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Antibacterial effects of natural tenderizing enzymes on different strains of *Escherichia coli* O157:H7 and *Listeria monocytogenes* on beef

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ABSTRACT

This study determined the efficacy of actinidin and papain on reducing *Listeria monocytogenes* and three mixed strains of *Escherichia coli* O157:H7 populations on beef. The average reduction of *E. coli* O157:H7 was greater than that of *L. monocytogenes* and higher concentrations of either protease yielded greater reduction in bacterial populations. For instance, actinidin at 700 mg/ml significantly ($p \leq 0.05$) reduced the population of *L. monocytogenes* by 1.49 log cfu/ml meat rinse after 3 h at 25 & 35 °C, and by 1.45 log cfu/ml rinse after 24 h at 5 °C, while the same actinidin concentration significantly reduced the populations of three mixed strains of *E. coli* O157:H7 by 1.81 log cfu/ml rinse after 3 h at 25 & 35 °C, and 1.94 log cfu/ml rinse after 24 h at 5 °C. These findings suggest that, in addition to improving the sensory attributes of beef, proteolytic enzymes can enhance meat safety when stored at suitable temperatures.

1. Introduction

Foodborne diseases have a major impact in the United States with estimated 48 million illnesses, 128,000 hospitalizations and up to 3000 deaths occurring each year from bacteria, viruses, parasites and fungi (CDC, 2011). *Escherichia coli* O157:H7 and *Listeria monocytogenes* are pathogens that have received special attention by federal agencies and food safety researchers due to their economic and human health impact. These pathogens are responsible for 3 billion dollars in economic losses each year (USDA, 2006), therefore, alternative interventions are being studied to control these microorganisms.

L. monocytogenes is a Gram positive, motile, microaerophilic and nonspore-forming rod that grows at a wide temperature (1.7 °C–50 °C) and pH range (4.5 to 7.0) (Junttila, Niemala, & Hirn, 1988; Walker & Stringer, 1987). *L. monocytogenes* is widely distributed in nature with some studies indicating that 1–10% of humans are intestinal carriers of *L. monocytogenes* (FDA, 2012). Its association with meat and slaughter environments is well established (Benkerroum, Daoudi, & Kamal, 2003). Consumption of raw, partially cooked or post-cook contaminated meat can result in listeriosis, especially among the immune-compromised populations, elderly and pregnant (Shrinithivihahshini, Sheelamary, Mahamuni, & Chithradevi, 2011). According to the US Centers for Disease Control and Prevention, the rate of listeriosis has fallen by 24% from 1996 to 2003 (Voetsch et al., 2007). Yet, *L. monocytogenes* causes an estimated 1600 cases of listeriosis and 255 deaths annually in the United States (Scallan et al., 2011; CDC, 2011). As *L. monocytogenes* is a ubiquitous organism able to multiply at refrigeration temperatures and under anaerobic conditions, they are of major concern especially in RTE meat and poultry products (Martin et al., 2009). The minimum infective dose of *L. monocytogenes* is unknown but thought to vary with strain and individual susceptibility (FDA, 2012) and while indications that intake of up to 100 cells does not affect a healthy adult mouse (Golnazarian, Donnelly, Pintauro, & Howard, 1989) and Gilbert and Pini (1988) reported that cheese containing 10^4–10^5/g *L. monocytogenes* had no known human illnesses reported, the major concern remains in the high mortality rate for consumers acquiring listeriosis.

E. coli O157:H7 is an emerging pathogen responsible for about 63,000 illnesses, 2000 hospitalizations, and 20 deaths each year in the United States (Kudva et al., 2012). Some of these illnesses are associated with eating undercooked, contaminated ground beef. *E. coli* O157:H7 was recognized as a significant foodborne pathogen in the early 1980s and continues to be a major cause of diarrheal illness in North America. *E. coli* O157:H7 infections are the primary cause of hemolytic uremic syndrome (HUS) in children (Banatvala, Griffin, & Greene, 2001). According to the CDC, 350 outbreaks were reported from 1982 to 2002 (Rangel, Sparling, Crowe, Griffin, & Swerdlow, 2005).

The US Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS, 2012) has updated the lethality regulations for meat and poultry products. A 5-log lethality of *E. coli* O157:H7 for ready-to-eat (RTE) products containing beef and a 3-log reduction of *L. monocytogenes* should be achieved, although a 5-log reduction or greater is desirable for providing an even greater safety margin for ensuring that *L. monocytogenes* doesn't grow during cold storage to detectable levels.

