LETHALITY OF *Escherichia coli* O157: H7 IN HAMBURGER TREATED WITH PURIFIED ALLYL ISOTHIOCYANATE AND MUSTARD FLOUR

By

Dharshini Nadarajah

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FACULTY OF GRADUATE STUDIES

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ABSTRACT

The consumption of undercooked ground beef contaminated with *Escherichia coli* O157:H7 has been a significant cause of hemorrhagic colitis and the hemolytic uremic syndrome in North America. This work examined the use of the volatile natural antimicrobial (allyl isothiocyanate, AIT) to eliminate *E. coli* O157:H7 from ground beef. An advantage of this approach was that AIT evaporated upon opening of the package, leaving minimal residue in the food. The objectives of the research were to examine ways to achieve effective headspace concentrations of AIT from 94% pure commercial product and use dry mustard flour as an alternate source of AIT to enable reduction or elimination of viable *E. coli* O157:H7 from ground beef. Experiments were designed to inoculate a five strain *E. coli* O157:H7 cocktail at levels of either 3 $\log_{10}$ or 6 $\log_{10}$ cfu/g into ground beef patties. AIT in corn oil added as either a volume of 0.5 or 1 ml was placed on a piece of filter paper, positioned on top of a ground beef patty (each 100g) placed singly in plastic bags (Nylon/EVOH/PE), backflushed with 100% N_2, heat-sealed and stored at 4, 10, and -18°C for 21, 8, or 35 days, respectively. The mustard treatment was performed similarly except that 5,10, or 20% (w/w) mustard was mixed with the ground meat and stored at 4°C for 21 days. Survival of very low inoculated levels of *E. coli* O157:H7 (14 to 40 cfu/g) in ground beef with 5% mustard was tested using Immunomagnetic Separation (IMS). During storage, the AIT levels in the package headspace were determined by gas liquid chromatography. Total viable bacteria were determined using trypticase soy agar whereas viable *E. coli* O157:H7 were counted on sorbitol MacConkey agar (CT-SMAC).

Results indicated that the natural microflora in ground beef patties was largely unaffected by the addition of AIT at all three temperatures and lengths of storage tested. At an initial population of 3 $\log_{10}$ cfu/g, *E. coli* O157:H7 was reduced by liquid AIT to undetectable levels after 18 days at 4°C and 10 days at –18°C. Increasing the *E. coli* numbers to 6 $\log_{10}$ cfu/g resulted in a >3 $\log_{10}$ reduction of *E. coli* O157:H7 at 4°C, while a 1 $\log_{10}$ reduction was observed at both 10 and –18°C over 8 and 35 days, respectively. The final AIT concentrations in the headspace samples after storage at 4,10, and –18°C were 456, 444, and 112 μg/ml at 21, 8, and 35 days, respectively. In the mustard treatment there were 0.5, 3, and 5.4 $\log_{10}$ decreases from the initial levels of 6 $\log_{10}$ cfu/g in meat containing 5, 10, and 20% mustard flour, respectively, after 21 days. At the low inoculum level 3 $\log_{10}$ cfu/g *E. coli* was reduced to undetectable levels after 18, 12 and 3 days at 5,10, and 20% mustard flour, respectively. The final AIT concentrations present in the headspace at 21 days from the mustard powder at 5, 10, and 20% were 19.6, 15.6 and 22.9 μg/ml, respectively. However, it was found that mustard at 5%(w/w) did not completely eliminate very low inoculated numbers ($\leq$40 cfu/g *E. coli*) from meat when IMS was used for *E. coli* detection. The natural microflora of the ground beef was unaffected by the addition of the mustard flour. Mustard powder treatments were more effective than liquid AIT in reducing viable *E. coli* O157:H7. It was thought that this greater effectiveness was due to the mustard powder being directly formulated with the meat. It is possible that other components present in the mustard may also have had a synergistic effect in reducing the number of viable *E. coli* O157:H7 in ground beef patties. The sensory evaluation of the cooked

ground beef showed that there were no significant differences in acceptability between 5 and 10% (w/w) mustard powder- amended meat. However panelists could distinguish untreated controls from mustard treatments. Nonetheless, panelist still considered the mustard-treated meat to be acceptable. Our results showed that it may be possible to use mustard at levels of 5-10% to eliminate *E. coli* O157:H7 from fresh ground beef.

Chapter 1

INTRODUCTION

Escherichia coli O157:H7 causes life threatening hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura in the young, old and immuno-compromised (Acheson et al., 1996; Rowe, 1995). Canada has the highest number of reported cases of *E. coli* O157:H7 illness in the world (Rowe, 1995). The Center for Disease Control estimates that *E. coli* O157:H7 causes 75,000 human intoxications infections every year. As a result of a low infectious dose in food (10 cfu/g), 2000 people will be seriously ill each year and require hospitalization, and of these 50 will die (Rasmussen and Casey, 2001). The majority of *E. coli* O157:H7 outbreaks have been linked to undercooked or raw hamburgers eaten during the summer months. Therefore this illness is also known as 'hamburger disease' or 'barbecue season syndrome' (Waters et al., 1994). In the US, ground beef accounts for nearly 50% of all beef consumption, and last summer 24 million pounds were recalled for possible *E. coli* O157:H7 contamination (FSIS, 2003). *E. coli* contaminated ground beef not only lowered consumer confidence, but also represented significant economic loss in the meat industry. *E. coli* O157:H7 has cost 2.8 billion dollars in direct and indirect losses for the beef industry in the US over the past decade (Kay, 2003).

Currently many researchers are investigating different ways to eliminate *E. coli* O157:H7 from ground beef which will be acceptable to regulatory authorities, the food industry, and consumers. One promising approach is the addition of natural antimicrobials from plant sources. Spices and essential oils have been reported in the scientific literature to have antimicrobial activity (Davidson, 2001). Allyl isothiocyanate (AIT) is a naturally occurring volatile compound found in plants belonging to the *Cruciferaeae* family (Lin et al., 2000a; Ohta et al., 1995). *Cruciferaeae* plants include horseradish, mustard, brussel sprouts, broccoli, kale and turnip (Clydesdale, 1999; Delaquis and Mazza, 1995). Studies in the past have demonstrated that purified AIT as well as crude plant extracts containing AIT inhibit *E. coli* O157:H7 in broth and agar model systems (Isshiki et al., 1992; Kanemaru and Miyamoto, 1990). Therefore, AIT has the potential to eliminate *E. coli* O157:H7 in food systems such as ground beef patties.

The objective of the research was three fold. The first objective was to control the release rate of AIT by altering its volatility using various fats and oils as carriers at different temperature and also to select a plastic packaging material that was impermeable to AIT vapors. The second part of the research was to kill *E. coli* O157:H7 from packaged ground beef through the incorporation of AIT on filter paper inserted in the package with the meat. Finally, the third objective was designed to kill *E. coli* O157:H7 in ground beef through incorporation of de-hulled, non-deheated mustard flour as an ingredient in hamburger and to evaluate the sensory acceptability of cooked ground beef patties containing added mustard flour.

Chapter 2

LITERATURE REVIEW

2.1 *Escherichia coli* O157:H7

Escherichia coli is a Gram negative, rod shaped and facultative anaerobic species that is normally found in the intestinal tract of humans and warm-blooded animals (Cassin et al., 1998; Padhye and Doyle, 1992). A few strains of *E. coli* are pathogenic and they can cause severe diarrheal symptoms (Padhye and Doyle, 1992). Serotype O157:H7 differs from other types of *E. coli* by producing one or two toxins, which can result in severe and potentially fatal illness (Acheson et al., 1996; Rowe, 1995).

E. coli O157:H7 differs biochemically from other types of *E. coli.* It is unable to ferment sorbitol within 24 h, does not produce the enzyme β-glucuronidase, and grows poorly at 44-45°C which is the standard temperature for growing fecal *E. coli* (Mermelstein, 1993; Padhye and Doyle, 1992; Silveira et al., 1999).

E. coli O157:H7 has been transmitted to humans through foods (67% of cases), direct contact from person-to person (22%), swimming water (8%), and drinking water (2%) (Griffin, 1998; Mead and Griffin,1998). The majority of *E. coli* O157:H7 outbreaks have been linked to undercooked or raw hamburgers eaten during the summer months (Chinen et al., 2001; Dorn, 1995; Rowe, 1995; Simmons, 1997). Therefore, this illness has been designated by the media as “hamburger disease” or “barbecue season syndrome” (Waters et al., 1994). Other foods that are also implicated with *E. coli* O157:H7 outbreaks as the result of either cross contamination with meat products or contamination in the field with animal feces include apple cider, cantaloupe, watermelon, dry cured salami, mayonnaise, drinking water, alfalfa sprouts, lettuce and radish sprouts. (Cassin et al., 1998; D'Sa et al., 2000; Hara-Kudo et al., 1999; Mead and Griffin, 1998). *E. coli* O157:H7 causes a high rate of mortality among those infected (1/12,000) and is therefore an organism of considerable concern. It is primarily associated with foods of bovine origin.

2.1.1 Emergence of *E. coli* O157:H7 as a foodborne illness agent

In 1975 the first reported case of *E. coli* O157:H7 with symptoms of bloody diarrhea occurred in California, however, *E. coli* O157:H7 was not identified as a human foodborne pathogen until 1982 (Getty et al., 2000; Padhye and Doyle, 1992). In 1977, Konowalchuck and co-workers discovered that some diarrheagenic *E. coli* strains produced cytotoxins that killed Vero cells from the kidney of the African Green monkey (Padhye and Doyle, 1992; Park et al., 1999). In 1982, researchers investigated a large outbreak of hemorrhagic colitis that occurred in Oregon and Michigan. A total of 47 individuals showed symptoms of abdominal pain and bloody diarrhea after eating hamburger from a fast food restaurant chain. Investigators were able to isolate a new *Escherichia coli* serotype, O157:H7, from the infected patients' stools and from the frozen beef patties (Park et al., 1999). The new *Escherichia coli* strain did not fit into the existing groups of *E. coli* types, which included those classified as enteropathogenic, enteroinvasive, or enterotoxigenic. Enteropathogenic *E. coli* causes neonatal and infantile diarrhea. Enteroinvasive *E. coli* affects the colon cells and result in bloody diarrhea. Enterotoxigenic *E. coli* produces enterotoxin that causes watery diarrhea also known as traveler's diarrhea (Riley, 1987). As a result of the discovery of the O157:H7 strain, a fourth new group of enterohemorrhagic *E. coli* was recognized for its capacity to cause hemorrhagic colitis (Padhye and Doyle, 1992). O'Brien et al. (1983) discovered that *E. coli* O157:H7 from the Oregon and Michigan outbreaks produced a shiga toxin. In 1985 Karmali and co-workers reported that *E. coli* that produces the shiga toxin also causes hemolytic uremic syndrome (HUS) (Park et al., 1999). Currently there are 200 different types of shiga toxin-producing *E. coli* (STEC) that have been found by researchers, and of these 60 have been documented as causing disease in humans (Acheson, 2000). *E. coli* O157:H7, which causes illness at a rate of 3 per 100,000 population is currently considered as the fourth most costly of foodborne illnesses in the US following *Campylobacter* spp. (25 per 100,000), *Salmonella* spp. (16 per 100,000), and *Shigella* spp. (9 per 100,000) (Nataro and Kaper, 1998).

2.1.2 Non-O157 *Escherichia coli*

Two years after the discovery of *E. coli* O157:H7, researchers noticed that there were other strains of Shigella-like toxin producing organisms that were non-O157 *E. coli* (STEC), and these also caused bloody diarrhea and HUS. The non-O157 *E. coli* include O111: NM, O26:H11, O103:H2 and O113:H21 (Uhitil et al., 2001). However the non-O157 *E. coli* cause fewer cases of bloody diarrhea and HUS, and have lower hospitalization rates compared to *E. coli* O157:H7. The Center for Disease Control (CDC) estimated that *E. coli* O157:H7 causes 75,000 cases whereas non-O157 STEC cause 37,000 human infections every year. The rate of hospitalization for *E. coli*

O157:H7 is estimated at 2000 per year, and of these 50 patients will die. For non-O157 STEC, 1000 will be hospitalized and 30 will die (Rasmussen and Casey, 2001). Both non-O157 and O157:H7 have been isolated from food, and while not the only source, cattle serve as a major reservoir for *E. coli* O157:H7. The organisms do not cause clinical symptoms in these animals. STEC such as O133:Hu O22:NM, O82:H8, O8:H9, O13:Hu have been isolated from ground beef after causing disease in humans (Neil, 1997). Acheson (2000) reported that STEC O111:NM caused 23 cases of HUS in Australia and 50 cases in Texas.

The majority of clinical and food tests are done for O157:H7. Non-O157 STEC testing is rarely done because these bacteria are phenotypically diverse and therefore the testing required is much more labor intensive. Since *E. coli* O157:H7 and other STEC are capable of causing HUS and death, testing for all types of STEC should be done in order to make sure the food supply is safe from these pathogenic bacteria (Acheson, 2000).

2.1.3 Verocytotoxins

E. coli O157:H7 does not cause illness by its mere presence in the intestine, but it causes illness through the production of toxins, which affect intestinal epithelial cells among others in the human (Rowe, 1995; Weeratna and Doyle, 1991). The toxins are called verocytotoxins because they are toxic to cultured Vero cells (Weeratna and Doyle, 1991). *E. coli* O157:H7 can produce two verotoxins, VT-1 and VT-2. The VT-1 is also known as shiga-like toxin-1 (SLT-I) because it is immunologically indistinguishable from the toxin produced by *Shigella dysenteriae* type 1, and it is neutralized by the antisera against shiga toxin. The VT-2 toxin has only 56% of its amino acids similar to shiga-like toxin 1, and it is not neutralized by anti-shiga toxin. Nonetheless, VT-2 is called shiga-like toxin-2 (SLT-II) (Getty et al., 2000; Mead and Griffin, 1998). The VT-1 toxin is found in cell lysates while VT-2 is found in culture filtrates. *E. coli* O157:H7 normally produces VT-2 alone or in combination with VT-1, but rarely produces VT-1 alone (Rowe, 1995).

The *E. coli* toxins (VT-1 and VT-2) are made up of five types of B subunits and one A subunit coded by the chromosome (Weeratna and Doyle, 1991). The B subunit binds to globotriasylceramide (Gb_3) normally found in eukaryotic blood vessel cells, smooth muscle cells, renal endothelial cells and red blood cells (Mead and Griffin, 1998; Rowe, 1995; Todd and Dundas, 2001). The A subunit attacks the 60S ribosomal subunit preventing protein synthesis and causing cell death (Doyle, 1991). *E. coli* O157:H7 causes serious illness when the toxin produced in the intestine attaches to the Gb_3 receptors on endothelial cells in the kidney. Children are more susceptible to illness from *E. coli* O157:H7 since they have more Gb_3 receptors than adults (Park et al, 1999).

It is still unclear whether it is toxins that are produced by *E. coli* O157:H7 after ingestion of contaminated food or preformed toxin in the food that causes illness. The VT-1 is a heat stable toxin, therefore it is not inactivated during mild heat treatment of food (Doyle, 1991).

2.1.4 Pathogenicity

As mentioned, *E. coli* O157:H7 can produce two cytotoxins that can affect all age groups, but it most commonly affects the young, old and immuno-compromised (Rowe, 1995). Much is known about the mechanism of *E. coli* O157:H7 pathogenicity, but it is still not fully understood (Getty et al., 2000). The infectious dose of *E. coli* O157:H7 can be low as 10 cfu/g (Uhitil et al., 2001). The symptoms of serious *E. coli* O157:H7 infection include hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP) (Mead and Griffin, 1998).

The clinical symptoms of HC are sudden abdominal pain followed by watery or non-bloody diarrhea, which then turns into bloody diarrhea (Riley, 1987). The symptoms of HC begin when *E. coli* O157:H7 attaches to epithelial cells of the intestinal wall. This results in local disruption of tissue causing the initial watery diarrhea (Todd and Dundas, 2001). Bloody diarrhea occurs when verocytotoxins from the bacteria damage the endothelial layer of small blood vessels (Monnens et al., 1998). The amount of blood in the stool can vary from streaks to grossly visible blood (Rowe, 1995). Almost 70% of the patients who suffered from HC reported that they had bloody diarrhea (Mead and Griffin, 1998). The HC patients suffer from abdominal pain of high intensity, which has been described as similar to labor pains (Padhye and Doyle, 1992). Another symptom is vomiting which occurs in about 30-60% of the patients. Fever is very rare, therefore this organism is considered not invasive (Padhye and Doyle, 1992; Riley, 1987). The incubation period ranges from 3 to 9 days. Illness usually lasts about

2 to 9 days (Padhye and Doyle, 1992). HC is normally self-limited and most patients require supportive care to recover (Riley, 1987).

