromelain was tested at concentrations from 1 to 4 mg/ml at 5, 25, and 35°C and was effective at all concentrations in reducing bacterial populations after 24 and 48 h compared to no added bromelain ($P \leq 0.0001$) (Figure 1). However, there was not a difference ($P > 0.05$) in *E. coli* populations among samples exposed to bromelain concentrations of 1, 2 or 4 mg/ml at 5 and 25°C. At 48 h, a bromelain concentration of 4mg/ml was the most efficient on *E. coli* reducing the log CFU/ml population by 5.5 ($P < 0.0001$). Similar results were observed by Sparso and Moller (2002) who added bromelain to soy protein films to inhibit *E. coli*. The exact mechanism by which bromelain inhibits the growth of *E. coli* is not completely understood but could be related to compromise of the Gram-negative outer membrane which also contains proteins. These surface proteins may be digestible by bromelain, weakening the cell wall to allow leakage, swelling of the cell and finally cell fracture.



1a. Bromelain against *E. coli* at 5°C



1b. Bromelain against *E. coli* at 25°C



1c. Bromelain against *E. coli* at 35°C

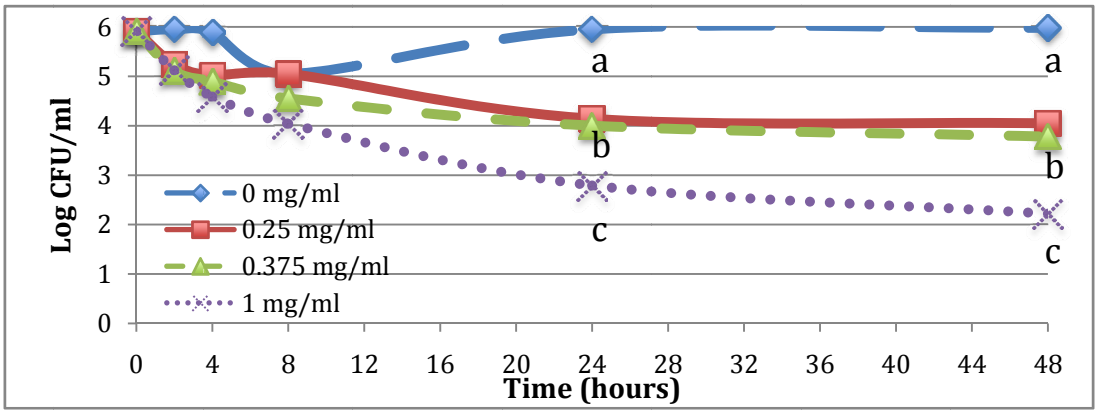
Figure 1. Effect of bromelain on *E. coli* at 5, 25 and 35°C over 48 hours

a, b, c data points or groups of points with the same superscripts are not significantly different ($p > 0.05$). Standard error of the mean = 0.18.

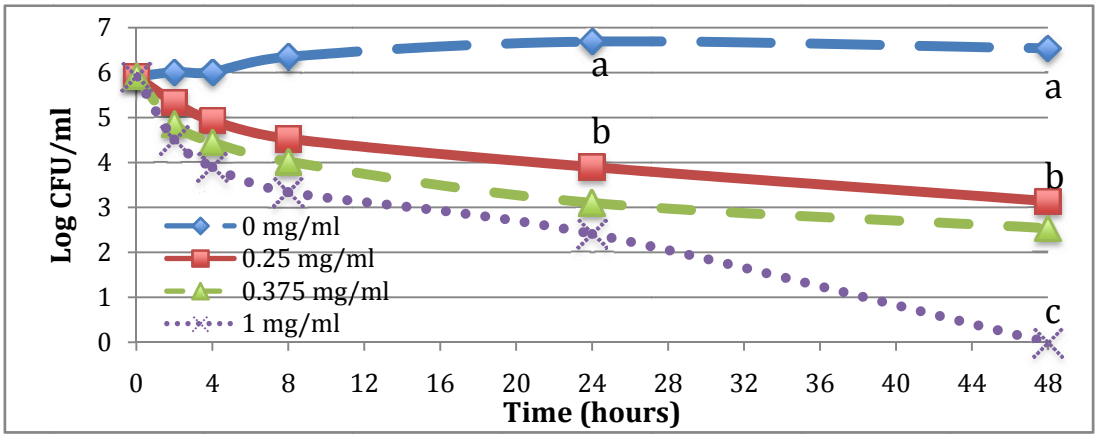
3.1.2 Effect of Bromelain on *L. monocytogenes*