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In spite the fact that foodborne pathogens are subjected to physical, chemical, and nutritional stresses during processing (Yousef & Courtney, 2003), their elimination and/or inhibition remains a big hurdle to processors. Remarkable advances have been made in developing thermal and non-thermal intervention technologies to eliminate foodborne pathogens from meat and poultry product, yet their ability to grow and proliferate at a wide range of temperatures and pH are major concerns during food preparation, storage or distribution (Pathania, McKee, Bilgili, & Singh, 2010). Consumers' increasingly demand for convenience foods of the highest quality has triggered the use of marinades to enhance food safety (Sabah, Juneja, & Fung, 2004; Shelef, 1984).

Using exogenous proteases to tenderize meat has been of considerable interest, with focus on some plant cysteine proteases such as papain, bromelain and actinidin (Ha, Bekhit, Carne, & Hopkins, 2012; Ketnawa & Rawdkuen, 2011; Koak, Kim, Choi, Baik, & Kim, 2011; Naveena, Mendiratta, & Anjaneyulu, 2004; Sullivan & Calkins, 2010). Papain is an important plant peptidase due to its powerful proteolytic activity, derived from the latex of unripe papaya fruit (*Carica papaya*, *Caricaceae*). 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 (Llerena-Suster, Priolo, & Morcelle, 2011). Studies found that papain and other papaya extracts possess antimicrobial activities against *Bacillus subtilis*, *Enterobacter cloacae*, *E. coli*, *Salmonella* Typhi, *Staphylococcus aureus*, and *Proteus vulgaris* (Emeruwa, 1982; Osato, Santiago, Remo, Cuadra, & Mori, 1993). Actinidin is another member of cysteine protease family present in kiwi fruit and belongs to the same class of enzymes as ficin, papain and bromelain. The important features of actinidin include a wide pH range for catalytic activity and stability at moderate temperatures, but the enzyme is susceptible to oxidation, a feature common with other plant thiol proteases (Kaur, Rutherford, Moughan, Drummond, & Boland, 2010). Actinidin has many applications in the food industry replacing other plant proteases like papain and ficin because of its mild tenderizing reaction even at high concentrations preventing surface mushiness. It has a relatively low inactivation temperature (60 °C) which makes it easier to control tenderization without overcooking (Tarté, 2009). Actinidin has potential pharmaceutical usage. Mohajeri et al. (2010) concluded that kiwi fruit extract has dramatic antibacterial and debridement effects when it is used as a dressing on deep second-degree burns, due to its potent proteolytic effects (Hafezi, Rad, Naghibzadeh, Nouhi, & Naghibzadeh, 2010). Moreover, Basile et al. (1997) found that kiwi fruit (*Actinidia chinensis*) extract has significant antibacterial activity against various Gram-positive and Gram-negative strains. Many studies have addressed the roles of these plant proteases as meat tenderizers (Ha, Bekhit, Carne, & Hopkins, 2013; Ha et al., 2012; Ketnawa & Rawdkuen, 2011; Koak et al., 2011; Naveena et al., 2004; Sullivan & Calkins, 2010). However, only a few of them have studied the antifungal, antioxidant and antibacterial properties of these plant proteases on meat and poultry products. Antibacterial activity depends on many factors such as pH, temperature and level of target microbial population (Bloomfield, 1991). Immobilized bacterial cells on solid surfaces behave differently in terms of growth rate and survival; thus liquid laboratory media are not suitable to simulate real food conditions (Brocklehurst, Mitchell, & Smith, 1997; Robins & Wilson, 1994). In the present study, *L. monocytogenes* and three mixed strains of *E. coli* O157:H7 were used to determine the effect of these proteolytic enzymes on bacteria on beef at different temperatures (5, 25 and 35 °C).