Five to ten percent of HC patients develop HUS when toxins enter the blood stream and attach to Gb_3 receptors found in the kidney (Simmons, 1997; Todd and Dundas 2001). Inside the kidney the verocytotoxins initiate the blood clotting mechanism, which results in clogging of the capillaries causing waste build up in the blood (Park et al., 1999). HUS affects all age groups, but is most severe in children under 10 years of age. Most cases of HUS result in acute renal failure in children. The clinical symptoms of HUS include a decrease in hemoglobin and platelets, an increase in urea/creatinine concentration in the blood, jaundice and hypertension (Padhye and Doyle, 1992; Todd and Dundas, 2001). Almost 50% of the patients who develop HUS may need dialysis and 75% need a blood transfusion (Mead and Griffin, 1998). In the most serious cases, patients may undergo heart failure, seizures, coma and stroke (Mead and Giffin 1988; Padhye and Doyle, 1992). About 3 to 5% of HUS patients die. A few patients who recover from HUS may suffer from permanent damage to the central nervous system, which leads to retardation, hemiparesis or learning disabilities which can remain unresolved for several years and even decades later (Rowe, 1995; Mead and Griffin, 1998).

A few cases of HUS may develop into TTP. TTP affects the central nervous system, whereas HUS attacks the renal system (Mead and Griffin, 1998; Padhye and Doyle, 1992). TTP symptoms mainly occur in adults and can include microangiopathic hemolytic anemia (intravascular destruction of red blood cells), thrombocytopenia (depressed platelet counts), fluctuating neurologic signs, fever and mild azotemia. TTP patients mostly develop blood clots in the brain, which results in death (Getty et al., 2000; Padhye and Doyle, 1992).

2.2 Link between *E. coli* O157:H7 and cattle

Because most early outbreaks of *E. coli* O157:H7 were associated with foods of bovine origin like undercooked hamburger and un-pasteurized milk, researchers came to believe that cattle might serve as a reservoir of *E. coli* O157:H7 that contaminated the food supply (Vold et al., 2000).

E. coli O157:H7 is found in the cattle intestine and is periodically shed in feces. Cattle appear to be clinically normal when shedding *E. coli* O157:H7 (Rasmussen and Casey, 2001). The amount of *E. coli* O157:H7 shed by the cow varies at different growth stages (Mermelstein, 1993). D'Sa et al. (2000) reported that *E. coli* O157:H7 could be found in 3.2% calves and 1.6% cattle in the USA. When positive, adult cows normally shed 10^2-10^5 cfu/g of *E. coli* O157:H7 (Uhitis et al., 2001). Cattle are more frequently found to shed *E. coli* O157:H7 during the summer and early fall months, but during the winter months the number of *E. coli* O157:H7 positive animals is almost reduced to zero (Park et al, 1999). Several researchers reported that *E. coli* O157:H7 may survive in water and feed over the winter months and re-infect cattle during the spring and summer months (Hancock et al., 1998; Mead and Griffin, 1998; Park et al., 1999). A study sponsored by the US Food and Drug Administration (FDA) found that 3.8% of water troughs and 1.8% of bulk feeds in the US are frequently contaminated with *E. coli* O157:H7 (Hancock et al., 1998). Ostroff et al. (1989) reported that the seasonal variation in *E. coli* O157:H7 shedding by cattle along with growth of the organism in the farm environment might explain the outbreaks associated with bovine foods during the summer and fall months.

E. coli O157:H7 from cattle feces are transmitted to the surface of the meat during the slaughtering, evisceration and skinning processes (Cassin et al., 1998; Mermelstein, 1993; Uhitil et al., 2001). During meat grinding the *E. coli* that contaminates the surface of the meat can be introduced throughout the meat as it is ground. Since ground beef is made with the combined meat from many carcasses, a small amount of *E. coli* O157:H7 from a single carcass can contaminate a large quantity of product (Mead and Griffin, 1998). Once the *E. coli* contaminates the ground meat it is capable of surviving when frozen (-18°C) up to 9 months and cause disease if the meat is not exposed to proper cooking times and temperatures (Ansay et al., 1999; Rowe, 1995). The FDA advises consumers to cook ground beef to internal temperatures of 160°F or 71.1°C for 2 min (Simmons, 1997). Beef steaks contaminated with *E. coli* O157:H7 do not normally cause illness since the bacterial contamination only occurs at the surface and the organisms can be easily killed by normal cooking temperatures (Mermelstein, 1993).

Attempts to reduce the incidence of *E. coli* O157:H7 contamination of carcasses have involved washes containing organic acids, chlorine dioxide, trisodium phosphate, and the use of steam or hot water washes or steam pasteurization. More recently irradiation of ground beef has been approved in the US and its use is becoming more frequent (Cutter, 2000; Sage and Ingham 1998; Vogel, 1995). Despite all the precautions taken by the industry, *E. coli* O157:H7 still manages to enter the food supply.

2.3 Outbreaks of *E. coli* O157:H7 in ground beef

Outbreaks of *E. coli* O157:H7 infections have been reported from more than 30 countries on 6 continents (Chinen et al., 2001). The majority of *E. coli* O157:H7 cases occur sporadically (Wilson et al., 1997). Higher rates of *E. coli* O157:H7 intoxication were reported in countries that have high cattle density and better surveillance tracing systems (Nataro and Kaper, 1998; Park et al., 1999). The developed world has higher cattle density than the developing world, therefore, more cases of *E. coli* O157:H7 illness caused by contaminated ground beef were reported in the US, Canada, Argentina and Scotland (Park et al., 1999).

E. coli O157:H7 was first reported as a cause of human foodborne illness in the United States in 1982 (Keskimaki et al., 1998). On average 2.1 cases per 100,000 population of O157:H7 infections were reported annually. Most of the O157:H7 intoxications occur in the northern part of the US (Park et al., 1999). There were 139 reported *E. coli* O157:H7 outbreaks in the US from 1982 - 1996. Of the 139 outbreaks, 85 were related to food and of the food outbreaks, 53 were related to ground beef. During the same time 3,000 people became ill, 22% of whom were hospitalized, 6% of whom developed HUS or thrombotic thrombocytopenic purpura and 0.6% died (Sparling, 1998). The largest outbreak of *E. coli* in ground beef occurred in 1993. In this outbreak 475 people became seriously ill and 4 children died after consuming hamburgers at fast food restaurants (Jack in the Box) in Washington, Idaho, California and Nevada (Getty et al., 2000; Mermelstein, 1993). Inquiry into this outbreak showed that hamburgers were contaminated with *E. coli* O157:H7 and the meat was undercooked because company officials were unaware that the FDA's recommended cooking temperatures for ground beef had been raised from 140 to 155°F (Mermelstein, 1993).

The United States Department of Agriculture has a zero tolerance policy for *E. coli* O157:H7 in ground beef (Venkitanrayanan et al., 1999). Table 2.1 shows the class 1 recalls of *E. coli* contaminated ground beef in the United States from 1997 to 2003. A class 1 recall is issued by the regulatory agency when there is a probability that consuming a food product will cause serious health problems or death. Recalls of *E. coli* contaminated meat in the US occur mostly during the summer and autumn months (Rasmussen and Casey, 2001). In August, 1997, 11 million kg of ground beef products contaminated with *E. coli* O157:H7 were recalled by the Food Safety and Inspection Service (FSIS, 2003). This has been designated as the largest voluntary recall of ground beef in the United States. A total of 15 people became ill in the Colorado area (James, 1998). In July, 2002 the second largest recall of ground beef involved 8.4 million kg that were contaminated with *E. coli* O157:H7. There were 16 cases of illness reported in the Colorado area (FSIS, 2003).

Canada has the highest reported rates of *E. coli* O157:H7 illness in the world (Rowe, 1995). Between 1991 - 1996, 3.0 to 5.3 cases per 100,000 population were reported (Park et al., 1999). The largest *E. coli* O157:H7 outbreak from ground beef in Canada occurred in six Inuit communities in the Northwest Territories. A total of 521 people were infected, 22 children developed HUS and 2 died (Wilson et al., 1997; Spika et al., 1998). *E. coli* outbreaks are more common in western Canada and Alberta has the highest incidence of *E. coli* intoxications in North America. A total of 387 cases were reported from 1980 to 1990 (16.3 cases per 100,000) (Waters et al., 1994). Ramotar et al. (1995) reported that the highest rate of HUS was also found in this region. Perhaps this is because the major *E. coli* reservoir (cattle) is unusually close to the population (Riley, 1987). The largest voluntary recall of *E. coli* O157:H7 contaminated ground beef (32,000 kg) occurred in August 2001. Table 2.2 shows the class 1 recall notifications of ground beef for possible contamination by *E. coli* O157:H7 issued by the Canadian Food Inspection Agency from 1999 to 2003 (CFIA, 2003).

Argentina has the highest rate of cases per year (300 to 400) of HUS among children in the world. Argentineans consume 60 kg of bovine meat per person each year (Chinen et al., 2001). This amount is the highest level of consumption reported in the world. Bovine meat is cheap and Argentineans consume meat, starting early in life. Almost 20% of Argentinean children consume meat as early as 5 months of age and 80% of the children at 8 months eat meat at least three times a week. It is not surprising that Argentina has exceptionally higher rates (22 per 100,000) of HUS among children <5 years of age (Lopez et al., 1998).

Table 2.1. Class 1 recall notification of ground beef for possible contamination of *E. coli* O157:H7 over 1000 kg by the Food Safety and Inspection Service (USDA) from 1997 to 2003

Date	Federal Establishment	Location	Quantity Recalled x 1000 kg
June 1997	Hudson	Colorado	11,339
April 1998	Joslin	Illinois	127
June 1998	Costco Wholesale Corp.	Washington	78
November 1998	IBP Inc.	Nebraska	252
November 1998	Glenmark Industries Ltd.	Chicago	273
November 1998	Boxed Beef Co.	Florida	136
August 1999	Jac Pac Foods	New Hampshire	113
December 1999	Supreme Beef Processors	Texas	81
June 2000	Packerland Packing Co. Inc.	Wisconsin	88
June 2000	IBP Inc.	Illinois	121
July 2000	Jac Pac Foods	New Hampshire	95
August 2000	Moyer Packer Co.	Pennsylvania	157
December 2000	Green Bay Dressed Beef Inc.	Wisconsin	50
May 2001	Emmpak Foods Inc.	Milwaukee	213
June 2001	Excel Corporation	Georgia	86
August 2001	Green Bay Dressed Beef Inc.	Wisconsin	240
August 2001	IBP Inc.	Nebraska	227
July 2002	Carneco Foods	Nebraska	59
July 2002	ConAgra	Colorado	8,618
August 2002	GFI American Inc.	Minnesota	325
September 2002	Moyer Packing Company	Pennsylvania	92
October 2002	Emmpak Foods Inc.	Wisconsin	1,043
November 2002	Skylark Meat Inc.	Nebraska	49
November 2002	Fairbank Reconstruction Corp.	New York	145
March 2003	American Foods Groups	Ohio	48
June 2003	Stampede Meat Inc.	Chicago	335

Adapted from FSIS (2003)

Table 2.2. Class 1 recall notification of ground beef for possible contamination of *E. coli* O157:H7 by the Canadian Food Inspection Agency from 1999 to 2003

Date	Meat Processing Company	Location
May, 1999	Costco Wholesale	Winnipeg
June, 2000	Costco Wholesale	NA[1]
August, 2000	IBP-Lakeside Packers	Alberta
May, 2001	Canada Safeway Ltd.	Surrey
April, 2001	IBP Lakeside Packer	Alberta
August, 2001	Belmont Meat Products Ltd.	Ontario
August, 2001	IBP-Lakeside Packer	Alberta
July, 2002	Canada Safeway Limited	NA
November, 2002	Co-Op Limited	Saskatoon

Adapted from CFIA (2003)

[1] Not available

More cases of *E. coli* related illness (4.0 cases per 100,000) have been reported in Scotland than anywhere else in the United Kingdom (Waters et al., 1994). In 1996 an *E. coli* O157:H7 outbreak occurred in central Scotland during which 496 people were infected and 20 elderly persons died (Park et al., 1999). More people died during this outbreak than in any of the other *E. coli* O157:H7 outbreaks that were related to food (Dundas et al., 2001). The source of the outbreak was identified as cooked meat products that were cross contaminated with raw meat product at a butcher shop (Smith et al., 1998). Waters et al. (1994) reported that ground beef was implicated in 57.7% of cases of *E. coli* contamination in Scotland.

Escherichia coli O157:H7 contaminated ground beef has caused significant human morbidity and mortality in many parts of the world. Currently many researchers are investigating procedures to eliminate *E. coli* O157:H7 from ground beef that are acceptable to regulatory authorities, the industry and consumers. One promising approach is to use natural antimicrobials from plant sources to kill *E. coli* O157:H7 should it occur in ground beef.

2.4 Natural antimicrobials from plant sources

Antimicrobial activities of plant extracts have been reported in the scientific literature since the beginning of the 19th century. At the middle of the century, however synthetic preservatives replaced natural preservatives because they were cheaper, easier to manufacture and more effective. Health concerns such as potential carcinogenicity and mutagenicity that arose following use of man made chemical preservatives in food, have led to renewed interest in substitution of synthetic preservatives by natural antimicrobials from plant sources (Delaquis and Mazza, 1995; Kim et al., 2001).

Natural antimicrobials from plants were initially used to extend shelf life by improving the sensory quality of food. Currently, food researchers are interested in studying whether natural antimicrobials from plants can be used to kill pathogenic bacteria and improve food safety. Spices and essential oils containing phenolic compounds such as cinnamic aldehyde (cinnamon), eugenol (cloves), and thymol (thyme) have been reported to have antimicrobial activities. These essentials oil components are only effective against certain microorganism at high concentrations, which detracts from the sensory quality of food (Davidson, 2001). Studies have shown that a volatile aliphatic sulfur containing extract from horseradish or mustard known as allyl isothiocyanate (AIT) effectively inhibits a variety of pathogenic microorganisms when used at low concentrations (Isshiki et al., 1992). Not only does purified AIT exhibit antimicrobial properties, but crude plant extracts containing AIT also inhibit bacteria (Kanemaru and Miyamoto, 1990). Improvements in barrier properties of plastic packaging films have enabled workers to investigate the antimicrobial effectiveness of

AIT in its vapor phase against pathogenic bacteria using model food systems (Sekiyama et al., 1995).

2.5 Allyl Isothiocyanate

Allyl isothiocyanate (AIT) is a naturally occurring volatile compound found in plants that belong to the family *Crucifereae* (Lin et al., 2000a; Ohta et al., 1995). AIT is also referred to as allyl mustard oil, allyl iso-rhodanide, synthetic oil of mustard, and oil of sinapis (Clark, 1992). AIT is one of many natural antimicrobials that are found in the seeds, stem, leaves, and roots of cruciferous plants (Clark, 1992; Okano et al., 1990). Cruciferous plants include horseradish, black and brown mustard, cabbage, brussels sprouts, broccoli, cauliflower, kohlrabi, kale, turnip, rutabaga, watercress, wasabi, radish and papaya (Clydesdale, 1999; Delaquis and Mazza,1995; Ono et al., 1998). Certain plants contain more AIT than others. For example, cabbage contains 3 to 17 mg/kg, horseradish contains 1.3 to 1.8 g/kg and mustard contains >7g/kg AIT (Delaquis and Mazza, 1995; Pechacek et al, 1997).

AIT from natural sources is permitted for use in Japan as a food preservative. However, AIT is not allowed as a food preservative in North America, though AIT from plant sources can be added to mayonnaise and horseradish to enhance the flavor (Isshiki et al., 1992; Delaquis and Massa, 1995). The United States recently added AIT to the Generally Recognized As Safe (GRAS) list administered by the FDA as a flavoring agent (Kim et al., 2001).

2.5.1 Formation of allyl isothiocyanate

Sinigrin, a glucosinolate found in the cell vacuoles of cruciferous plants, is the precursor of allyl and other isothiocyanates in *Crucifera* (Shofran et al., 1998). There are over 100 different types of glucosinolates in plants and they differ by their carbohydrate side chain (R) groups (Delaquis and Mazza, 1995). The glucosinolate structure includes a central thiocyanate group adjacent to a molecule of D-glucose attached by β-linkage (Depree et al., 1999). Glucosinolates are stable, water-soluble and they comprise 1% (w/w) of cruciferous plant tissue (Fahey and Stephensen, 1999). Different parts of the plant (root, leaves, and stem) have different amounts of glucosinolates (Delaquis and Mazza, 1995). Formation of isothiocyanates begins when plant tissue is damaged or injured; glucosinolates are hydrolyzed by a cell wall bound enzyme known as myrosinase (thioglucoside glucohydrolase EC 3.2.3.1) in the presence of water (Ahn et al., 2001; Delaquis and Mazza 1995; Shofran et al., 1998). Myrosinase is stable at neutral pH and has maximum activation above 30°C. Myrosinase reacts with glucosinolates to produce glucose, isothiocyanate, nitriles, thiocyanates and sulfuric acid (Fig. 2.2).