Bromelain reduced *L. monocytogenes* populations after 4 h for all 3 temperatures tested ($P < 0.0001$) (Figure 2) however, there was not a difference in populations at concentrations of 0.25 and 0.375 mg/ml after 4 h. After 2 and 4 h at 35°C, the 1 mg/ml bromelain level reduced *L. monocytogenes* by 3 log cycles which was significantly greater than the other concentrations tested. After 8 h, *L. monocytogenes* population exposed to 1mg/ml was significantly lower compared to bacteria exposed to concentrations of 0.25 and 0.375 mg/ml at all temperatures tested. This finding contradicts Sparso and Moller (2002) who concluded that bromelain is more efficient against

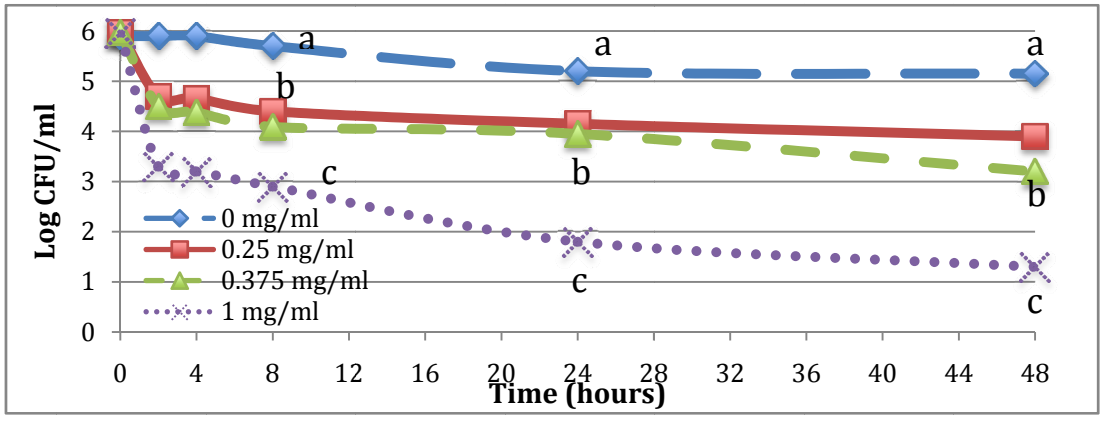
Gram-negative than Gram-positive bacteria. Overall, bromelain at the 1 mg/ml level was more effective in reducing *L. monocytogenes* populations than all other levels tested ($P < 0.0001$).



2a. Bromelain against *L. monocytogenes* at 5°C



2b. Bromelain against *L. monocytogenes* at 25°C



2c. Bromelain against *L. monocytogenes* at 35°C

Figure 2. Effect of bromelain on *L.monocytogenes* at at 5, 25 and 35°C over 48 h

a, b, cdata points or groups of points with the same superscripts are not significantly different ($p > 0.05$). Standard error of the mean = 0.24.

3.1.3 Effect of Temperature on Bromelain Efficiency

The 48 hours exposure time was used as a comparison point for the temperature effects of the enzymes on bacteria. As temperature increased from 5 to 35°C, the efficacy of bromelain to reduce both *E. coli* and *L. monocytogenes* increased (Figures 3 and 4). The optimum temperature conditions for proteolytic activity of

bromelain are in range of 50-60°C. (Corzo et al., 2012) thus as this optimum temperature was approached the greater activity yielded greater cell destruction. At 5°C, bromelain had no effect on *E. coli* populations after 24 hours at all concentrations tested. However at 25°C, bromelain concentrations were reduced at least 3 log cycles after 24 h at all concentrations. At 25°C, *E. coli* populations were reduced by 5 log cycles using 1 mg/ml and by 6 logs (below detection) at 4 mg/ml. The effect of increasing temperature and increasing activity against *E. coli* by bromelain was not observed against L. monocytogenes (Figure 4). Bromelain reduced *L. monocytogenes* by 1 log cycle at 0.25 and 0.4 mg/ml and by 3 logs at 4.0 mg/ml after 24 hours at 5°C. These reductions were only 1-1.5 logs greater at 25 and 35°C, respectively after 24 hours for bromelain against *L. monocytogenes*.