2. Materials and methods

2.1. Inoculum preparation

Three strains of *E. coli* O157:H7 (*E. coli* O157:H7 E-380-94:CDC, *E. coli* O157:H7 C-7929 and *E. coli* O157:H7-0654: supplied by Dr. Mike Doyle at UGA) were preserved by freezing the culture at −70 °C in vials containing trypticase soy broth (TSB) (DIFCO, Detroit, MI) 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 10 ml of enrichment TSB (DIFCO, Detroit, MI) in screw capped tubes and incubated aerobically for 16–18 h 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 10 ml tube of enrichment TSB (DIFCO, Detroit, MI), and incubated aerobically for 16–18 h at 37 °C with shaking. After incubating overnight, the washed cell suspension of the organism was harvested by centrifugation for 10 min at 1107 $\times g$ (IEC HN-SII Centrifuge, International Equipment Co., Inc., Needham Heights, MA), then the pellet was resuspended in 10 ml of sterile 0.1% (w/v) peptone water (Bacto peptone, Becton Dickinson, Sparks, MD) to obtain a population of approximately 8–9 log CFU/ml. Three ml of each strain was mixed together then 1 ml of the suspension was transferred into 99 ml of sterile 0.1% (w/v) peptone water to obtain population of approximately 5–6 log CFU/ml. Initial cell populations were verified by enumeration of the cells following surface-plating in TSA (DIFCO, Detroit, MI) and incubating at 37 °C for 24 h. The same procedure was followed for *L. monocytogenes* (ATCC 15313) which was surface-plated on Listeria Enrichment Agar (DIFCO, Detroit, MI) and incubated at 37 °C for 48 h.

2.2. Preparation of enzyme concentrations

The concentrations of actinidin (KFPE OT1005X, Ingredient Resources Pty Ltd, Australia) used with *L. monocytogenes* and three mixed strains of *E. coli* O157:H7 were 0 mg/ml, 175 mg/ml, 350 mg/ml, and 700 mg/ml, while the concentrations of papain (P4762-500MG, Sigma Chemicals, St. Louis, MO) used with *L. monocytogenes* and three mixed strains of *E. coli* O157:H7 were 0 mg/ml, 5 mg/ml, 8 mg/ml and 10 mg/ml. These concentrations were chosen based on preliminary experiments using a greater range of enzyme concentrations.

2.3. Meat sample preparation and inoculation

Chunk beef meat was purchased from a local store, transported to the laboratory under refrigerated conditions (0–4 °C) and stored at 4 °C until use. Proximate composition was determined using AOAC procedures to be approximately 72.5% water, 6.5% fat, 19.4% protein and 1.6% ash. The beef meat was cut using a sterile sharp knife and a stainless steel square template into approximately 3 × 3 cm^2 and thickness of 1.5–2 cm (~10 g). Meat samples were transferred into individual sterile plastic bags (WHIRL-PAK®, Nasco, CA, USA). A 0.5 ml aliquot of the inoculum was pipetted on meat surface in the bags, giving a surface inoculum of 5–6 log CFU/cm^2 and allowed to remain undisturbed for 5 min at room temperature to permit bacterial cell attachment before subjecting enzyme treatments. After inoculation, 1 ml of each enzyme concentrations was pipetted on the meat surface. The bags were then held at three different temperatures at 5, 25, and 35 °C.

2.4. Enumeration of surviving bacteria and sampling time

Meat samples held at 25 and 35 °C were sampled at 0, 1, and 3 h while those at 5 °C were sampled at 0, 6, and 24 h. 20 ml 0.1% (w/v) peptone water was added to each bag and massaged for 1 min then 0.1 ml was aseptically removed, 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* O157:H7, in duplicate. Non-inoculated control samples without enzyme added and with enzyme added were also used to determine if any *L. monocytogenes* or *E. coli* O157:H7 were present on the beef.

Plates were incubated (Model 2300 incubator, VWR Scientific Product) at 37 °C for 48 h for *L. monocytogenes* and 24 h for *E. coli* O157:H7. Dilution plates with 25–250 colonies were counted (LEICA,

QUEBEC DARK FIELD colony counter, Buffalo, NY 14240, USA; model 3325) and populations were reported as a CFU/ml and log CFU/ml (of meat rinse). All experiments were repeated three times.

2.5. Determination of enzymatic activity and protein content

The enzyme activity of actinidin and papain was measured spectrophotometrically according to the modified method of Robinson (1975). The assay mixture contained 1 ml of actinidin (KFEP OT1005X) and papain dilutions (2.5, 5 and 10 mg/ml) and 5 ml of 0.65% (w/v) of substrate, casein solution (Sigma, St. Louis, MO) dissolved in 50 mM potassium phosphate buffer, pH 7.5 at 37 °C. All reaction mixtures were incubated at 37 °C for 10 min. The reaction was stopped by adding 5 ml of 110 mM trichloroacetic acid (TCA) and precipitated protein was removed by filtration through a 0.45 μm syringe filter (0.45 μm Supor® membrane, Pall Corporation, Ann Arbor, MI). The absorbance of filtrate was measured at 660 nm (Spectrophotometer GENESYS 20 4001-4, 100–240 V 50/60 Hz, Madison, WI). Blank samples were prepared by adding the enzyme at the end of the incubation time, just before TCA addition and precipitation. One unit of the enzyme activity was defined as the amount of enzyme which releases 1 μmol of tyrosine per minute under the assay conditions. Specific activity was expressed as enzyme units per mg protein. Protein content of actinidin (KFEP 0T1005X) and papain were measured using a modified Lowry protein assay method (Lowry, Rosebrough, Farr, & Randall, 1951) with bovine serum albumin (Pierce Biotechnology, modified Lowry protein assay kit, Rockford, IL) as the protein standard (detection range = 0.5–20 μg/ml).