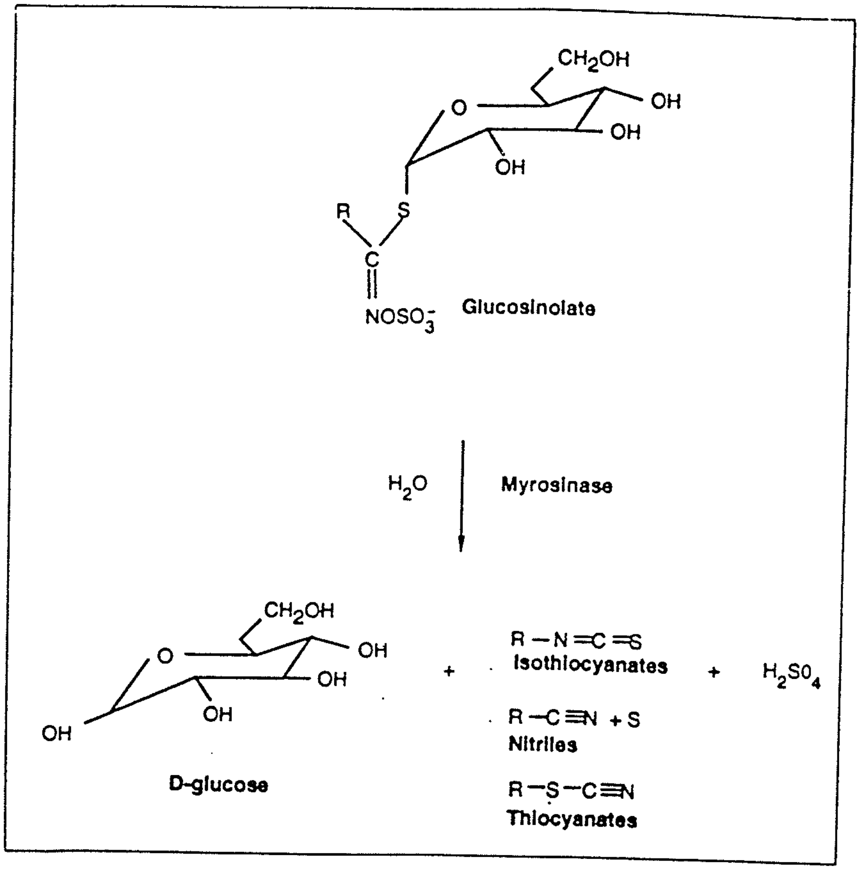

Figure 2.2. Formation of glucose, allyl isothiocyanate, nitriles and thiocyanates from glucosinolates hydrolyzed by the enzyme myrosinase (adapted from Delaquis and Mazza, 1995).

Glucose and a small amount of sulfuric acid are always formed, but the formation of isothiocyanate, nitriles and thiocyanates depend on the environmental conditions (Ahn et al., 2001). Isothiocyanate is formed under neutral pH conditions while nitriles and elemental sulphur are formed under acidic conditions or in the presence of Fe^{+2} (Depree et al., 1999). Conditions favoring the formation of thiocyanates are unclear (Shofran et al., 1998). There are many different types of isothiocyanates that can be formed and these depend on the nature of the R-side group present in the original glucosinolate. Allyl isothiocyanates are formed when the R-side group in the glucosinolate consists of an (CH_3-CH_2-CH_2) aliphatic chain (Delaquis and Mazza, 1995).

2.5.2 Properties of AIT

AIT is a colorless or pale yellow liquid (Verghese et al., 2000). Its melting and boiling points are –80°C and 150°C, respectively, thus, it is highly volatile at refrigeration temperature (Clark, 1992). AIT is stable in organic solvents such as propylene glycol, esters, alcohols, ketones, chlorinated solvents, aliphatic and aromatic hydrocarbons (Cejpek et al., 2000; Clark, 1992). It is fat-soluble and dissolves well in oils. AIT stability is not affected by high relative humidity or light (Sekiyama et al., 1994). AIT disintegrates rapidly at room temperature or at 37°C in water and it gives off a garlic-like odor (Chen and Ho, 1998). The unique properties of AIT such as volatility, fat solubility, stability at high relative humidity and lower temperatures provides opportunity for its use as a natural antimicrobial in food systems.

2.5.3 Antimicrobial activities of AIT

In previous studies zone inhibition or broth dilution were commonly used to determine the effectiveness of antimicrobial agents. Since AIT is a volatile antimicrobial, it has been necessary to use enclosed atmosphere model systems to overcome the difficulties associated with its volatility in order to measure antimicrobial activity (Delaquis and Sholberg, 1997).

Although unreacted sinigrin does not demonstrate any antimicrobial properties, its hydrolysis products including AIT have shown promising results (Kyung and Fleming, 1997). Studies have demonstrated that AIT is more effective in killing Gram-negative than Gram-positive bacteria (Shofran et al., 1998). Antimicrobial properties of AIT depend on its concentration, exposure time and temperature. The vapor form of AIT is more effective in controlling microorganisms than the liquid form (Lin et al., 2000b). AIT has been reported in the literature as having a broad range of antimicrobial effects and can be bactericidal, bacterostatic, fungicidal or fungistatic. Aerobic bacteria are more sensitive to AIT than facultatively anaerobic bacteria. The mechanism whereby AIT inhibits or kills bacteria is still not fully understood (Ahn et al., 2001; Delaquis and Sholberg, 1997). Studies in the past have shown that AIT may inhibit bacterial growth by altering protein structures or by disrupting metabolic functions by inhibiting oxygen uptake (Lin et al., 2000b; Kojima and Ogawa, 1971). Isshiki (1992) reported that the minimum inhibitory concentrations of AIT in the gaseous phase for bacteria was 34 - 110 ng/ml; for yeast it was 16 - 37 ng/ml; and for mold it was 16 to 62 ng/ml. Both the liquid and vapor form of AIT were previously tested against *E. coli* O157:H7 using agar, broth and model food systems (Table 2.3). In broth cultures approximately 6 to 8 $\log_{10}$ reductions of *E. coli* O157:H7 occurred using 12.3 ppm of AIT in the liquid phase (Kanemaru and Miyamoto, 1990; Lin et al., 2000a). In an agar model 4 to 7 $\log_{10}$ reductions of *E. coli* O157:H7 were achieved using 10 to 1500 µg/L AIT applied in the vapor phase (Delaquis and Sholberg, 1997; Isshiki et al., 1992; Park et al., 2000; Ward et al., 1998). The antimicrobial effectiveness of AIT has been tested on various food models such as roast beef, cucumber, alfalfa seed, lettuce and apples. Ward et al. (1998) were able to reduce *E. coli* O157:H7 from 3.03 to 2.26 log cfu/cm^2 on agar plates using 20 000 nl/l of AIT in the vapor phase. These researchers also reported that AIT did not inhibit the growth of Gram-positive lactic acid bacteria. Lin et al. (2000a) reported a 4 $\log_{10}$ reduction of *E. coli* using 88 µl/l of AIT as a liquid in lettuce and a 3 $\log_{10}$ reduction using 133 µl/l of AIT as a liquid in tomatoes. Ogawa et al. (2000) reported that a 5 $\log_{10}$ reduction was achieved using 80 µl/l of AIT as a liquid along with high pressure treatment at 200 MPa for 10 min at 4°C. Park et al. (2000) were able to reduce *E. coli* from 2.0 $\log_{10}$ to less than 0.70 $\log_{10}$ in alfalfa seed with 50 µl/l of AIT in the vapor phase. AIT in the vapor phase has been shown to be an effective antimicrobial against Gram-negative pathogenic bacteria such as *E. coli* O157:H7 while it did not appear to significantly affect the viability of the naturally occurring Gram-positive lactic acid bacteria (Park et al., 2000).

Table 2.3. Summary of studies done on the antimicrobial activity of various levels of allyl isothiocyanate applied as liquid or vapor against *E. coli* O157:H7 in broth, agar, and model food.

Model system	AIT state	AIT concentration	Reduction of viable bacteria by AIT ($\log_{10}$ cfu)	Exposure time	Temp.	Reference
Broth	liquid	12.3 ppm	6/ml*	24 h	30°C	Kanemaru and Miyamoto, 1990
Broth	liquid	1,000 µg/ml	8/ml*	2 h	37°C	Lin et al., 2000a
Agar disk	vapor	0.034 µg/ml	4/ml*	2 d	37°C	Isshiki et al., 1992
Agar disk	vapor	1500 µg/l	6/cm^{2*}	48 h	40°C	Delaquis and Sholberg, 1997
Agar disk	vapor	2µl/l	7 /cm^2	7 d	12°C	Ward et al., 1998
Agar disk	vapor	10 µl/l	7/ml*	24 h	37°C	Park et al., 2000
Roast beef	vapor	20µ/l	2/cm^2	7 d	12°C	Ward et al., 1998
Lettuce	liquid	88 µl/l	4/cm^2*	4 d	37°C	Lin et al., 2000a
Apple	liquid	133 µl/l	3/ml	2 d	4°C	Lin et al., 2000a
Cucumber	liquid	80 µl/l &200 MPa	5 /ml	10 min	4°C	Ogawa et al., 2000
Alfalfa seed	vapor	50 µl in 950 cm^3 jar	2.2/g	24 h	37°C	Park et al., 2000

* to undetectable levels

2.5.4 Advantages and disadvantages of using AIT in food systems

An additional advantage of using AIT in food systems other than its beneficial antimicrobial properties is that AIT is believed to play a significant role in preventing cancer (Pechacek et al., 2000). Isothiocyanates have been shown to prevent cancer of the lung, esophagus, forestomach, colon, mammary gland, and pancreas in many animal models (Jiao et al., 1998).

A disadvantage of using AIT in food systems is that it has been shown to generate a burning sensation on the tongue and serve as an irritant in the nasal cavity, of individuals from some ethnic groups, while members of other group find the taste highly desirable (Coogan et al, 2001; Delaquis and Mazza 1995). It is worthwhile noting that its low water solubility limits AIT use in food systems (Lin et al., 2000b).

Consumption of large quantities of cruciferous plant material can lead to development of goitre. The thiocyanate ion can bind to iodine leading to nutritional deficiency. This problem mostly occurs in regions where iodine is low in the diet (Delaquis and Mazza, 1995) and is not likely to be a problem in North America. Other components that are produced by decomposition of glucosinolates such as cyanides, oxazolidinethiones, epithionitriles, and thiocyanate ions have resulted in growth retardation, damage to kidney, liver and pancreas tissue in experimental animals (Pechacek et al., 2000). AIT at high concentration exhibits strong lachrymatory and skin vesicant properties (Pechacek et al., 1997).

In spite of having potentially harmful effects at high concentration, it is likely that if AIT is highly effective against *E. coli* O157:H7 the advantages associated with its use will outweigh the disadvantages.

2.6 Antimicrobial effectiveness of AIT in mustard flour

Mustard is the second most common spice consumed in the human diet after pepper. Written records of the use of mustard seed go back as far as 530 BC in the Greek literature (Clark, 1992). The name mustard comes from the Latin "must" referring to grape juice. Romans mixed ground mustard into grape juice as a preservative (Delaquis and Mazza, 1995). There are two types of mustard available- white and black, but only black mustard has significant amounts of the essential oil AIT. Two species of black mustard commonly grown are *Brassica nigra* and *Brassica juncea*. Canada produces the largest amount of mustard (*B. juncea)* in the world and it is also known as oriental mustard (Remaud et al., 1997).

AIT occurs at approximately 0.5 to 1% of the mustard seed by weight (Clark, 1992). AIT is extracted from the mustard after oil has been removed by cold press methods. The oil accounts for 28-36% of the seed weight. The remaining product is known as presscake. AIT is extracted from the press cake using steam distillation. In this process the enzyme myrosinase hydrolyses glucosinolates and releases the AIT in the presence of water (Farrell, 1985; Verghese et al., 2000). Whole mustard seed has been used in pickles and salads as a spice and preservative (Raghavan et al., 1971). Mustard flour is prepared by removing the hull and then grinding the endosperm to finer particles without water. These products can be used in the meat industry as binders especially in sausage, but first must be prepared to eliminate the potential to form AIT which is the source of bitter and pungent flavors. One way this can be done is by raising the moisture content of the seed to $\leq$10% and holding at room temperature for several days, then milling and drying the flour. However, deheating or removal of the hot (pungent) characteristic of mustard removes its antimicrobial activity. In order to maintain the highly potent antimicrobial properties of mustard, low moisture mustard seed is milled to flour under low humidity. This "non-deheated" flour still contains glucosinolates unreacted with myrosinase which can form AIT upon contact with moisture.

Kanemaru and Miyamoto (1990) compared the antimicrobial effects of mustard and purified AIT at equal concentrations of AIT. They found that mustard was more antimicrobial against *E. coli* than purified AIT. Their study showed that 0.1% mustard with 9.4 ppm of AIT was able to inhibit the growth of *E. coli* in culture medium within 24 h but 12.3 ppm of purified AIT solution was required to achieve the same level of inhibition (Kanemaru and Miyamoto, 1990). Another study done by Mayerhauser (2001) reported that retail style mustards eliminated 6 $\log_{10}$ of *E. coli* O157:H7 within a few hours in trypticase soy broth incubated at either refrigerated or room temperatures. More recently, Rhee et al. (2002) showed that mustard flour alone or in conjunction with acetic acid reduced 6 $\log_{10}$ of *E. coli* O157:H7 to <0.3 $\log_{10}$ after incubation at room temperature for 24 h.

AIT from mustard provides an alternative natural approach to kill pathogenic *E. coli* O157:H7 in food products since mustard has been used as spice in foods for several centuries. AIT from mustard is most effective in food systems when closed atmosphere models are used to control its volatilization. One approach is to use impermeable plastic packaging films or glass containers to prevent mustard AIT loss.

2.7 Application of AIT in food systems using plastic packaging materials

Traditionally, food packaging materials were only used to extend the shelf life and maintain the quality of food products (Han 2003). Currently, there are many substances that can be added to packing materials to increase the shelf life of food products by actively preventing deterioratio, and at the same time without having effects on the nutritional quality the food. The active substances can be oxygen scavenging, have antimicrobial activity, show moisture or ethylene scavenging ability, or can be ethanol emitting (Han 2000). The antimicrobial activity of the packaging systems can result from an added antimicrobial agent(s). These agents kill microorganisms directly or they can generate an inhospitable environment, which in turn reduces the survival of microorganisms (Appendini and Hotchkiss, 2002; Kim et al., 2002). A variety of natural antimicrobials have been used in active packaging systems in the recent past, but the extremely volatile nature of AIT has limited its use in food systems. Recent research in plastic packaging material has renewed interest in using AIT in small quantities as a vapor (Kim et al., 2002). Isshhiki et al. (1992) first discovered that adding small amounts of AIT inside plastic bags preserved foods by killing yeast, molds, and pathogenic bacteria. Since AIT is volatile, it can diffuse throughout the package contents to inhibit bacteria and yet can evaporate after the package is opened, leaving no residue (Isshhiki et al., 1992). Sekiyama et al. (1995) reported that 100 ppm of AIT vapor had no corrosive effects on plastic. However, at 3000 ppm, AIT vapors deformed most types of plastic except polyethylene (PE), polypropylene (PP), nylon, polyacetal, and teflon (Lim et al., 1999; Sekiyama et al.,1995). Lim and Tung (1997) reported that a conventional plastic film (PVDC/PVC) did not retain enough AIT vapor to be antimicrobial, but these plastic films can still be used in situations where the initial residence time of AIT is sufficient to kill undesirable microorganisms and quickly dissipate in a short time. AIT cannot be directly formulated into the plastic films as they are formed because plastics are formulated at high temperature and AIT breaks down under these conditions. To solve this problem, it has been suggested that AIT be introduced in the package after the packaging material is formed or introduced as coating on the film or food. Subsequently, AIT is allowed to permeate through the packaged materials. Currently the food industry is also using polyolefin type plastics which are not affected by AIT (Sekiyama et al.,1995). AIT-containing pouches are commercially produced (Wasa Ouro®) in Japan using polypropylene or polyethylene films. These films are designed to release 95% of AIT within 3 h at 25°C (Worfel et al., 1997). Wasa Ouro® films are currently used to preserve non-refrigerated lunch box meals in vending machines, packaged bread and fresh vegetables (Conca and Yang, 1996).