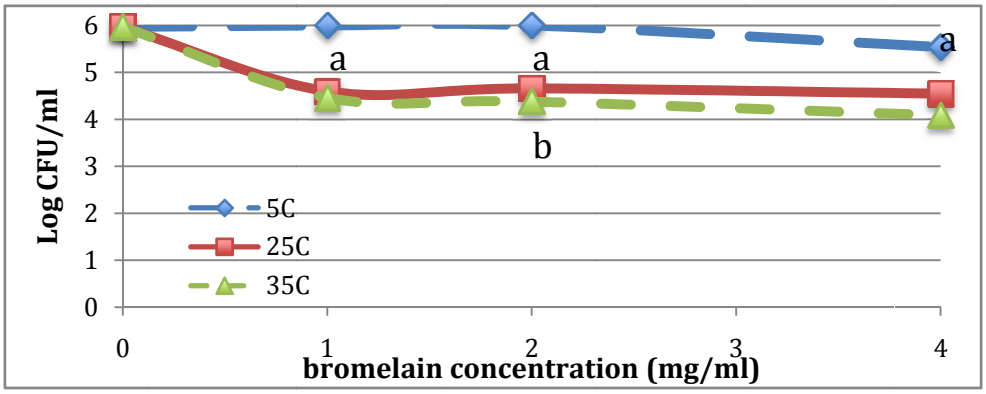


Figure 3. Effect of temperature on bromelain efficiency against on *E. coli* after 48h

a, b, c data points or groups of points with the same superscripts are not significantly different ($p > 0.05$). Standard error of the mean = 0.25.

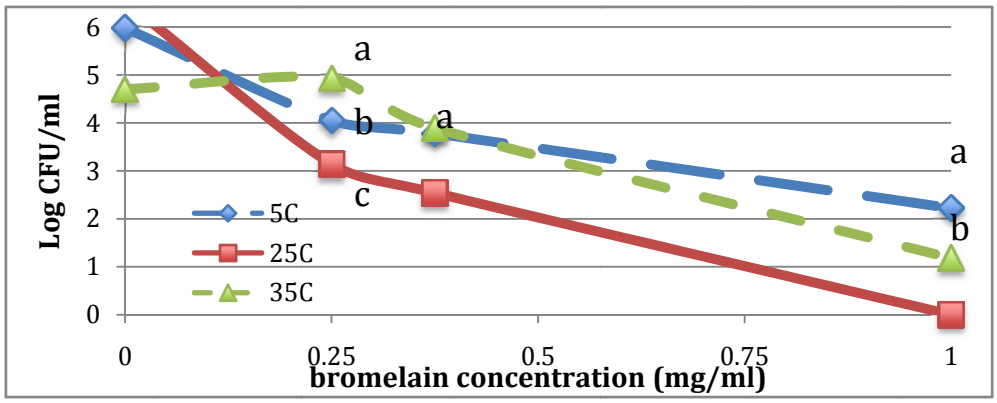


Figure 4. Effect of temperature on bromelain efficiency against *L. monocytogenes* after 48h