2.6. Statistics analysis

A split–split–split plot design was used for the study. The factor at the highest level was the bacterium type (*L. monocytogenes* and *E. coli* O157:H7). The next level was the concentration level for papain (0, 5, 8 and 10 mg/ml) and for actinidin (0, 175, 350 and 700 mg/ml) and the third level was temperature (5, 25 and 35 °C). The fourth level was time (6 h and 24 h for 5 °C and 1 h and 3 h for 25 and 35 °C). The response variable was the log increase in the bacterium count compared to time 0. Treatment effects are to be evaluated using analysis of variance techniques and linear contrasts. Mean was separated using Fisher's protected least signification difference (LSD) test with alpha = 0.05.

3. Results

No detectable *L. monocytogenes* or *E. coli* O157:H7 were found on control meat samples with and without enzyme added.

3.1. Effect of actinidin on L. monocytogenes and E. coli O157:H7 at 5 °C

The average reduction of *E. coli* O157:H7 was greater than that of *L. monocytogenes* and higher concentrations of actinidin yielded greater reduction in bacterial populations.

The population of both *L. monocytogenes* and *E. coli* O157:H7 was reduced significantly at actinidin concentrations greater than or equal to 175 mg/ml from 6 h to 24 h (Table 1).

L. monocytogenes populations were reduced significantly at actinidin concentrations greater than or equal to 350 mg/ml after 6 h and for all concentrations after 24 h compared to starting populations. Whereas, for *E. coli* O157:H7 there was no significant difference in log reductions at any concentration tested. The reductions when averaged for all concentrations were 1.21 log cfu/ml after 6 h and 1.74 log cfu/ml after 24 h (Table 1).

Table 1
Reduction in *E. coli* O157:H7 and *L. monocytogenes* populations at 5 °C exposed to various concentrations of actinidin on beef.

Concentration (mg/ml)	*E. coli* O157:H7		*L. monocytogenes*	
	Time		Time	
	6 h	24 h	6 h	24 h
	Log cfu/ml rinse		Log cfu/ml rinse	
0	0.02^{b}	-0.019^{b}	0.05^{b}	**0.55^{ab}**
175	**1.08^{a}**	**1.52^{a}**	0.32^{ab}	**0.53^{b}**
350	**1.19^{a}**	**1.77^{a}**	**0.43^{ab}**	**1.03^{ab}**
700	**1.34^{a}**	**1.94^{a}**	**0.77^{a}**	**1.45^{a}**

Bold value indicates that the reduction in the mean log bacterium count was significantly greater than 0. Means with the same superscripts are not significantly different within the same bacterial type and time (column). Level of significance used was 0.05. The standard error of the mean was 0.18.

3.2. Effect of actinidin on L. monocytogenes and E. coli O157:H7 at 25 °C and 35 °C

There was no significant difference in the log reduction for *L. monocytogenes* or *E. coli* O157:H7 at 25 and 35 °C; therefore, the data were pooled for these two temperatures (Table 2).

There was no significant difference in *L. monocytogenes* population between 1 and 3 h at actinidin concentrations of 0 and 175 mg/ml; however the population of *L. monocytogenes* at actinidin concentrations greater than or equal to 350 mg/ml decreased between 1 and 3 h. Actinidin concentrations greater than or equal to 350 mg/ml after 1 h and actinidin concentrations greater than or equal to 175 mg/ml after 3 h significantly reduced *L. monocytogenes* compared to the initial population (Table 2).

On the other hand, there were differences in the population of *E. coli* O157:H7 between 1 h and 3 h with all actinidin levels tested. Also, the average population of *E. coli* O157:H7 was reduced significantly for actinidin concentrations greater than or equal to 175 mg/ml by 1.15 log cfu/ml average after 1 h and by 1.56 log cfu/ml average after 3 h (Table 2).