2.8 Summary

Among pathogenic *E .coli* strains, O157:H7 is the leading cause of HC, HUS, and TTP in North America. The majority of *E. coli* O157:H7 outbreaks have been linked to consumption of undercooked or raw hamburgers. One possible way of killing *E. coli* in ground beef is to introduce a volatile natural antimicrobial, which can diffuse through and dissolve in the meat-fat matrix during storage. Such an antimicrobial would evaporate after the package is opened and during cooking, leaving no residue. Allyl isothiocyanate, a naturally occurring volatile compound found in members of the *Cruciferae* family has been reported to be an effective antimicrobial agent against *E. coli* O157:H7. AIT from crude plant extracts has been shown to have antimicrobial activity against these pathogenic bacteria in agar or broth model systems. AIT has an appropriate spectrum of activity and chemical characterteristics which should prove valuable in reducing the food safety hazard associated with *E. coli* O157:H7 in fresh ground beef.

Chapter 3

MATERIALS AND METHODS

3.1 Controlled release of AIT from fats and oil

Controlled release of AIT from fats and oil was analyzed using the modified methods of Lim and Tung (1997) and Kim et al (2002). In this experiment, 5 ml of melted butter, shortening, lard, beef tallow (from a local butcher shop), and corn oil were mixed with 0.5 ml of 95% pure allyl isothiocyanate (Aldrich Chemical Company, WI) and vortexed for 30 s. Then 0.4 ml of each mixture was placed inside ten 2 ml volume screw-capped vials (Aglilent, USA). The vials were tightly closed using caps with rubber septum provided and stored at -18, 4 and 22°C. At five min intervals a single vial from each treatment was removed and headspace gas was immediately injected using an airtight syringe (Varian 03-918986-ob, Walnut Creek, CA) into the gas chromatograph with an auto sampler as noted below.

3.1.1 GC Analysis of AIT in the headspace

A gas chromatograph (Varian Star 3400cx Varian Chromatography Systems, Walnut Creek, CA) equipped with a flame ionization detector and column measuring 30 m x 0.25 mm internal diameter, 0.25 μm wall thickness (J&W DB5MS) was used to determine the amount of AIT released into the headspace from the fats and oil. An air sample of 500 μl was withdrawn from the headspace of each vial and injected immediately into the GC. The operating conditions used were a column temperature of 60°C, initially, which was increased at a rate of 12.5°C/min to 90°C and maintained for

45 s. The injector and the flame ionization detector temperature were programmed to operate at 250°C. Hydrogen was used to fuel the flame at the detector. Helium was used as the carrier gas. Total running time was 4 min. Resulting peaks including that of AIT were identified using Varian star chromatography software (Walnut Creek, CA).

3.2 Identification of fatty acids in fats and oil

Identification of fatty acids in fats and oils were determined by using modified methods of Bligh and Dyer (1959) and Kramer et al. (1997). Butter, lard, beef tallow, and shortening were melted in a microwave oven for 30 s. One ml of each of the melted fats was pipetted into a teflon lined tube (28.5 x 104 mm). Chloroform:methanol (2:1) was added. The corn oil was not subjected to the chloroform extraction because of its liquid nature. All the solutions were then homogenized for 30 s with a polytron (Macalaster Bicknell Co. New Haven, CT). After homogenization, 6 ml of 0.7% aqueous NaCl was added and the mixture centrifuged at 1000 ppm for 10 min. The supernatant was removed using a Pasteur pipette. The remaining liquid was transferred to a teflon lined tube (30 ml). The tubes were then heated at 50°C for 20 min to evaporate the solvent. Following evaporation, 1 ml of iso-octane was added and vortexed to solubilize the lipids. Subsequently, 6 ml of 0.5 N NaOH was added and the samples were placed inside an oven at 50°C for 5 min. Solutions were then vortexed and heated further at 50°C for 10 min. The solutions were then cooled to room temperature and 2 ml of iso-octane plus 3 ml of 10% acetic acid were added. The bottles were centrifuged at 2000 rpm (Beckman L8-M refrigerated centrifuge, Lab Trader, Vista CA) for 5 min. Following centrifugation the supernatant was carefully removed using a Pasteur pipette and placed inside GC analysis glass vials (2ml). The lids were tightened and placed on the GC trays.

3.2.1 GC analysis for fatty acids

A Hewlett-Packard model 5890 GC equipped with an FID detector was used to analyze the fatty acids. The column used (SP 256) was 100m long and 0.25 μm in diameter. The gases used in this experiment were nitrogen, helium and hydrogen. The operating condition for the GC was initially 70°C for 2 min which was increased at a rate of 15°C/min to 155°C for a total of 25 min. The temperature was then increased to 215°C at a rate of 3°C/ min and maintained for a maximum of 8 min. Peaks obtained from the GC were recognized using an internal standard (methyl heptadeceanoate in iso-octane solution) (NU-Chek-Prep, Inc., Elysian, MN).

3.3 Permeability of AIT through various plastic packaging materials

Three types of plastic materials were used to analyze the permeability of AIT. Plastic bags used were Deli*1, composed of Nylon/EVOH/Polyethylene and a commercial bag made with polymers ESXE 120R and MESE 1250R (both from Winpak, Winnipeg, MB), also Ziplock® freezer bags (Dupont, ON). The thickness of the Deli*1 bag wall was 75 μm, oxygen transmission rate over 24 h at 23°C was 2.3 cc/m^2 and the moisture vapour transmission at 24h, 37.8°C, 90% RH was 7.8 g/ m^2. The ESXE 1250 and MESE 1250 bags were composed of 1.27 x 10^{-2} mm

Polyester/PVDC/5.08 x 10^{-2} mm EVA copolymer with 1.27 x 10^{-2} mm metalized Polyester/5.08 x 10^{-2} mm EVA copolymer. The thickness of the bag wall was 62 µm, its oxygen transmission rate at 24 h/23°C was 7.8 g/m^2 and its moisture vapour transmission at 24 h, 37.8°C, 90% RH was 6.0 g/m^2. Ziplock® freezer bags were purchased from a local supermarket. A mixture of 1 ml of AIT and 5 ml of corn oil were vortexed for 30 s, then 0.1 ml of AIT and corn oil mixtures were poured in a glass Petri dish, placed inside the plastic bags and heat-sealed with a vacuum packaging machine (Model GM-2000, Bizerba Canada Inc, Mississauga, ON). The bags were stored at 4 or 22°C for 41 days. At days 1, 2, 5, 7, 12,16, 23, 30 and 41, 3 bags of each treatment were removed from storage and 0.5 ml of headspace was withdrawn by piercing the bag with an airtight syringe (Precision Sampling Corp, Baton Rouge, LA) and injected into the Varian gas chromatograph.

3.4 Ground beef preparation

Fresh beef (chuck roast) was purchased from local butcher shop. The meat was stored at 4°C overnight. On the day of the experiment the meat was kept at -18°C for 3h until the outer surface was frozen. The outer layer (2 mm) of meat was then trimmed away using a stainless steel knife sanitized with 200 ppm aqueous chlorine solution. The meat was then cut into 5x5 cm pieces and coarse ground through a 1.2 cm diameter die plate affixed to an electric meat grinder (Model 84142, Hobart Manufacturing Company, Tory, Ohio). After coarsely ground beef was held at 4°C for 1 h, it was inoculated and re-ground as described in section 3.6. All meat contact surfaces including aluminum trays were washed with detergent, and rinsed with distilled water. Grinder parts and trays were wrapped in aluminum foil and sterilized at 121°C at 17 psi for 15 min and cooled to room temperature before each use.

3.5 Bacterial strain preparation

E. coli O157:H7 strains 7128, 7110, 7220 (human isolates), and 7282 and 7283 (hamburger isolates) were provided by the Laboratory Centre for Disease Control, Ottawa, Canada. All the strains were routinely preserved in glycerol and stored at –80°C. Strains were activated by two consecutive transfers in 5 ml portions of trypticase soy broth (BBL, Becton Dickinson) and incubated at 35°C for 24 h. Following incubation, 30 ml of each *E. coli* culture was centrifuged at 12,000 rpm for 15 min at 10°C (Sorvall RC-5 refrigerated centrifuge, Du Pont, Newtown, CT). The supernatants were discarded and each precipitate was washed once in 30 ml 0.1% peptone water (Sigma, St. Louis, MO) and re-centrifuged at 12,000 rpm for 15 min, at 10°C. The cultures were then re-suspended in peptone water and diluted to an optical density (OD) of 0.3 at 600 nm (Ultrospec 2000, Pharmacia Biotech Inc., Baie d'Urfe, QC). The final inoculum consisting of 50 ml TSB was inoculated with 50 µl of each of the strains (at 12 h growth) and incubated at 35°C without shaking for a further 12 h. The approximate bacterial population at 0.3 OD and 600 nm was 8 $\log_{10}$ colony forming units per ml (cfu/ml).

3.6 Preparation of inoculated patties for AIT

Coarsely ground beef weighing approximately 5 kg was inoculated with a mixture of an equal concentration of each 5 *E. coli* strains (approximately 20 ml of each strain) to yield a final concentration of either 3 $\log_{10}$ cfu/g or 6 $\log_{10}$ cfu/g. The meat was then re-ground using a 0.8 cm diameter die plate affixed to a manual meat grinder to achieve uniform distribution of *E. coli* in the meat. Each hamburger patty was formed by hand with 100g of meat (approximately 10 cm diameter and 1 cm height) and placed singly inside the plastic bag. Each experimental treatment was replicated three times.

3.7 Treatment of ground beef patties with AIT

A mixture of 0.7 ml AIT (94% pure allyl isothiocyanate, Acros organics, Geel, Belgium) was made with 0.3 ml of corn oil and vortexed for 20 s and then added to a filter paper of 10 cm diameter (Whatman No.1, Struers Kebo Lab, Denmark), which was then placed on top of a ground beef patty. The patty was placed in a Deli*1 bag and gas flushed with 100% N_2. The bags were heat sealed with the vacuum packaging machine and stored at 4, 10 and –18°C for 8, 21 and 35 days, respectively.

3.8 Inoculation of bacteria for the mustard experiment

Ground beef was prepared similarly as in the AIT experiment except that de-hulled non-deheated mustard powder at a final concentration of 5, 10, and 20% (Newly Wed Foods, UFL Division, Edmonton, AB) was added to 5 kg of coarse ground beef then mixed by hand using sterile gloves and ground again through the 1.2 cm diameter die plate using the electric meat grinder. The meat weight was adjusted to 5 kg and inoculated with an equal number of each of 5 strains of *E. coli* (about 20 ml of each strain) to yield a final concentration of 3 $\log_{10}$ cfu/g or 6 $\log_{10}$ cfu/g. The meat was then re-ground using a 0.8 cm diameter die plate equipped manual meat grinder to achieve an even distribution of *E. coli*. The ground beef was formed into 100g patties and placed singly in Deli*1 bags, which were backflushed with 100% N_2, heat sealed and stored at 4°C for up to 21 days.

3.9 Microbial analysis of patties

Eleven g of meat from each patty was randomly chosen, weighed in a sterile stomacher bag (Fisher Scientific Ltd., Nepean, ON) mixed with 99 ml of peptone water (0.1%) and homogenized in a stomacher (Model 400, Seward, London) for 30 s. Each sample was serially diluted with peptone water and total plate counts were determined using trypticase soy agar (BBL, Becton Dickinson). Viable *E. coli* O157:H7 were determined using Sorbitol MacConkey (Difco) agar supplemented with cefixime (50 µg/l) and potassium tellurite (2.5 mg/l) (Dynal Inc., Lake Success, NY) (CT-SMAC), while lactic acid bacteria were determined on MRS lactobacilli agar (Difco). The trypticase soy agar (TSA) plates were incubated at 22°C for 48 h while CT-SMAC agar plates were incubated at 35°C for 48 h. The MRS agar was incubated anaerobically using Gas Pak Jars (BBL, Becton-Dickinson) with the Gas Pak plus Anaerobic system using a palladium catalyst at 25°C for 48 hours. All colonies were counted on a Quebec colony counter. At each time point three samples were plated in duplicate.

3.10 Detection of low levels of *E. coli* in ground beef using Immunomagnetic Separation (IMS)

Ground beef patties were prepared similarly as in the earlier mustard experiments except that only 5% mustard was used in this test. Coarse ground meat weighing 2.5 kg was inoculated with an equal mixture of 5 *E. coli* strains (approximately 15 ml of each strain) to yield a final concentration of 14 cfu/g to 40 cfu/g. The meat was then re-ground using a 0.8 cm die plate attached to a manual meat grinder to achieve uniform distribution of *E. coli* in the meat. Each hamburger patty was made with 100g of meat (approximately 10 cm dia and 1 cm thick). Patties were placed individually in Deli*1 bags, backflushed with 100% N_2, heat-sealed and held 6 d at 4°C. At each time point samples were analyzed in triplicate. For analysis of *E. coli*, 25g of ground beef was added to 225 ml of buffered peptone (BBL, Becton Dickinson) in a stomacher bag and pummeled for 1 min. The bags were then incubated at 35°C for 6 h. After incubation the bag was re-stomached for 1 min. One ml of liquid was transferred into the IMS with magnetic beads coated with antibody against O157:H7 (Dynal Oslo, Norway) according to the manufacturer's instructions. Concentrated samples were plated on CT-SMAC and plates were incubated at 35°C for 24 h.

3.11 Determination of fat (%) content in ground beef

Ground beef samples were dried in a freeze dryer (Virtis Co., Gardiner, NY) for 72 h. The crude fat was determined using the Soxhlet method (AOAC 1975). Five grams of pre-dried samples were placed in the thimbles and covered with glass wool. The thimbles were fixed inside the extracting tubes. Two hundred fifty ml flat bottom flasks containing several boiling chips were heated at 125°C for 30 min. Flasks were then cooled to room temperature in a desiccator then weighed on an analytical balance. One hundred fifty ml of hexane was poured into the bottom flask. The extraction tube was assembled with the round bottom flask and the Soxhlex system was refluxed for 1 h. Following reflux, the round bottom flask was placed in a heating mantle and the left over hexane was evaporated. The flasks were then placed inside a drying oven for 1 h at 100°C. After drying, flasks were placed inside a desiccator to reach room temperature. The flasks were weighed on an analytical balance. The fat content was determined based on the following calculation on a dry matter basis

$$\% \text{ crude fat} = (\text{g}_{\text{crude fat}} / \text{g}_{\text{samples}}) \times 100$$

3.12 Sensory Evaluation

3.12.1 Sample preparation

Fresh beef (chuck roast) was purchased from local supermarket. The meat was trimmed to remove visible fat and coarse ground using an electric meat grinder. As a control 4 teaspoons of salt was added to the ground beef. For the 5% mustard treatment, 50g of mustard powder (Newly Wed Foods, Edmonton, AB) along with condiments including four teaspoons of salt, 1 teaspoon of sugar, and 4 tablespoons of vinegar were mixed with 1 kg of ground beef. For the 10% mustard treatment, 100 g of mustard powder along with condiments including, 4 teaspoons of salt, 2 teaspoons of sugar, and 12 tablespoons of vinegar was mixed with 1 kg of ground beef. Condiments were evenly distributed by hand mixing. Hamburger patties were made from these mixtures and weighed 100g. The samples were then placed in a convection oven, which was preheated to 450°F (232°C). The patties were baked in the oven until the internal temperate reached 71°C. After baking, the hamburger patties were held at 60°C until they were served to the panelists. Each panelist received 3 samples of 10g of cooked hamburger served in a plastic cup coded with a three digit random number.

3.12.2 Sensory analysis

Seventy five students and staff from Faculty of Agriculture, University of Manitoba who had received no training in sensory evaluation took part in the study. A 9 point hedonic scale was used to evaluate overall acceptability where 1 = dislike extremely and 9 = like extremely. The samples were presented under white light. Each panelist received three separate samples each labeled with a three digit random number, plus water, an unsalted cracker, a fork and a napkin. The panelists were asked to taste the samples and record their impression of overall acceptability of each product on an accompanying ballot sheet (see Appendix 20). The data were analyzed using the ANOVA and t-test for mean difference.

3.13 Statistical analysis

All the chemical and microbial analysis were performed in triplicate. Data were analyzed using the Statistical Analysis System software program, version 8.1 (SAS Institute, Inc., Cary, NC). Microbiological data were analyzed by the general linear model (GLM) procedure and Duncan's multiple range tests with examination for significant differences ($p<0.05$) at each storage interval for individual treatments.

Chapter 4

RESULTS

4.1. Controlled release of AIT in fats and oil stored at –18, 4 and 22°C

The release or volatilization rates of AIT from corn oil, lard, shortening, beef tallow, and butter at –18, 4, and 22°C are presented in Fig. 4.1. AIT was released and achieved average levels of 79, 100, and 242 μg/ml in the headspace in 50 min at -18, 4 and 22°C, respectively. At –18°C beef tallow released the highest amount of AIT in the headspace while the corn oil released the least amount. At 4°C both beef tallow and shortening released the highest amounts. As temperatures were increased to 22°C lard permitted release of the highest amount of AIT followed by corn oil, shortening, butter, and beef tallow. Differences between lard and beef tallow were substantial. AIT released from fats and oil reached equilibrium in the headspace after 10 min and levels were maintained up to 50 min at all three temperatures.