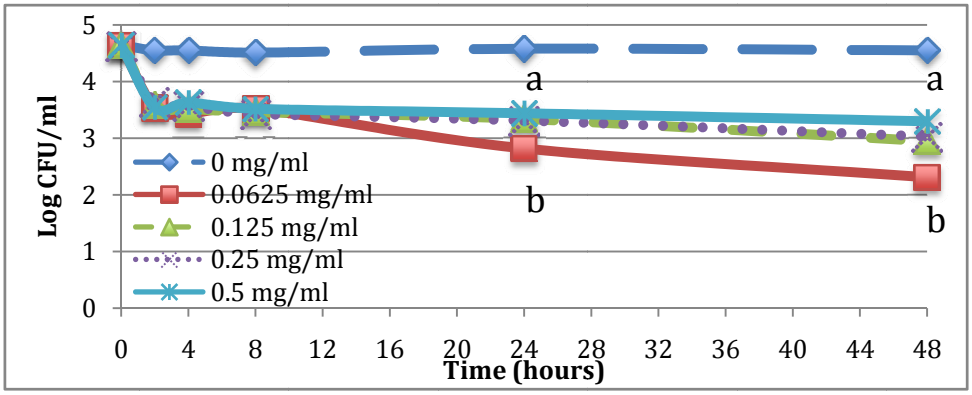
a, b, c data points or groups of points with the same superscripts are not significantly different ($p > 0.05$). Standard error of the mean = 0.24.

3.2 Papain

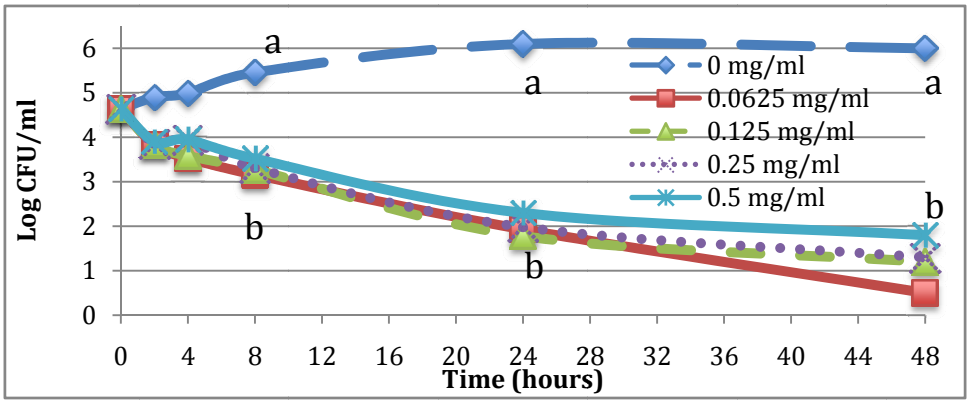
3.2.1 Effect of Papain on *E. coli*

Papain was tested in concentrations from 0.0625 to 0.5 mg/ml at 5, 25 and 35°C with all concentration reducing *E. coli* populations significantly compered to the control (no papain). At room temperature (25°C), the lower papain concentration of 0.0625 mg/ml was the most efficient on *E. coli* (Figure 6b). It reduced the log of CFU/ml by 4.94 ($P < 0.0001$), while the higher papain concentration of 0.5 mg/ml was the less effective. At 5°C the activity of papain against *E. coli* was reduced only yielding a 1-log reduction compared to the control except for the lowest concentration (0.0625 mg/ml) that showed nearly a 2.5 log reduction after 48 h. The most significant reduction in *E. coli* population with papain was at 35°C where all concentration reduced populations below detection after 48 h while the 0.0625 mg/ml level achieved this in 24 hours while other concentrations

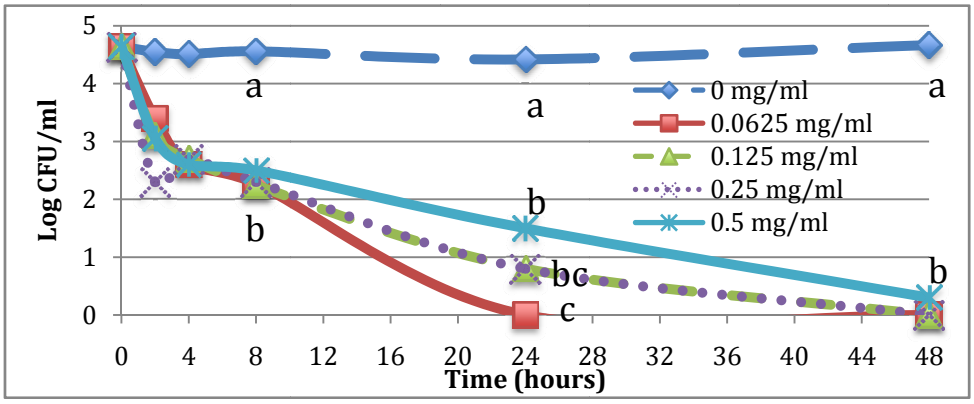
lagged behind this rate. Papain did not have any effect on *E. coli* at 35°C (Figure 5) until 4 h and the lower papain concentration (0.0625 mg/ml) was most effective. The antibacterial effect of papain found in this study is similar to those of Sparso and Moller (2002) where *L. plantarum* was significantly reduced by lower concentrations of papain. They explained that the relatively high concentration of protein in enzymes might inhibit or even destroy the enzyme because of the proteolytic properties.