3.3. Effect of papain on L. monocytogenes and E. coli O157:H7 at 5 °C

As found in actinidin, higher concentrations of papain resulted greater population reductions ($\alpha \leq 0.05$) for *L. monocytogenes* (Table 3). For papain concentrations tested with *L. monocytogenes*, 10 mg/ml was the most effective concentration in reducing bacterial counts after 6 h and 24 h, whereas *E. coli* O157:H7 population was reduced significantly with papain levels greater than or equal to 5 mg/ml by 1.13 log cfu/ml after 6 h and by 1.4 log cfu/ml after 24 h (Table 3).

Table 2
Reduction in *E. coli* O175:H7 and *L. monocytogenes* populations at 25 and 35 °C exposed to various concentrations of actinidin on beef.

Concentration (mg/ml)	*E. coli* O157:H7		*L. monocytogenes*	
	Time		Time	
	1 h	3 h	1 h	3 h
	Log cfu/ml rinse		Log cfu/ml rinse	
0	-0.18^{b}	-0.36^{c}	0.08^{c}	0.07^{c}
175	**1.06^{a}**	**1.35^{b}**	0.20^{bc}	**0.34^{c}**
350	**1.10^{a}**	**1.51^{ab}**	**0.49^{b}**	**0.80^{b}**
700	**1.28^{a}**	**1.81^{a}**	**1.11^{a}**	**1.49^{a}**

Bold value indicates that the reduction in the mean log bacterium count was significantly greater than 0. Means with the same superscripts are not significantly different within the same bacterial type and time (column). Level of significance used was 0.05. The standard error of the mean was 0.11.

Table 3
Reduction in *E. coli* O175:H7 and *L. monocytogenes* populations at 5 °C exposed to various concentrations of papain on beef.

Concentration (mg/ml)	*E. coli* O157:H7		*L. monocytogenes*	
	Time		Time	
	6 h	24 h	6 h	24 h
	Log cfu/ml rinse		Log cfu/ml rinse	
0	−0.02[b]	0.28[b]	−0.04[b]	−0.21[b]
5	**1.08[a]**	**1.29[a]**	−0.07[b]	−0.01[b]
8	**1.12[a]**	**1.40[a]**	0.04[ab]	0.15[ab]
10	**1.18[a]**	**1.57[a]**	**0.38[a]**	**0.46[a]**

Bold value indicates that the reduction in the mean log bacterium count was significantly greater than 0. Means with the same superscripts are not significantly different within the same bacterial type and time (column). Level of significance used was 0.05. The standard error of the mean was 0.17.

3.4. Effect of papain on L. monocytogenes and E. coli O157:H7 at 25 °C and 35 °C

There was no significant difference in the population reduction for *L. monocytogenes* or *E. coli* O157:H7 at 25 and 35 °C; therefore, the data for these two temperatures were pooled (Fig. 4). Log reductions for both 25 and 35 °C were significantly different from 5 °C (Fig. 3).

At papain concentrations greater than or equal to 5 mg/ml, there was a significant decrease in the population of *L. monocytogenes* and *E. coli* O157:H7 between 1 h and 3 h (Table 4).

As with actinidin, where higher concentrations were more effective in reducing bacterial populations, papain at 10 mg/ml reduced the average population of *L. monocytogenes* after 1 h and 3 h, while for *E. coli* O157:H7, there was no difference in the reductions for concentrations greater than or equal to 5 mg/ml. The average reductions at these concentrations were 1.06 log cfu/ml after 1 h and 1.38 log cfu/ml after 3 h (Table 4).

4. Discussion

4.1. Temperature effects on enzyme activity

The general effect of temperature on enzyme activity was in agreement with Aminlari, Shekarforoush, Gherisari, and Golestan (2009) who found that actinidin increased protein solubility by 20% at 37 °C for 2 h, indicating that the optimum temperature of actinidin ranges between 30 and 50 °C. Moreover, Katsaros, Katapodis, and Taoukis (2009) found that actinidin did not show any activity at temperatures higher than 55 °C. Similar results were reported for papain having an optimum temperature range between 65 and 80 °C (Ming, Awang, Duduku, & Sing, 2002).

Table 4
Reduction in *E. coli* O175:H7 and *L. monocytogenes* populations at 25 and 35 °C exposed to various concentrations of papain on beef.