4.2 Fatty acid analysis of fats and oil

The fatty acid composition of corn oil, lard, shortening, beef tallow and butter are shown in Table 4.1. All of the fats and oil analyzed for fatty acids contained greater than 10% of oleic (C18:1 9c) and palmitic (C16:0) acids. Stearic acid (C18:0) was found at levels of <5% in lard, shortening, beef tallow, and butter. Cis-oleic acid (C18:1 9c) was present at the highest level in lard (39.8%), shortening (32.6%), and beef tallow (46.3%). Corn oil and shortening contained large amounts of linoleic (C18:2) 57.2% and

26.7%, respectively. Palmitic acid (C16:0) was present in the highest amount among saturated fatty acids present in all the oils tested. Corn oil contained the highest total concentration of unsaturated fatty acids of all the fats examined, followed by shortening, beef tallow, lard and butter.

Parameter estimate of linear regression between AIT concentration (µg/ml) and fatty acids composition (%) of five different lipids (Table 4.2). Using the linear regression procedure we attempted to find a correlation(s) between specific fatty acid presence and AIT levels in package headspace. Positive values represent repulsion (volatilization) while negative values represent stronger interaction between AIT molecules and fatty acids. At 22°C, C4:0 was positively and C8:0 was negatively correlated with high levels of AIT. However, at both 4°C and –18°C the trend was reversed.

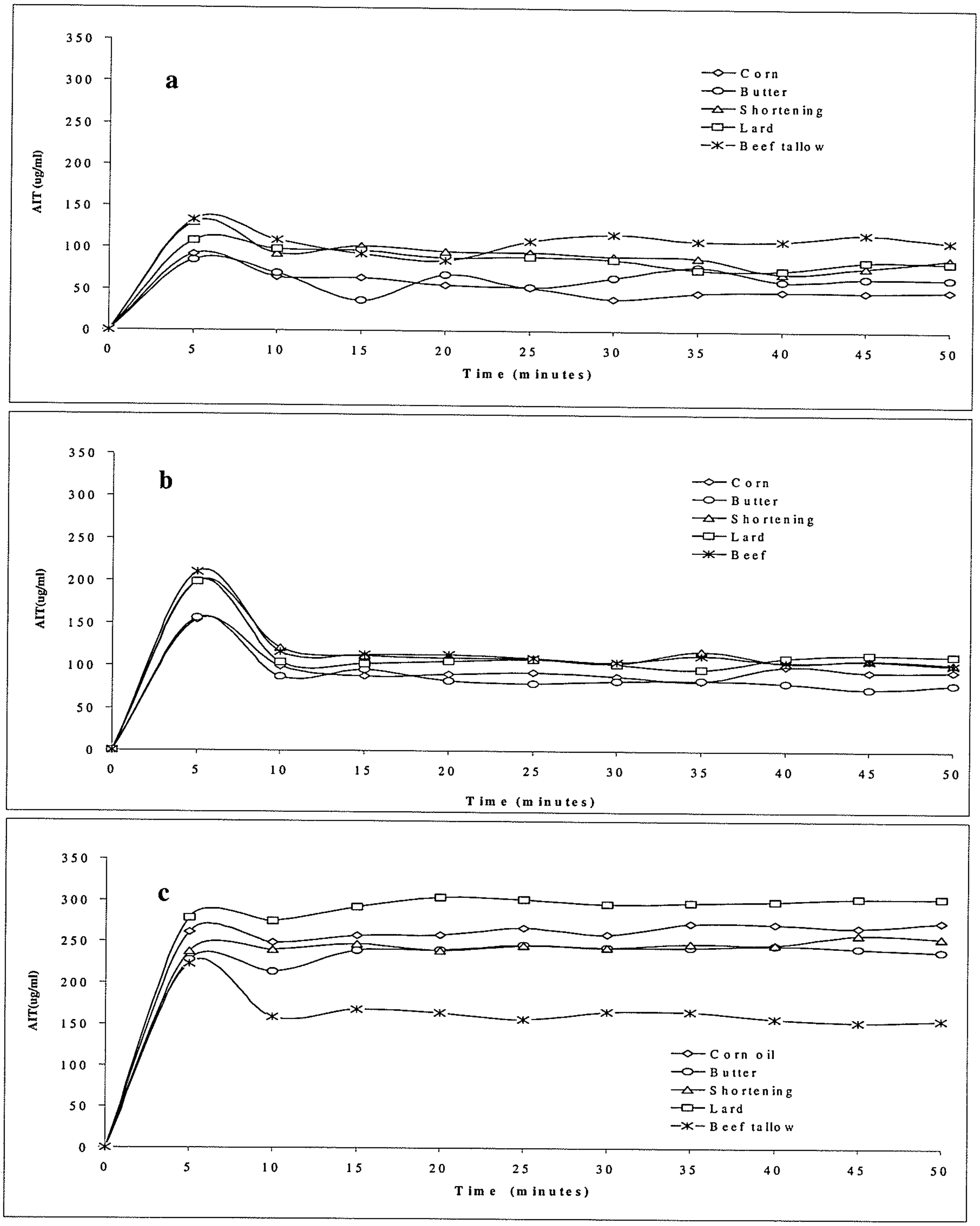

Figure 4.1. AIT released in the headspace from corn oil, lard, shortening, beef tallow, and butter stored at –18 (a), 4 (b), and 22°C (c). Headspace samples were analyzed at 5 min intervals for 50 min.

Table 4.1. Fatty acid analysis of various fats and oil

Fatty acids	Common name (acids)	Corn oil	Lard	Shortening	Beef tallow	Butter
				Fatty acids[1](%)		
C4:0	Butyric	-	-	-	-	2.37
C6:0	Caproic	-	-	-	-	2.88
C8:0	Caprylic	-	-	-	-	2.40
C10:0	Capric	-	0.12	-	0.07	3.60
C12:0	Lauric	-	0.12	0.12	0.08	3.72
C14:0	Myristic	0.04	1.47	0.37	2.55	11.28
C14:1	Myristoleic	-	-	-	0.83	1.05
C15:0		-	0.09	-	0.72	1.30
C16:0	Palmitic	10.36	25.15	18.92	23.16	28.72
C16:1	Palmitoleic	0.11	2.21	0.11	3.93	1.45
C17:0	Margaric	0.08	0.49	0.16	2.24	0.78
C17:1		1.73	1.73	2.33	1.89	2.33
C18:0		1.67	15.67	11.44	14.04	11.47
C18:1 11t		-	0.38	4.51	1.44	1.55
C18:1 9c	Cis-oleic	27.81	39.8	32.62	46.32	22.1
C18:1 11c		0.58	2.93	2.48	2.22	0.98
C18:1i		-	-	0.27	0.19	0.36
C18:2	Linoleic	57.20	9.17	26.70	0.92	2.34
C20:0	Arachidic	0.52	0.40	1.62	0.21	0.58
C18:3	γ-linolenic	0.92	0.91	-	0.20	0.19
C20:1	Eicosenic	0.29	0.97	0.25	0.38	0.09
C18:2 10t 9c		0.17	-	-	-	-
C18:2 29c 11c		0.23	-	-	-	-
C18:29c 11t		-	0.15	0.39	0.47	0.78

[1] Mean value of total fatty acids from C4:0 to C20:0, n=3

Table 4. 2. Parameter estimate of linear regression between AIT concentrations (μg/ml) and fatty acids composition (%) of five different lipids.

	Temperature		
Fatty acid	-18°C	4°C	22°C
C4:0	-89.8	-101.8	977.8
C6:0	81.9	-90.8	412.3
C8:0	171.6	192.8	-1331.4
C15:0			-267.7
C16:0		0.6	
C16:1	5.2	-3.1	31.4
C20:1	-18.9	-10.8	55.5
C18:0			-7.9
C18:2 10t 9c	-209.5		
C18:2		-0.40	
R^2	0.99	0.99	0.99

Model: $[AIT]_{Temp} = \beta_0+\beta_1 C_{4:0}+\beta_2 C_{6:0}+\beta_3 C_{8:0}+\ldots\ldots\ldots\ldots\ldots\ldots+\beta_{11} C_{18:12C11}+\varepsilon$
(SAS Model)

4.3 Analysis of permeability of AIT through various plastic packaging materials

Permeability of AIT through different plastic packaging materials is shown in Fig. 4.2. Nylon/EVOH/Polyethylene bags retained the greatest amount of AIT at 22 and 4°C over 41 d while Ziplock® bags retained the least amount under these conditions. AIT was retained significantly better by the nylon/EVOH laminate than by the other films (PET/PVDC) at 4°C. At 22°C the nylon/EVOH bags retained significantly higher ($p<0.05$) amounts of AIT in the headspace compared to polyester/PVDC laminate and Ziplock® bags. After 41 d, the nylon/EVOH film retained 74% and 73% of that added at time 0 when stored at 4°C or 22°C, respectively.

4.4. Analysis of AIT in the headspace from commercial AIT liquid and mustard powder

4.4.1 Commercial AIT liquid

AIT levels from a 94% pure concentrated liquid commercial preparation of AIT released into the headspace of ground beef patties packaged in Nylon/EVOH/Polyethylene film and stored at 4, 10, and –18°C for 21, 8 and 35 d are presented in Figs. 4.3, 4.4, and 4.5. In treatments where the lower volume (0.5 ml) of AIT was used, its levels decreased from an initial concentration of 662 μg/ml to 120.7 μg/ml during storage at 4°C for 21 d. When the higher volume (1 ml) was used, the initial levels of 783.1 μg/ml were reduced to 456 μg/ml after 21 d of storage at 4°C (Fig. 4.3). At 10°C when 1 ml was used, AIT decreased from 1666.8 μg/ml to 444.4μg/ml after 8 d of storage (Fig. 4.4). At –18°C when 0.5 ml was used, AIT decreased from

515.6 to 76.7 μg/ml. At the higher volume of 1 ml, levels of AIT decreased from 540 μg/ml at day 0 to 112.6 μg/ml after 35 d (Fig. 4.5). Temperature had a drastic effect on the volatilization of AIT with the greatest response at the higher temperature.

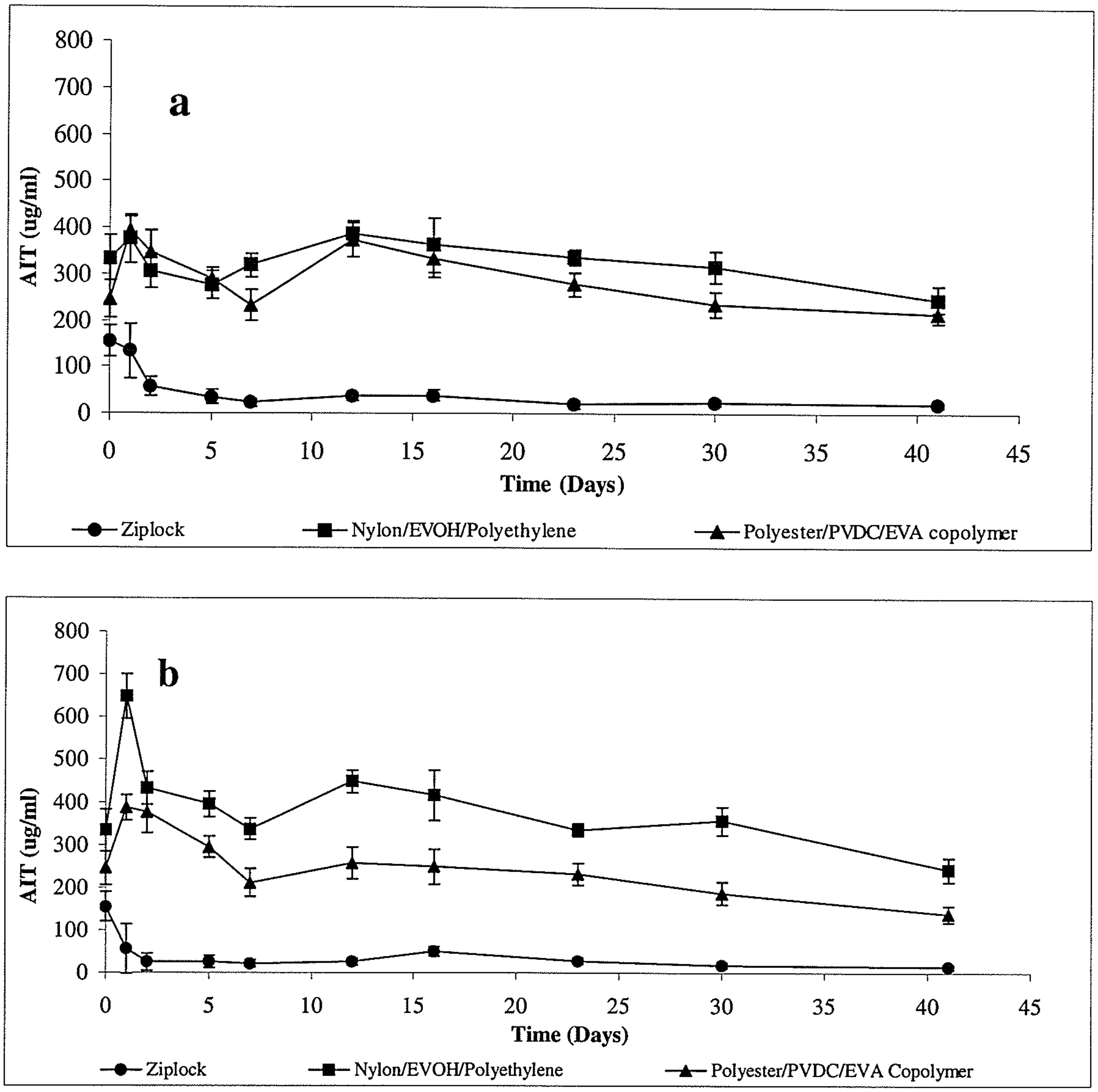

Figure 4.2. Permeability of AIT dissolved in corn oil through three types of plastic packaging materials stored at 4°C (a) and 22°C (b) for 41 days.

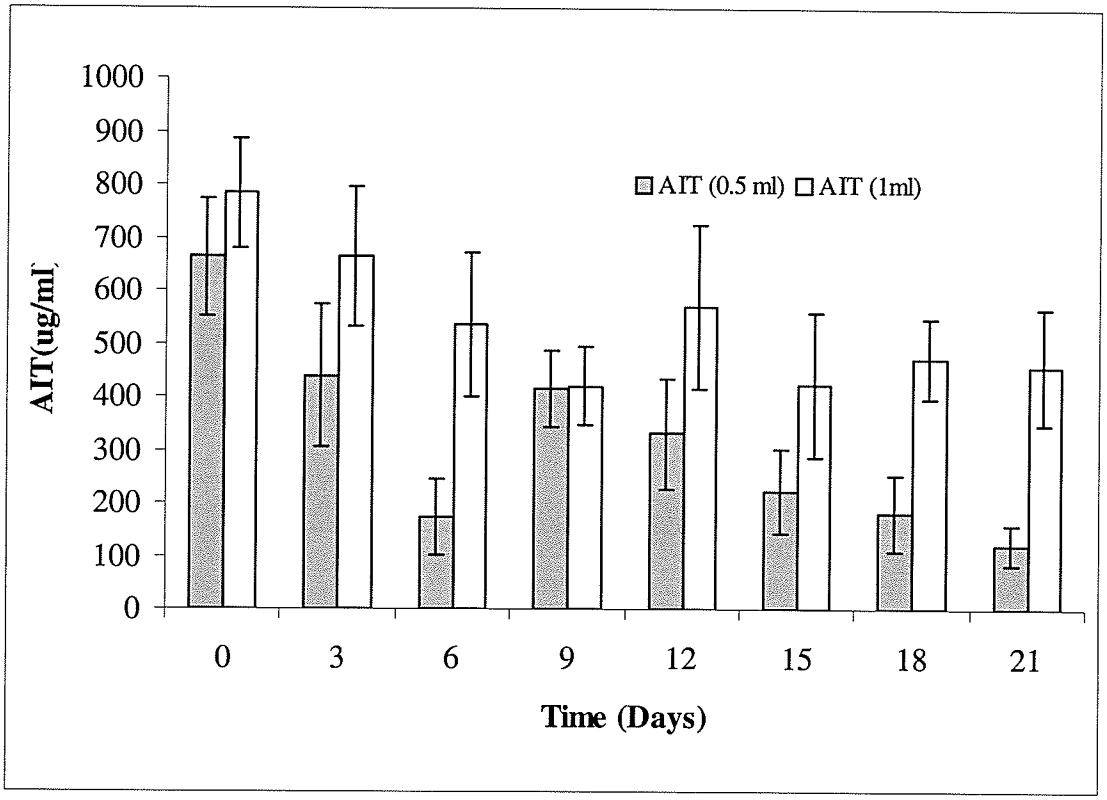

Figure 4.3. AIT levels from 94% pure commercial liquid AIT released into the headspace of packaged ground beef patties stored at 4°C for 21 days.