5a. Papain against *E. coli* at 5°C



5b. Papain against *E. coli* at 25°C



5.c. Papain against *E. coli* at 35°C

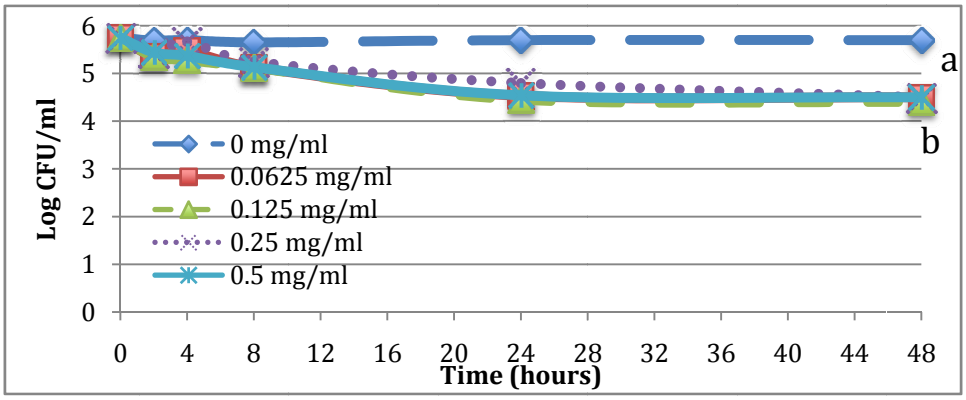
Figure 5. Effect of papain on *E. coli* at 5, 25 and 35°C

a, b, c data points or groups of points with the same superscripts are not significantly different ($p > 0.05$). Standard error of the mean = 0.46.

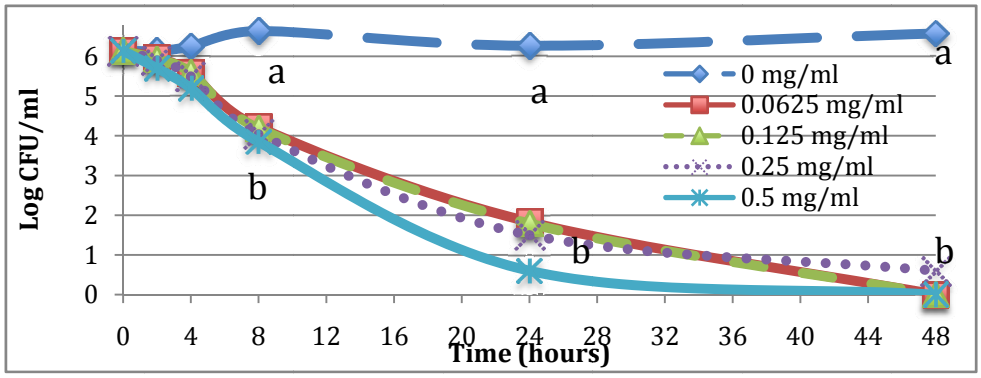
3.2.2 Effects of Papain on *L. monocytogenes*

At 5°C papain slowly reduced *L. monocytogenes* populations at all concentrations tested yielding about a 2-log