Concentration (mg/ml)	*E. coli* O157:H7		*L. monocytogenes*	
	Time		Time	
	1 h	3 h	1 h	3 h
	Log cfu/ml rinse		Log cfu/ml rinse	
0	0.07[a]	−0.38[b]	0.03[bc]	−0.2[c]
5	**0.98[a]**	**1.29[a]**	−0.15[c]	−0.01[bc]
8	**1.02[a]**	**1.37[a]**	0.12[ab]	0.14[b]
10	**1.19[a]**	**1.48[a]**	**0.33[a]**	**0.56[a]**

Bold value indicates that the reduction in the mean log bacterium count was significantly greater than 0. Means with the same superscripts are not significantly different within the same bacterial type and time (column). Level of significance was 0.05. The standard error of the mean was 0.14.

4.2. Effects of proteolytic enzyme concentrations

As was found in the present study, Eyob, Martinsen, Tsegaye, Appelgren, and Skrede (2008) reported that increasing concentrations of the active antimicrobial substance from plant extracts yielded greater antimicrobial activity. In the current study, antibacterial activities of papain and kiwi fruit extract (actinidin) were dependent on concentration. For instance, actinidin at 700 mg/ml reduced *E. coli* O157:H7 population by 1.81 log cfu/ml after 3 h at 25 and 35 °C and by 1.94 log cfu/ml after 24 h at 5 °C, while the same concentration reduced *L. monocytogenes* population by 1.49 log cfu/ml after 3 h at 25 and 35 °C, and by 1.45 log cfu/ml after 24 h at 5 °C (Figs. 1 & 2). On the other hand, papain at 10 mg/ml reduced *E. coli* O157:H7 population by 1.48 log cfu/ml after 3 h at 25 and 35 °C, and by 1.57 log cfu/ml after 24 h at 5 °C, while the same concentration reduced *L. monocytogenes* population by 0.56 log cfu/ml after 3 h at 25 and 35 °C, and by 0.46 log cfu/ml after 24 h at 5 °C (Figs. 3 & 4).

Interestingly, *L. monocytogenes* showed a nearly linear response in log reduction to actinidin concentration at 6 h ($R^2 = 0.97$) and 24 h ($R^2 = 0.94$) for 5 °C while *E. coli* O157:H7 log reduction response to actinidin concentration was not linear at the concentrations tested ($R^2 = 0.64$ and 0.61 for 6 and 24 h, respectively) (Fig. 1). This trend was also evident in the pooled 25 and 35 °C temperatures with *L. monocytogenes* having log reduction/actinidin concentration R^2 values of 0.98 (1 h) and 0.99 (3 h) while *E. coli* O157:H7 displayed R^2 values of 0.58 (1 h) and 0.64 (3 h) (Fig. 2). These different responses to enzymes by the two bacteria may be at least partially attributed to the difference in cell wall structure between Gram + (*Listeria*) and Gram − (*E. coli*) with Gram − normally more resistant to antimicrobials. This trend does not hold for papain (linear response to concentration by *L. monocytogenes*).

Consequently, it is important to determine enzyme activity for enzyme preparations. Papain concentration of 10 mg solid/ml had 0.3 activity units/ml while actinidin at the same concentration had 0.01 activity units/ml (Table 5). The specific activity of the two protease preparations, expressed as A_{660} min/mg of bovine serum albumin, was 0.1 and 0.02 for papain and actinidin enzyme preparations, respectively, showing that the papain protease preparation displayed the highest specific activity towards the bovine serum albumin substrate. This is in agreement with Foegeding and Larick (1986) and Ha et al. (2012) who demonstrated that actinidin possessed the minimal activity compared with other plant proteases such as papain, bromelain and zingibain.

4.3. Effects of bacterial species

The present study also reported that the average log reduction of *E. coli* O157:H7 was greater than that of *L. monocytogenes* for both enzymes and at all temperatures used in this study (Figs. 1–4). This could be due to the structural differences of bacterial cell wall between Gram-negative and Gram-positive bacteria. According to Volk and Wheeler (1988), bacteria have a three-layer cell wall structure. This

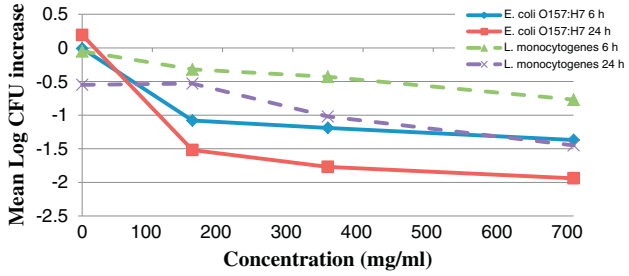

Fig. 1. Effect of different concentrations of actinidin on *L. monocytogenes* and *E. coli* O157:H7 at 5 °C. The standard error of the mean was 0.18.