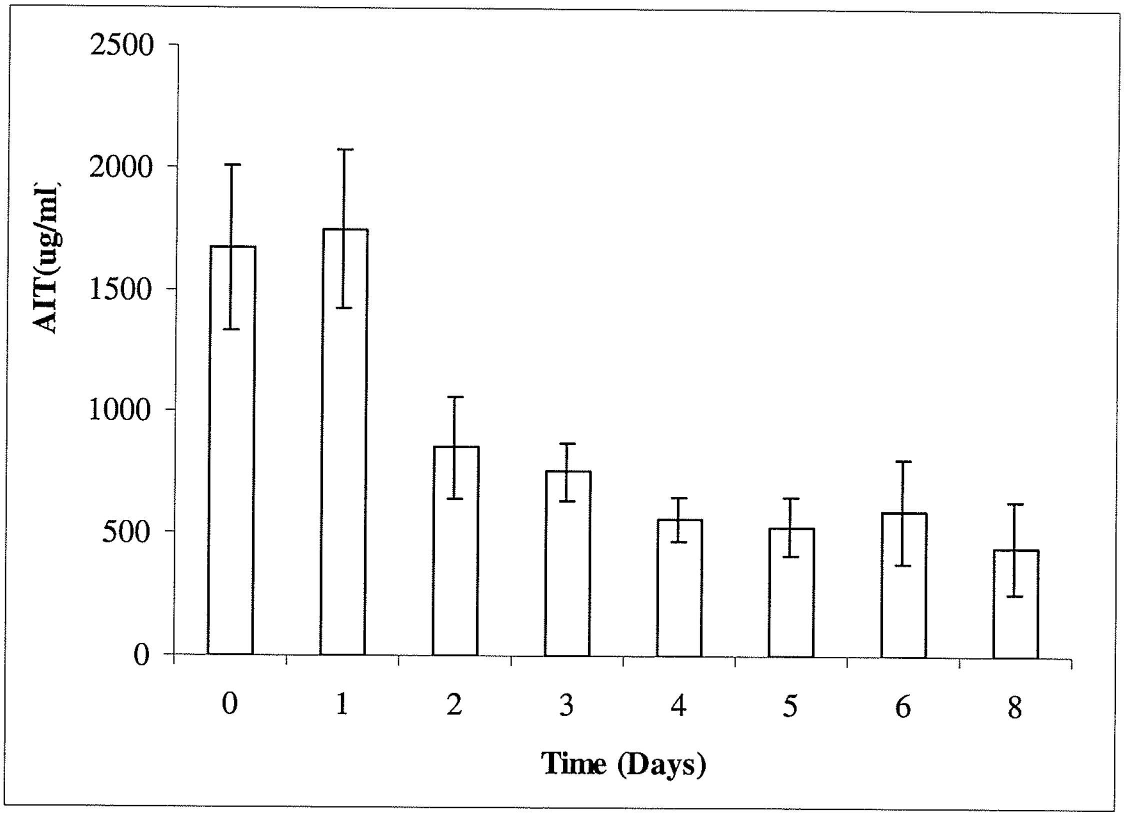

Figure 4.4. AIT levels from 94% pure commercial liquid AIT released into the headspace of packaged ground beef patties stored at 10°C for 8 days.

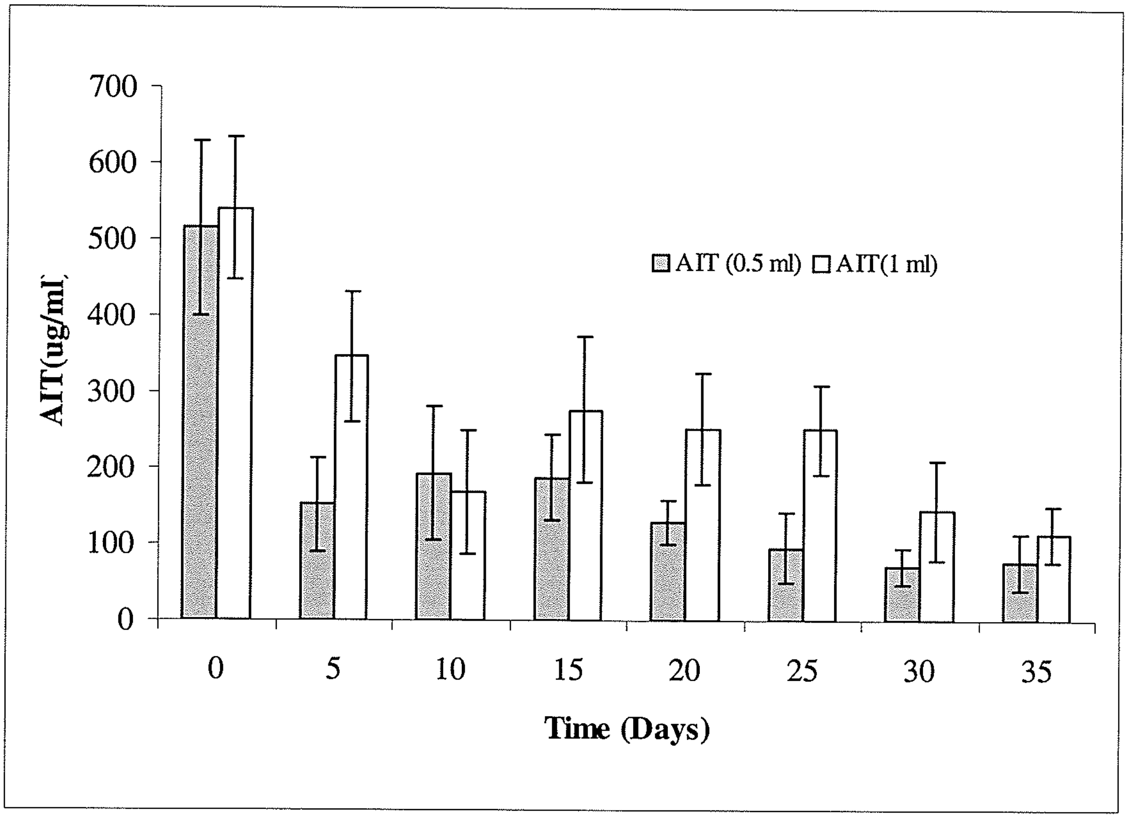

Figure 4.5. AIT levels from 94% pure commercial liquid AIT released into the headspace of packaged ground beef patties stored at –18°C for 35 days.

4.4.2. Non-deheated mustard powder

AIT levels from non-deheated mustard powder released into the headspace of ground beef patties packaged in Nylon/EVOH/Polyethylene film and stored at 4°C for 21 d are presented in Fig. 4.6. The initial AIT levels of 14.7, 19.0 and 57.2 μg/ml from 5, 10 and 20% mustard were found to be 19.6, 15.6, and 22.9 μg/ml after 21 d, respectively. Data indicated that in all three treatments, AIT headspace concentrations after 9 - 12 days reached an equilibrium of 17-30 μg/ml which was maintained up to 21 days of storage.

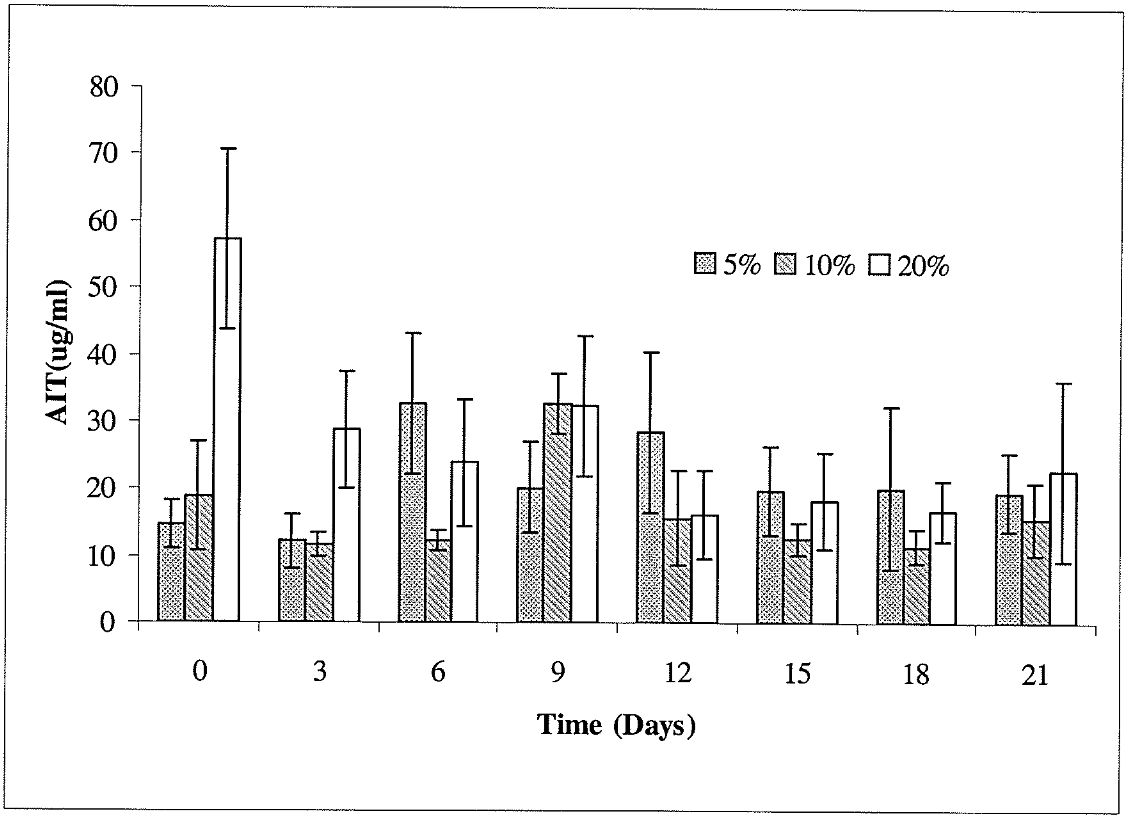

Figure 4.6. AIT levels released into the package headspace from non-deheated mustard powder formulated in ground beef patties stored at 4°C for 21 days.

4.5 Antimicrobial effects of AIT at 4, 10 and –18°C in ground beef patties

4.5.1 Natural microflora

Overall the average values of the total fat (%) content in ground beef were 26%. The antimicrobial effects of AIT on the natural microflora in ground beef patties stored at 4, 10, and –18°C are presented in Figs. 4.7, 4.8, and 4.9, respectively. At 4°C the lower AIT volume used (0.5 ml) did not show any significant effect on the natural microflora (Fig. 4.7a). At an AIT volume of 1 ml (Fig. 4.7b), numbers of naturally occurring bacterial cells present from day 6 to 18 were significantly lower ($p<0.05$) than the control. The antimicrobial effects of AIT (1 ml) in ground beef at 10°C were similar, but AIT was slightly less antimicrobial than at 4°C. At 10°C, the growth rate of natural microflora was reduced by AIT and the population was significantly lower except on days 3, 4, and 5 (Fig. 4.8). At –18°C, however, there was no significant difference between 0.5 and 1 ml AIT-treated samples at most points during 35 d of storage (Fig. 4.9). AIT did not affect the growth of the natural microflora in ground beef stored at 10 or –18°C, however, at the higher concentration of AIT at 4°C there was a delay in their growth (Fig. 4.7b). By 21 d of storage there was no difference between the control and the AIT treatment at each temperature.

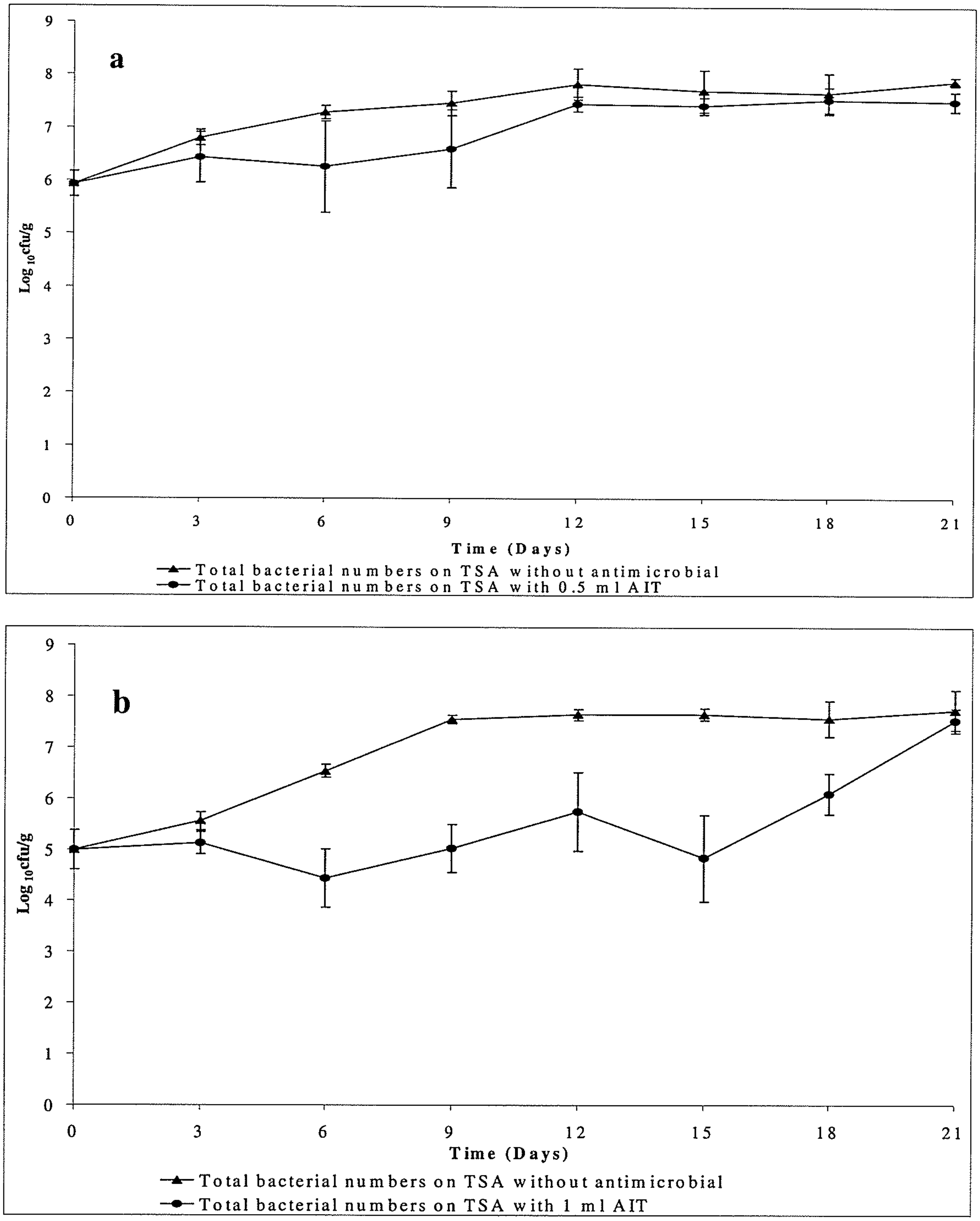

Figure 4.7. Antimicrobial effects of AIT (0.5 ml (a) and 1 ml (b)) on natural microflora in vacuum packed ground beef patties stored at 4°C and plated on TSA. Six replicates were used to generate the standard deviation bars.

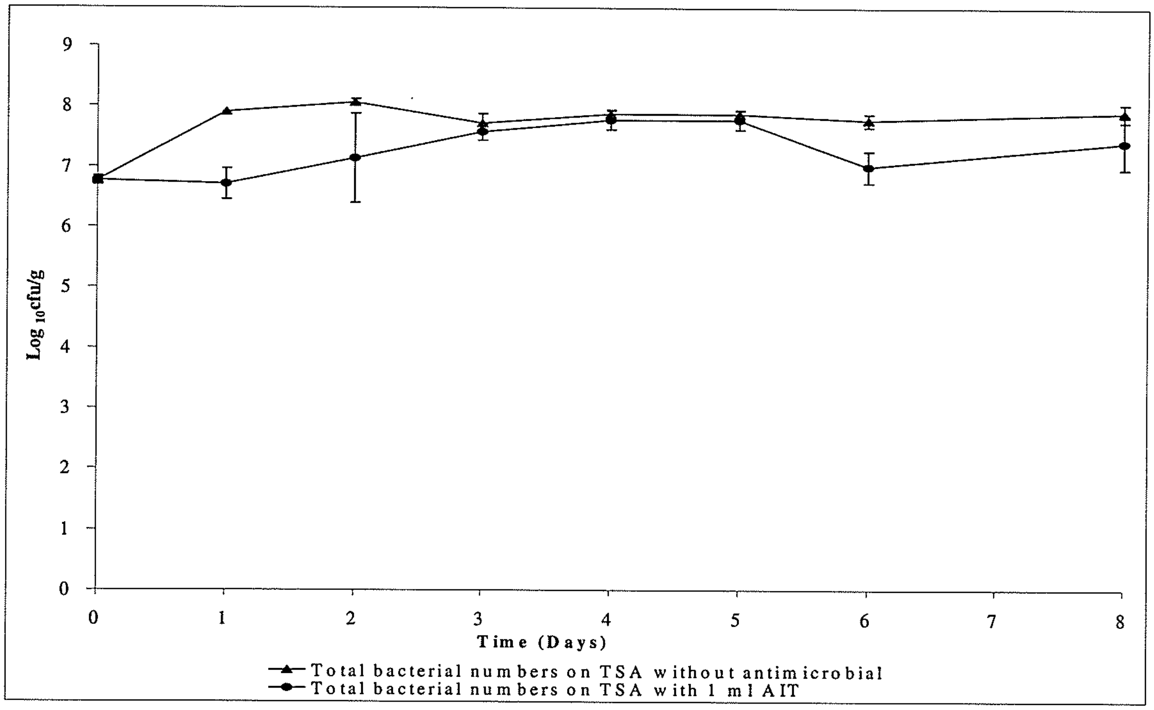

Figure 4.8. Antimicrobial effects of AIT (1 ml) on natural microflora in vacuum packed ground beef patties stored at 10°C and plated on TSA. Six replicates were used to generate the standard deviation bars.