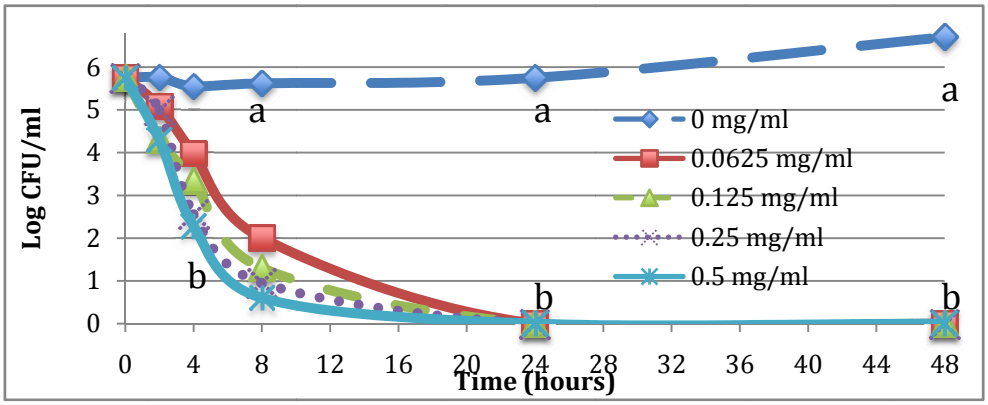
cycle reduction after 48 h compared to controls (Figure 6a). Papain concentrations of 0.0625 to 0.5 mg/ml at room temperature (25°C) reduced *L. monocytogenes* population by 2 log cycles after 8 h and 4 to 6 logs after 24 and 48 h, respectively, (Figure 6b) compared to the control 0mg/ml, which increased in population (log 0.44 CFU/ml) after 48 h. At 35°C, the rate of *L. monocytogenes* inactivation was greatest reaching a 4.5- to 5-log reduction in 8 h and complete elimination of detectable cells by 24 h. All papain concentrations were equally effective in reducing *L. monocytogenes* with the 0.5 mg/ml level significantly reducing bacterial numbers after 24 h at 5°C and 8 h at 35°C compared to the 0.0625 mg/ml concentration. The antibacterial effect of papain would be similar to bromelain and was theorized to inhibit bacterial cell wall synthesis or general protein synthesis (Osato et al., 1993).



6a. Papain against *L. monocytognes* at 5°C



6b. Papain against *L. monocytogenes* at 25°C



6c. Papain against *L. monocytogenes* at 35°C

Figure 6. Effect of papain on *L.monocytogenes* at 5, 25, and 35°C

a, b, c data points or groups of points with the same superscripts are not significantly different ($p > 0.05$). Standard error of the mean = 0.26.

3.2.3 Effect of Temperature on Papain Efficiency

There was no difference in the effect of papain concentration (0.0625, 0.125, 0.25 and 0.5 mg/ml) on *E. coli* population after 24 h at 5°C (Figure 7). However, all papain concentrations reduced *L. monocytogenes* population after 24 h at 5°C compared to controls. At 25°C, there was at least a 3-log reduction of *E. coli* and a 4-log reduction of *L. monocytogenes* at all concentrations tested compared to controls (Figure 8). The 24-hour reduction in E. coli held at 25°C and 35°C did not differ among the concentrations tested. However for *L. monocytogenes*, the 25°C was about 1-log higher in populations after 24 hours than the 35°C treatment at each concentration.

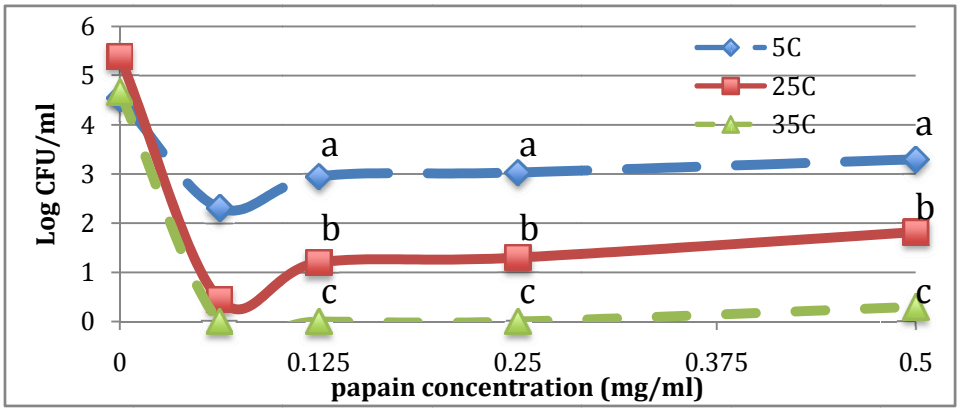


Figure 7. Temperature effect of papain on *E. coli* after 48 h

a, b, c data points or groups of points with the same superscripts are not significantly different ($p > 0.05$). Standard error of the mean = 0.23.