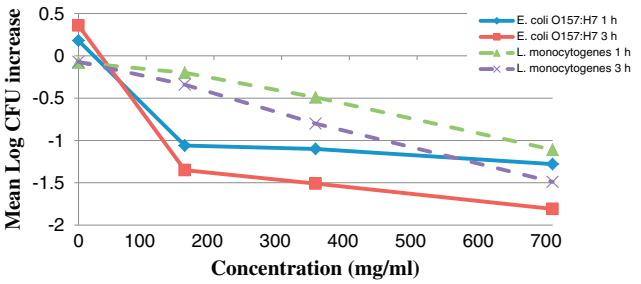

Fig. 2. Effect of different concentrations of actinidin on *L. monocytogenes* and *E. coli* O157:H7 at 25 and 35 °C. The standard error of the mean was 0.11.

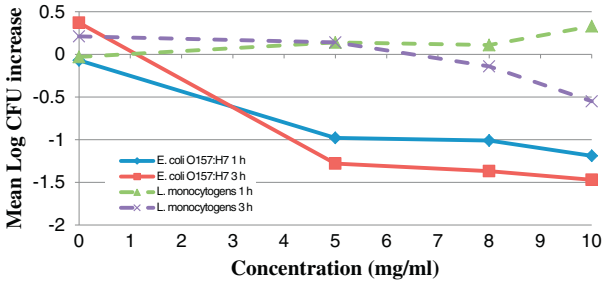

Fig. 4. Effect of different concentrations of papain on *L. monocytogenes* and *E. coli* O157:H7 at 25 and 35 °C. The standard error of the mean was 0.14.

structure is composed of: (1) cytoplasmic membrane, (2) thicker peptidoglycan membrane, and (3) varied outer membrane that is mainly composed of proteins and lipids that are susceptible to proteolytic nature of enzymes. Volk and Wheeler (1988) also explained that the bacterial destruction by papain and actinidin is due to proteolytic enzyme precipitation of the outer protein membranes, rupture of the cell wall and coagulation resulting in the loss of cell contents and energy through cell wall leakages. In addition, Conner and Beuchat (1984) concluded that antimicrobial compounds might change the functions of the microbial cell membrane and sensitivity of the cell to various antimicrobial compounds to increase the inactivation of membrane-bound enzymes. Therefore, effective antimicrobial preservatives might act on more than one target site on the bacterial membrane, resulting in leakage or autolysis and inhibition of growth or even death of the cell.

4.4. Effects of media

The inhibitory effect of papain against foodborne pathogens used in this study was less on beef than that in laboratory buffer (0.1% w/v peptone water) (Eshamah, Han, Naas, Rieck, & Dawson, 2012). Eshamah et al. (2012) used papain concentrations up to 0.5 mg/ml to obtain a 4-log reduction within 48 h at room temperature. This is in agreement with the results of Shelef, Jyothi, and Bulgarellii (2006) and Stecchini, Sarais, and Giavedoni (1993) who found that the potency of natural antimicrobial extracts decreases in complex food systems.

Moreover, Hao, Brackett, and Doyle (1998) suggested that the differences in results could be due to the complexity of the food system tested and/or the specific characteristic of the natural antibacterial used. Shelef et al. (2006) also concluded that the antimicrobial activity of plant extracts increases by increasing its solubility in meat systems. Cutter (2000) and Hsieh, Mau, and Huang (2001) both reported that the activity also increases under acidic conditions, high water contents, high salt and low fat contents of meat products. However, Robins and Wilson (1994) concluded that the growth of foodborne pathogens in liquid culture provides a baseline for their behavior in complex structures.