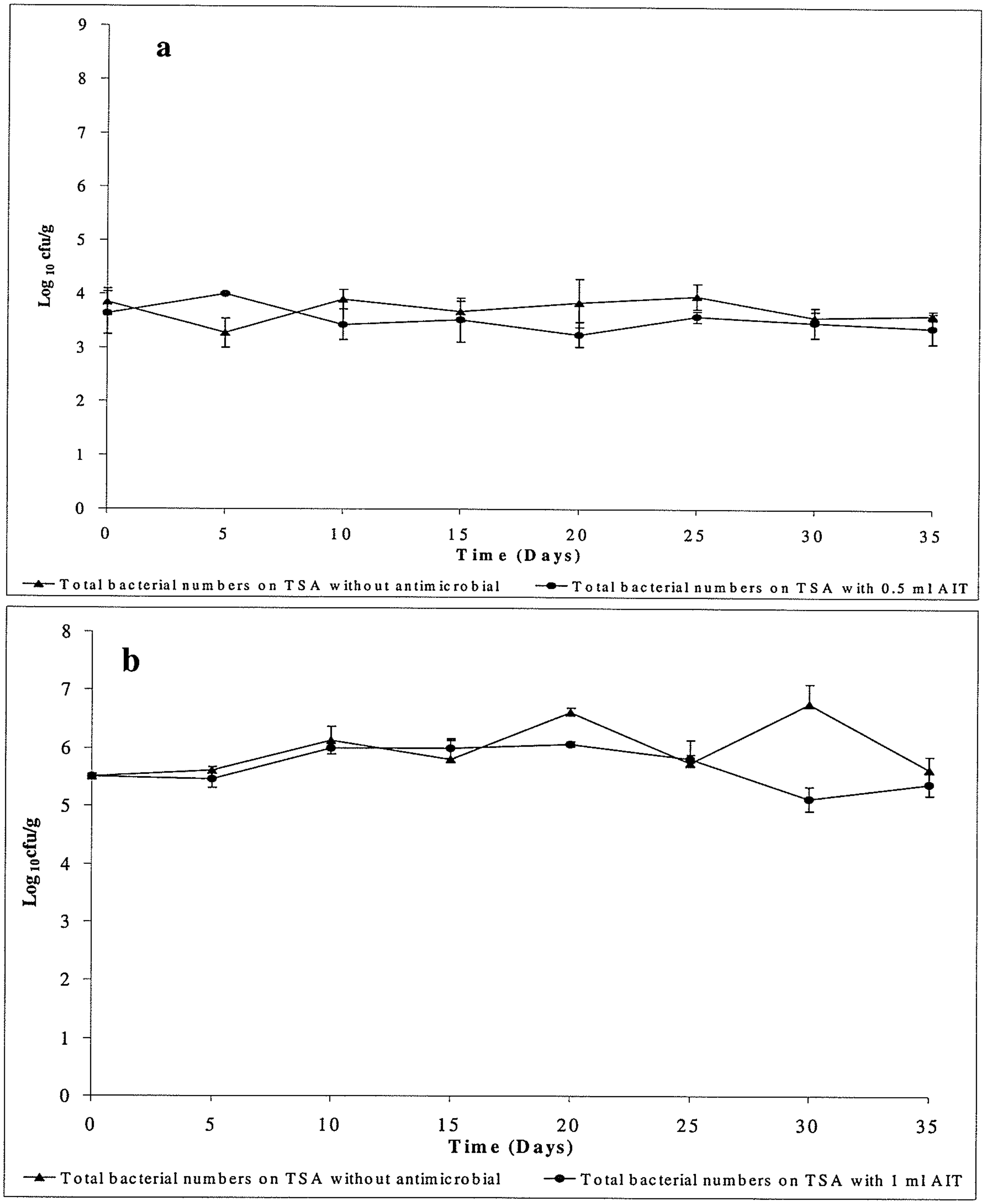

Figure 4.9. Antimicrobial effects of AIT (0.5 ml (a) and 1 ml (b)) on natural microflora in vacuum packed ground beef patties stored at –18°C and plated on TSA. Six replicates were used to generate the standard deviation bars.

4.5.2. Natural microflora and inoculated levels of 3 $\log_{10}$ cfu/g of *E. coli* O157:H7

The antimicrobial effects of AIT (0.5 and 1 ml) on the natural microflora of ground beef patties inoculated with 3 $\log_{10}$ cfu/g of *E. coli* O157: H7 during storage at 4, 10 and −18°C are presented in Figs. 4.10, 4.11, and 4.12, respectively.

At 4°C (Fig. 4.10a) the total bacterial population increased from 3.6 $\log_{10}$ cfu/g to 7.3 $\log_{10}$ cfu/g in the control and reached 6.5 $\log_{10}$ cfu/g in the AIT (0.5 ml) treated samples after 21 d. In AIT (0.5 ml) treated meat *E. coli* O157:H7 decreased from 3.3 $\log_{10}$ cfu/g initially to undetectable levels after 18 d. Increasing the AIT volume used to 1 ml also significantly ($p<0.05$) reduced the initial 3.6 $\log_{10}$ cfu/g of *E. coli* O157:H7 at a slightly faster rate and undetectable levels were observed as early as 12 d of storage with subsequent recovery of a small number at 15 d (Fig. 4.10b). AIT, at both 0.5 and 1 ml, was able to kill an average of 3.5 $\log_{10}$ cfu/g of *E. coli* O157:H7 while having only a slight inhibitory effect on natural microflora in the ground beef patties over 21 d when stored at 4°C. *E .coli* was able to survive for 21 d at 4°C in the untreated meat with only a slight reduction in its numbers.

At 10°C the total bacterial population was slightly reduced by AIT (1 ml) during 8 d of storage, but the *E. coli* O157:H7 levels on days 3 to 8 were significantly lower ($p<0.05$) than in the control. AIT reduced the *E. coli* O157:H7 population by 1 $\log_{10}$ cfu/g after 8 days of storage at 10°C (Fig. 4.11).

Antimicrobial effects of AIT (0.5 and 1 ml) at −18°C on total bacterial numbers were similar to those at 4°C. The lower volume of AIT reacted slowly, but 1.0 ml was more effective at –18°C in eliminating *E. coli.* The initial *E. coli* levels of 3.2 $\log_{10}$ cfu/g dropped and *E. coli* was undetectable after 35 and 10 days in 0.5 and 1 ml AIT treatments, respectively (Fig. 4.12).

AIT (1 ml) was able to eliminate greater than 3 $\log_{10}$ cfu/g of *E. coli* in ground beef patties stored at 4°C and –18°C within 18 and 10 d, respectively. At 10°C, *E. coli* were still detectable at 8 d when the test was terminated.

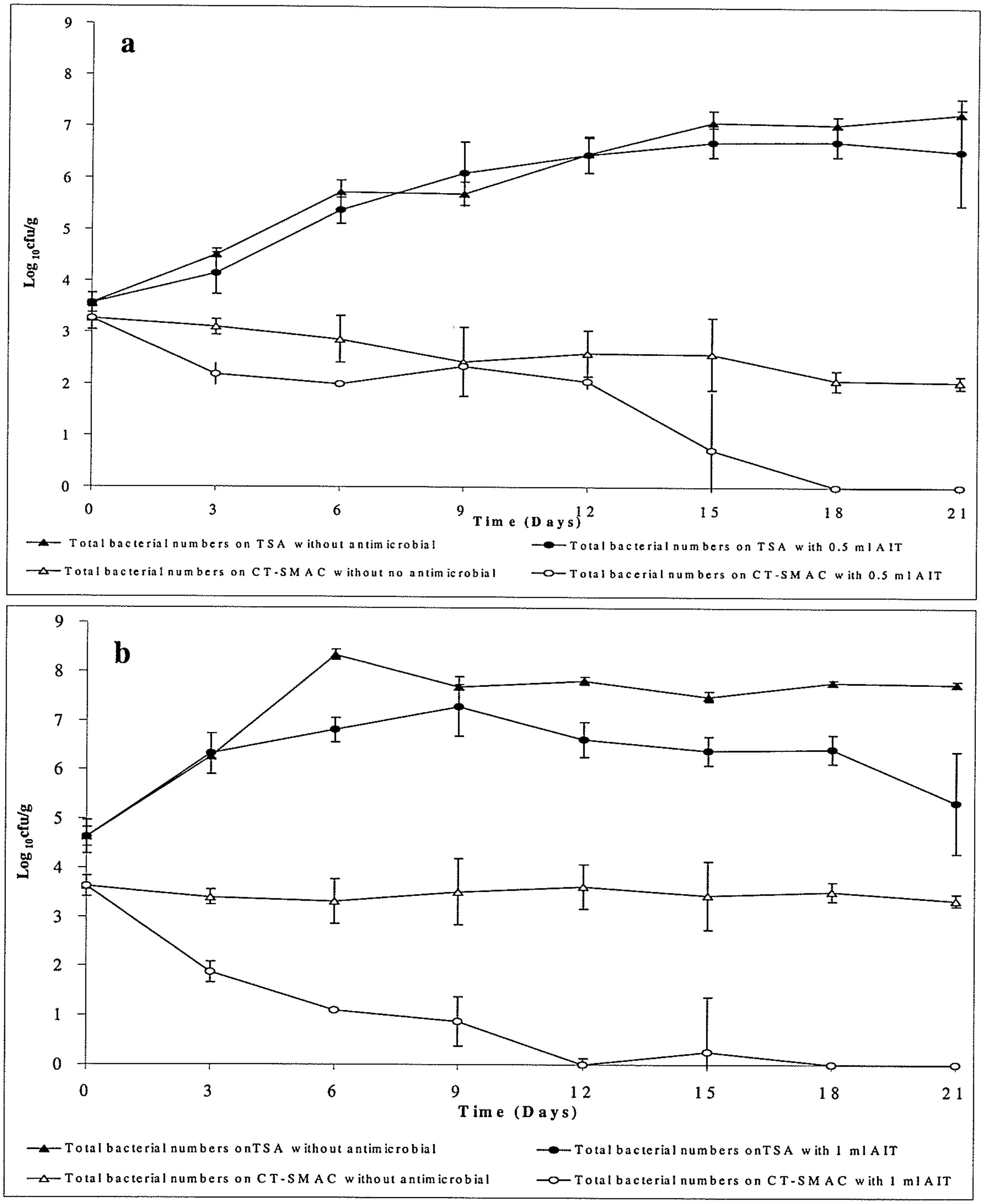

Figure 4.10. Antimicrobial effects of AIT (0.5 ml (a) and 1 ml (b)) on natural microflora and inoculated levels of 3 $\log_{10}$ cfu/g of *E. coli* O157:H7 in vacuum packed ground beef patties stored at 4°C and plated on TSA and CT-SMAC. Six replicates were used to generate the standard deviation bars.

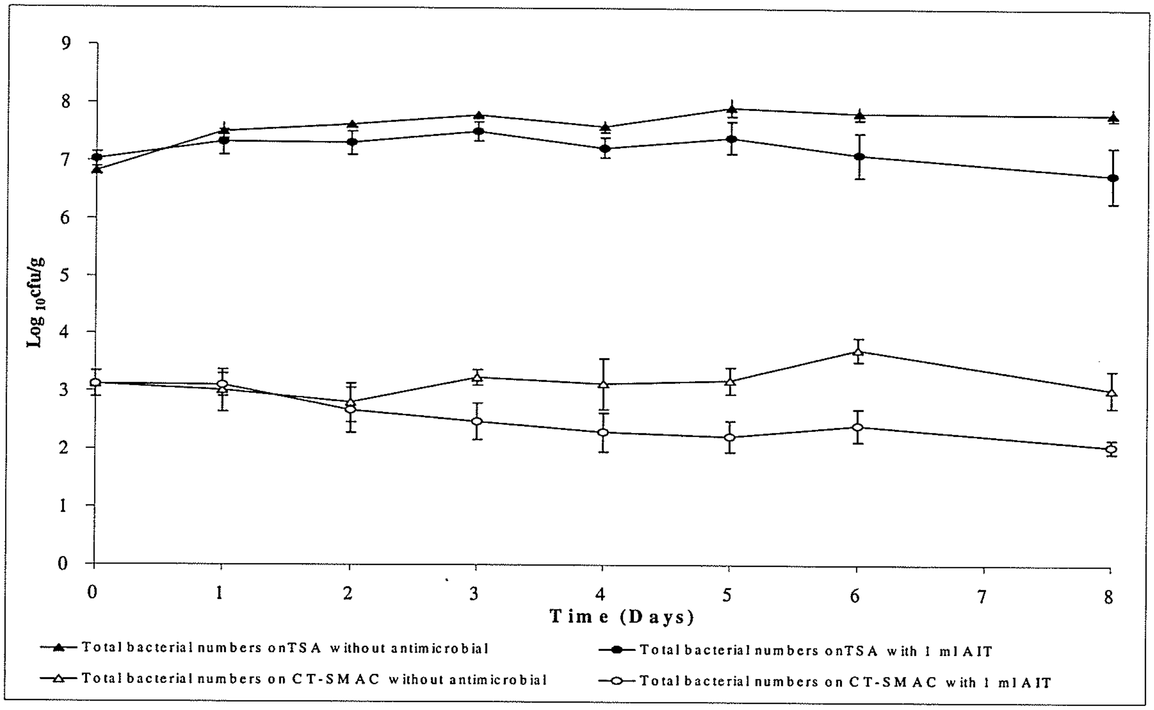

Figure 4.11. Antimicrobial effects of AIT (1 ml) on natural microflora and inoculated levels of 3 $\log_{10}$ cfu/g of *E. coli* O157:H7 in vacuum packed ground beef patties stored at 10°C and plated on TSA and CT-SMAC. Six replicates were used to generate the standard deviation bars.