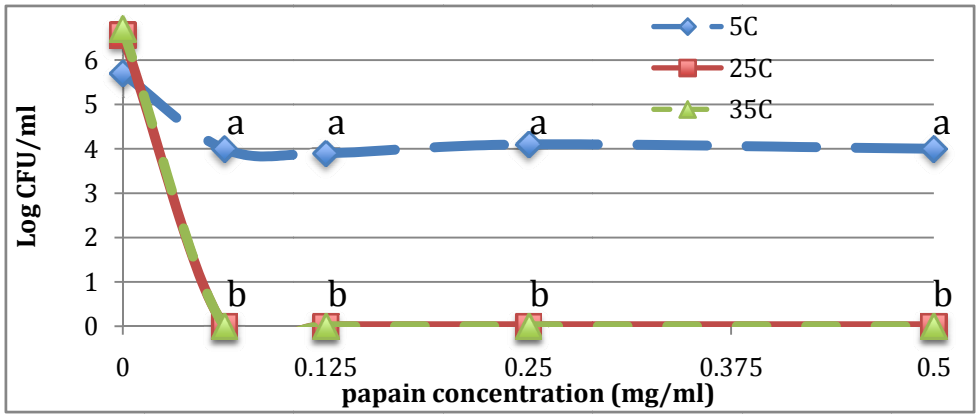


Figure 8. Effect of temperature of papain on *L. monocytogenes* after 48 h

a, b, c data points or groups of points with the same superscripts are not significantly different ($p > 0.05$). Standard error of the mean = 0.35.

4. Discussion

In general, all enzyme treatments were most effective at 35°C. However, 25°C and 35°C did not differ forpapain but did differ in antimicrobial activity of bromelain.Temperature effects on enzyme activity was demonstrated by Ming et al. (2002) as the optimum temperature conditions for proteolytic activity of bromelain and papain are in range of 50-60°C and 65-80°C, respectively. Moreover, Anibijuwon and Udeze, 2009 reported that the increase in temperature enhances papain activity. Both bromelain and papain belong to a family of cysteine proteases that are activated by cysteine, which is located at the active site of the enzyme. Bromelain preferentially cleavesat amino acid sites involving lysine, alanine, tyrosine and glycine while papain prefers hydrophobic sites that include valine and also lysine. The enzyme breaks bonds at selected locations dividing the protein chain into fragments.

Gram-negative bacterial cell walls differ from Gram-positive cell walls containing an outer membrane comprised of protein, lipoprotein and lipopolysaccharides, a peptidoglycan layer then a plasma membrane that also contains proteins. Gram-positive bacteria have a thick peptidoglycan layer and an inner plasma membrane. The surface layer of both Gram positive and Gram-negative bacteria contain protein components that can be targeted by proteases to compromise cell wall structure to varying degrees. For example, the peptidoglycan layer (outer layer of Gram-positive bacteria) consists of subunits that are joined by crosslinks between the amino group of one amino acid and the carboxyl group of alanine (Prescott et al., 1990), a preferred scission site for bromelain. In contrast, Gram-negative bacteria have an outer lipopolysaccharide layer that contains porin proteins that a lined with exclusively charged amino acids to facilitate passage of molecules through the membrane (Schirmer, 1998); papain prefers uncharged (hydrophobic) amino acid sites. The different responses observed to bromelain and papain for *E. coli* and *L. monocytogenes* are likely to be due, in part to the differences in cell wall/membrane structure amino acid presence between Gram-negative (*E. coli*) and Gram-positive (*L. monocytogenes*). The presence and availability of amino acids in bacterial cell wall proteins that are enzyme targets will enhance or inhibit protease antibacterial activity. The antimicrobial activity of proteolytic enzymes used as meat-tenderizing agents reported here and in other sources may enhance the safety and shelf life of marinated meat products.

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