Uhart, Marks, and Ravishankar (2006) also observed the differences in the efficacy of natural antimicrobials when studied in vitro versus

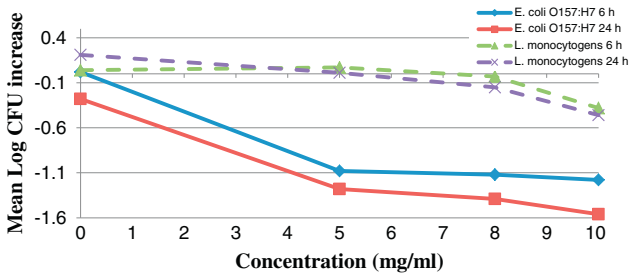

Fig. 3. Effect of different concentrations of papain on *L. monocytogenes* and *E. coli* O157:H7 at 5 °C. The standard error of the mean was 0.17.

when added to a food matrix. They concluded that fat, oil droplets, and/or protein interaction are responsible for this reduction in activity. Essential oils may also be affected by food composition. Farbood, MacNeil, and Ostovar (1976) explained that a high lipid fraction in meat may absorb rosemary extract and decrease its concentration in the aqueous phase and consequently its antibacterial effect. They also mentioned that a potential decrease in the penetration of the spice into the microbial cell could be due to the formation of a fat coat around the cell which could also reduce the efficacy of enzyme treatments. Regardless of the antimicrobial effects achieved, if these natural antimicrobials are used as a part of a hurdle system, higher pathogen reductions may be attained.

4.5. Effects of proteolytic enzymes

Bacterial cell walls contain peptides with some amino acids in the L configuration; therefore, proteolytic enzyme is able to hydrolyze these peptides. Most of the cell-wall lytic enzymes are characterized by ability to hydrolyze bonds between the amino sugars of the glycosaminopeptide (Ensign & Wolfe, 1966). Both papain and actinidin belong to a family of cysteine proteases that are activated by cysteine, which is located at the active site of the enzyme. Cysteine-25 attacks the carbonyl carbon in the backbone of the peptide chain freeing the amino terminal portion, breaking the protein chain (Mamboya, 2012).

Papain shows extensive proteolytic activity towards proteins, short chain peptides, amino acid esters and amide links. It preferentially cleaves peptide bonds involving basic amino acids, particularly arginine, lysine and phenylalanine (Menard, Khouri, Plouffe, Dupras, & Ripoll, 1990). Actinidin has a similar pattern of hydrolysis to that of papain. Actinidin also prefers hydrophobic sites including Leucine, Valine, or Phenylalanine but not Tyrosine (Boland & Moughan, 2013).

According to industry recommendations (Enzyme Development Corporation, 1999) typical commercial-home application levels of bromelain and papain are in the 3 g-enzyme/500 g meat (0.6%) range. Based on the approximate weight of our sample (10 g) and the concentrations used for actinidin (175–700 mg) and papain (5–10 mg) the levels used in the present study were higher than commercial levels for actinidin (1.75–7.0%) but substantially lower for papain (0.05–0.1%). The actinidin enzyme preparation was a kiwi extract and not as purified as the papain enzyme thus levels of enzyme may

Table 5
Total and specific activity of actinidin and papain measured by spectrophotometer.

	Concentration (mg solid/ml)	Protein [a](mg/ml)	Activity (units/ml)[b]	Unit activity/mg protein
Actinidin	10	0.58	0.01	0.02
Papain		3	0.3	0.1

[a] Measured by recording absorbance at 750 nm.
[b] One unit actinidin or papain activity is the amount of enzyme causing 1 unit change in absorbance at 660 nm/min.

have been similar. In fact the measured enzyme activity for papain was about 5 times greater than that measured for the kiwi extract containing actinidin. Ha et al. (2012) evaluated 4 commercial protease preparations (papain, bromelain, actinidin and zingibain) and reported that based on SDS-PAGE analysis, these preparations contained several proteins not previously found in synthetic substrates thus it may differ in activity from purified enzyme preparations. These researchers used the same source for actinidin as the present study, which was a crude extract. The difference between purified enzyme (papain) and the kiwi extract of actinidin may also have impacted the differences in results.

5. Conclusion

This study used a short time between inoculation and enzyme application and did not utilize cold-adapted bacteria, thus results may differ if resident bacteria were cold adapted. Furthermore, a 5-minute "attachment time" was used and longer exposure times prior to enzyme application may impact the bactericidal effect of the enzyme. There would likely be difference in results if standard industry injection and tumbling were evaluated for enzyme effects on bacteria. Since internal meat tissues are usually considered nearly sterile, inclusion of enzyme in the marinade would likely reduce bacterial populations there since little interference would be present. Once injected, the enzyme would probably have minimal bactericidal effect due to the enzyme interaction with the meat. These findings suggest that, in addition to improving the sensory attributes of beef, tenderization with proteolytic enzymes may enhance the safety and shelf life when stored at suitable temperatures. The findings also propose a promising approach in developing antimicrobial systems for meat products.

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