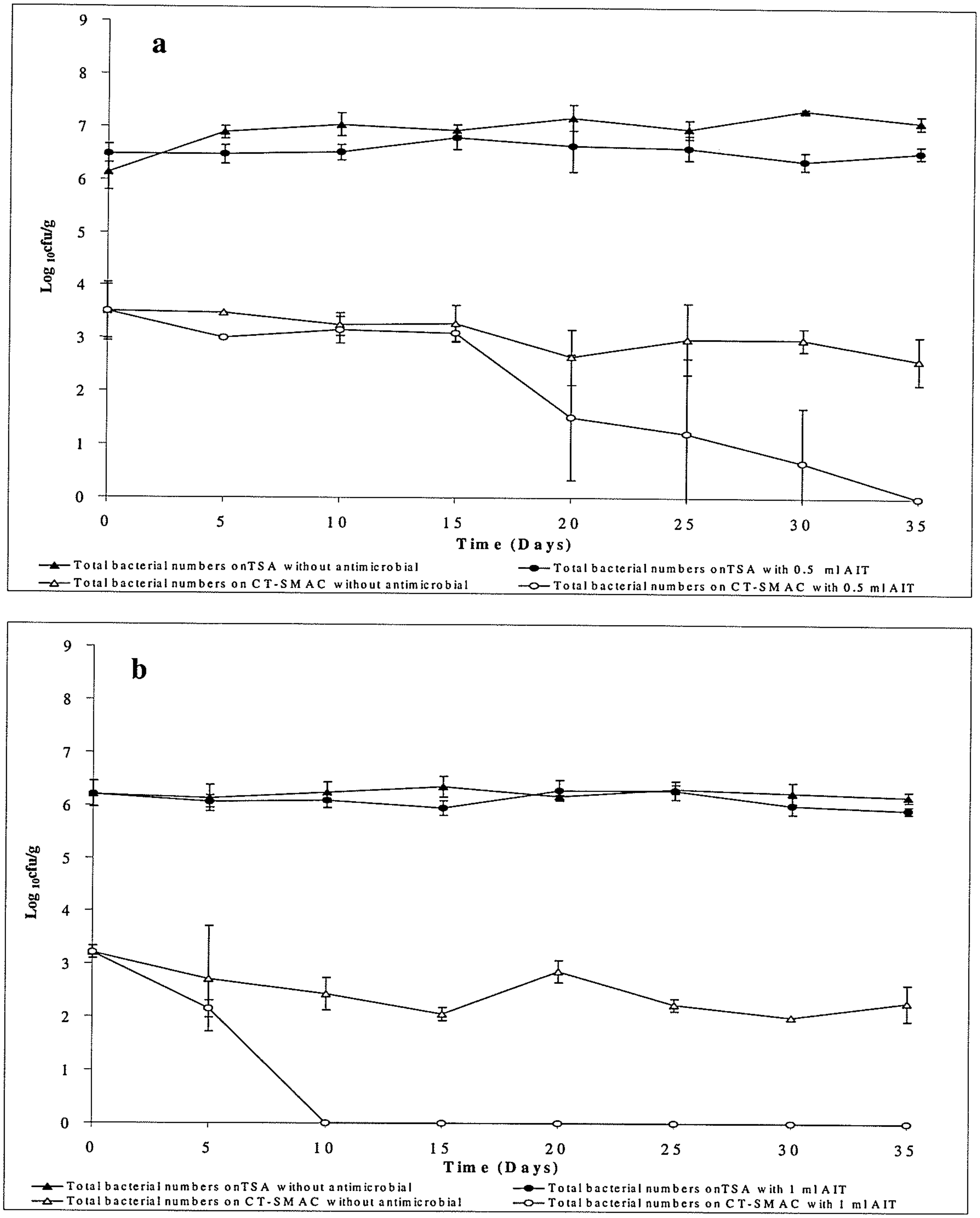

Figure 4.12. Antimicrobial effects of AIT (0.5 ml (a) and 1 ml (b)) on natural microflora and inoculated levels of 3 $\log_{10}$ *E. coli* O157:H7 in vacuum packed ground beef patties stored at –18°C and plated on TSA and CT-SMAC. Six replicates were used to generate the standard deviation bars.

4.5.3. Natural microflora and inoculated levels of 6 $\log_{10}$ cfu/g of *E. coli* O157:H7

The antimicrobial effects of AIT (0.5 and 1 ml) on the natural microflora and inoculated levels of 6 $\log_{10}$ cfu/g of *E. coli* O157:H7 in ground beef patties stored at 4, 10, and -18°C are presented in Figs. 4.13, 4.14, and 4.15, respectively.

AIT (0.5 ml) did not significantly affect the natural microflora or inoculated levels of 6 $\log_{10}$ cfu/g of *E. coli* O157:H7 in ground beef patties stored at 4°C. Increasing the volume of AIT to 1.0 ml caused a 3.4 $\log_{10}$ cfu/g reduction over 21 d when the initial level of *E. coli* O157:H7 was 6.2 $\log_{10}$ cfu/g. The total bacterial population in the AIT (1 ml) treatment was also significantly lower ($p<0.05$) than the control from days 6 to 21 (Fig. 4.13).

At 10°C, a 1 $\log_{10}$ reduction in *E. coli* O157:H7 occurred after 8 d of storage in the presence of AIT (1 ml) when the initial inoculum level of *E. coli* O157:H7 was 6 $\log_{10}$ cfu/g. The total bacterial number increased from 7.1 to 7.9 $\log_{10}$ cfu/g in the control, but dropped to 6.1 $\log_{10}$ in the AIT-treated samples at day 21 (Fig. 4.14).

At –18°C AIT (0.5 and 1 ml) did not reduce the natural microflora, but did inactivate more than 1 $\log_{10}$ cfu/g of *E. coli* O157:H7 after 35 d of storage (Fig. 4.15).

AIT was able to kill greater than 3 $\log_{10}$ of *E. coli* O157:H7 during 4°C storage but only 1 $\log_{10}$ at –18°C after 21 and 35 d, respectively. At 10°C the reduction was almost 1 $\log_{10}$ over the 8 d storage period.

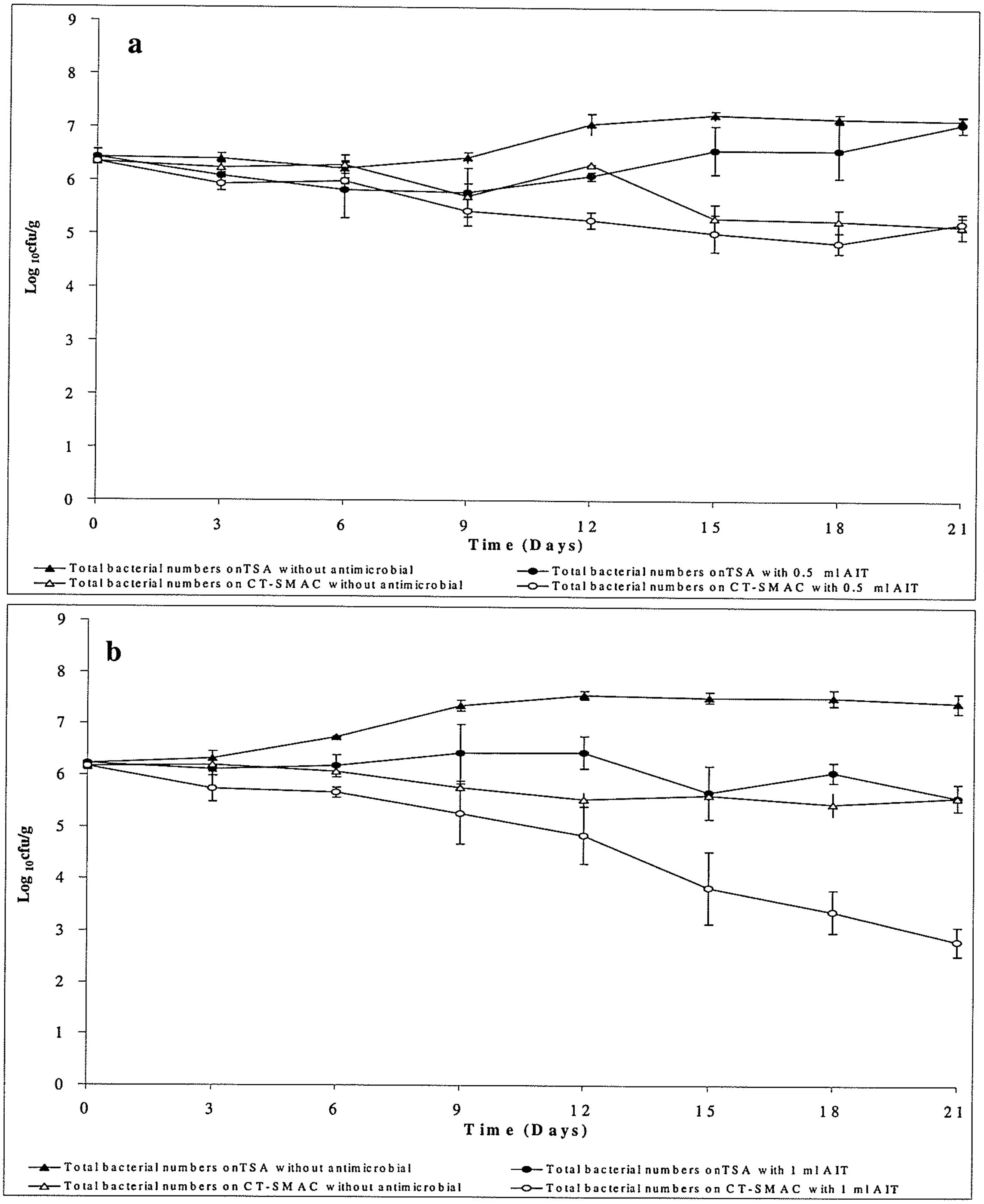

Figure 4.13. Antimicrobial effects of AIT (0.5 ml (a) and 1 ml (b)) on inoculated levels of 6 $\log_{10}$ cfu/g of *E. coli* O157:H7 in vacuum packed ground beef patties stored at 4°C and plated on TSA and CT-SMAC. Six replicates were used to generate the standard deviation bars.

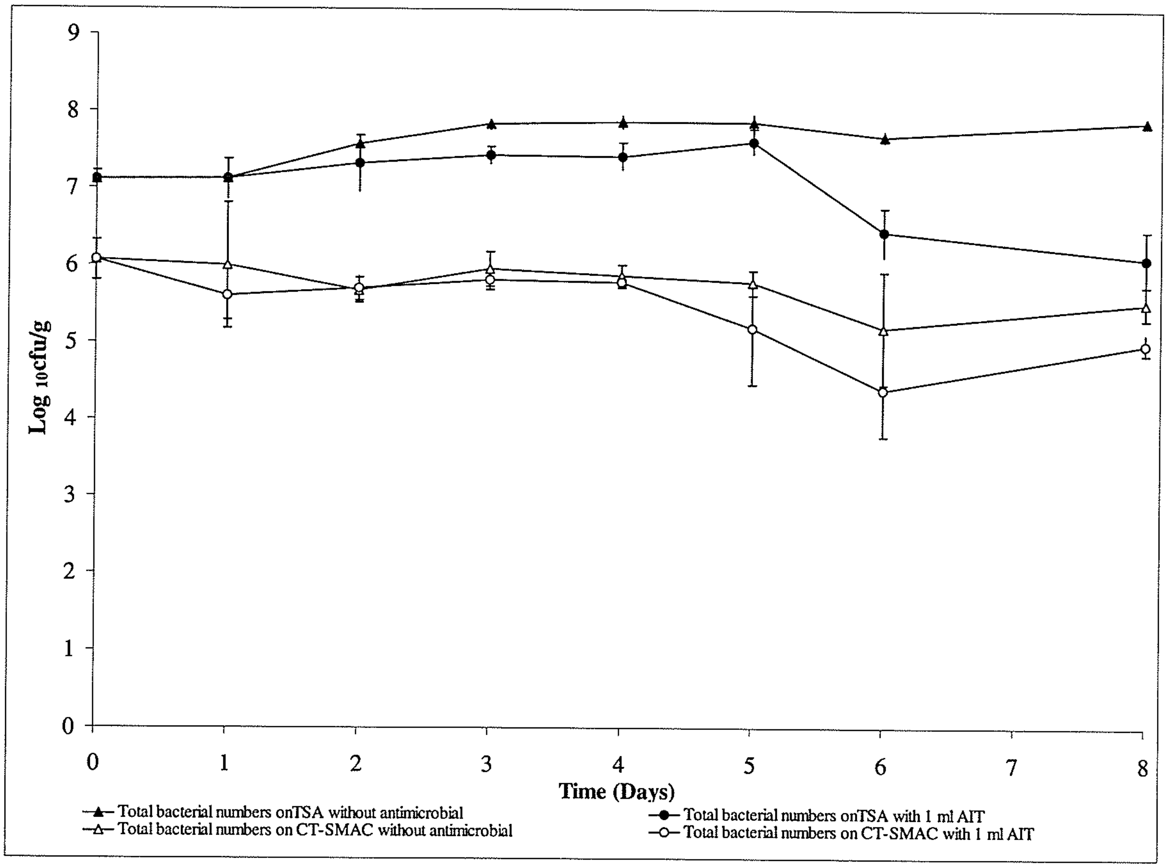

Figure 4.14. Antimicrobial effects of AIT (1 ml) on natural microflora and inoculated levels of 6 $\log_{10}$ cfu/g of *E. coli* O157:H7 in vacuum packed ground beef patties stored at 10°C and plated on TSA and CT-SMAC. Six replicates were used to generate the standard deviation bars.

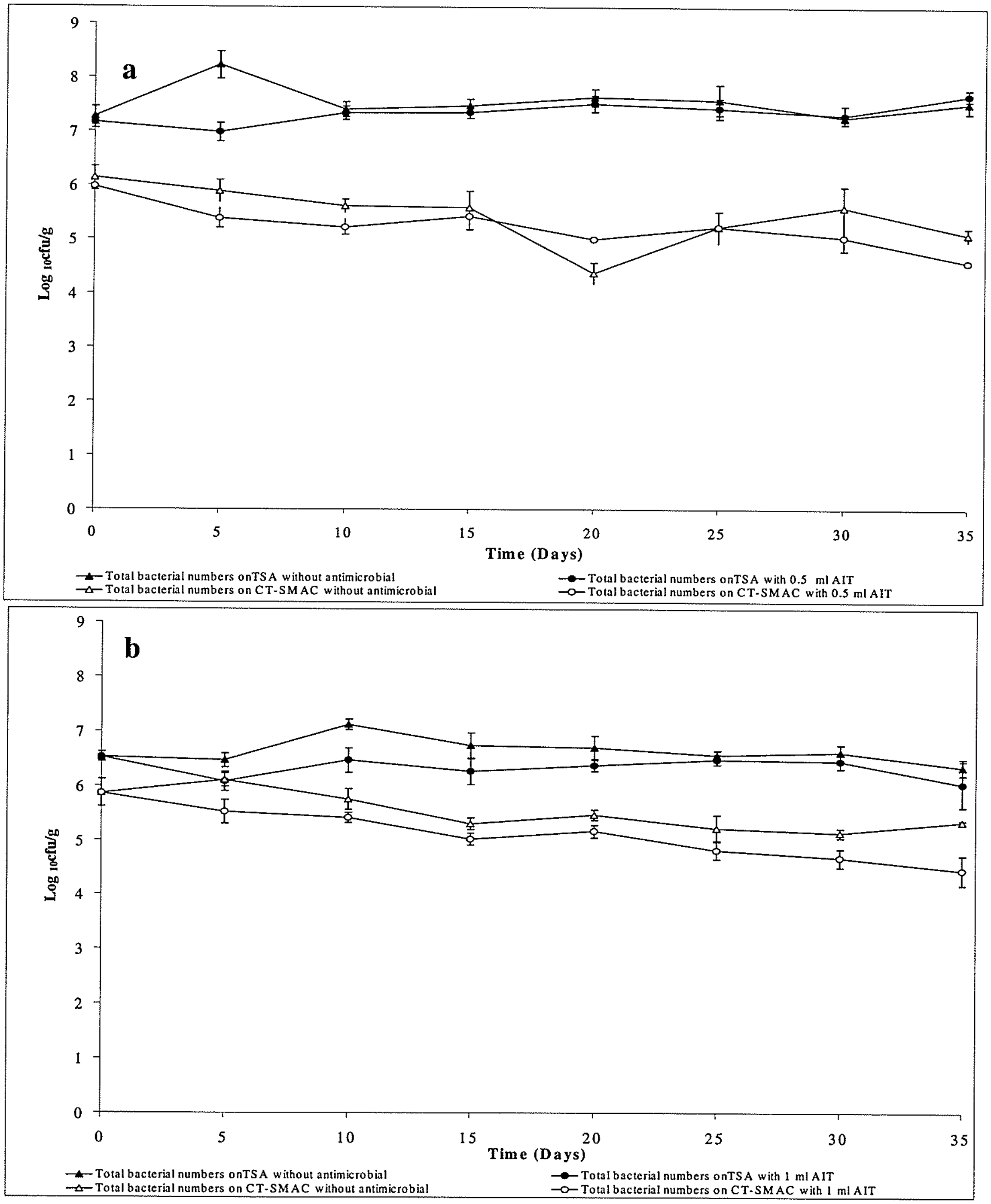

Figure 4.15. Antimicrobial effects of AIT (0.5 ml (a) and 1 ml (b) on natural microflora and inoculated levels of 6 $\log_{10}$ cfu/g *E. coli* O157:H7 in vacuum packed ground beef patties stored at –18°C, plated on TSA and CT-SMAC. Six replicates were used to generate standard deviation bars.

4.6. Antimicrobial effects of mustard powder at 4°C

4.6.1. Natural microflora

The antimicrobial effects of mustard powder at 5, 10, and 20% (w/w) on the natural microflora in ground beef patties stored at 4°C and plated on TSA are presented in Fig. 4.16.

The natural microflora in ground beef patties formulated with 5 and 20% mustard powder were significantly lower ($p<0.05$) on day 0 to 3 while in the 10% mustard treatment, numbers present on days 3 to 21 were significantly lower.

The antimicrobial effects of mustard powder at 10% (w/w) on the natural microflora in ground beef patties stored at 4°C and plated on MRS are presented on Fig. 4.17. The natural microflora in 10% mustard powder-treated meat plated on MRS was significantly lower on day 3, 12 and 21.

It is clear that mustard powder initially delayed growth of the spoilage microflora. However the mustard powder did not significantly affect the overall growth of the natural microflora in ground beef.

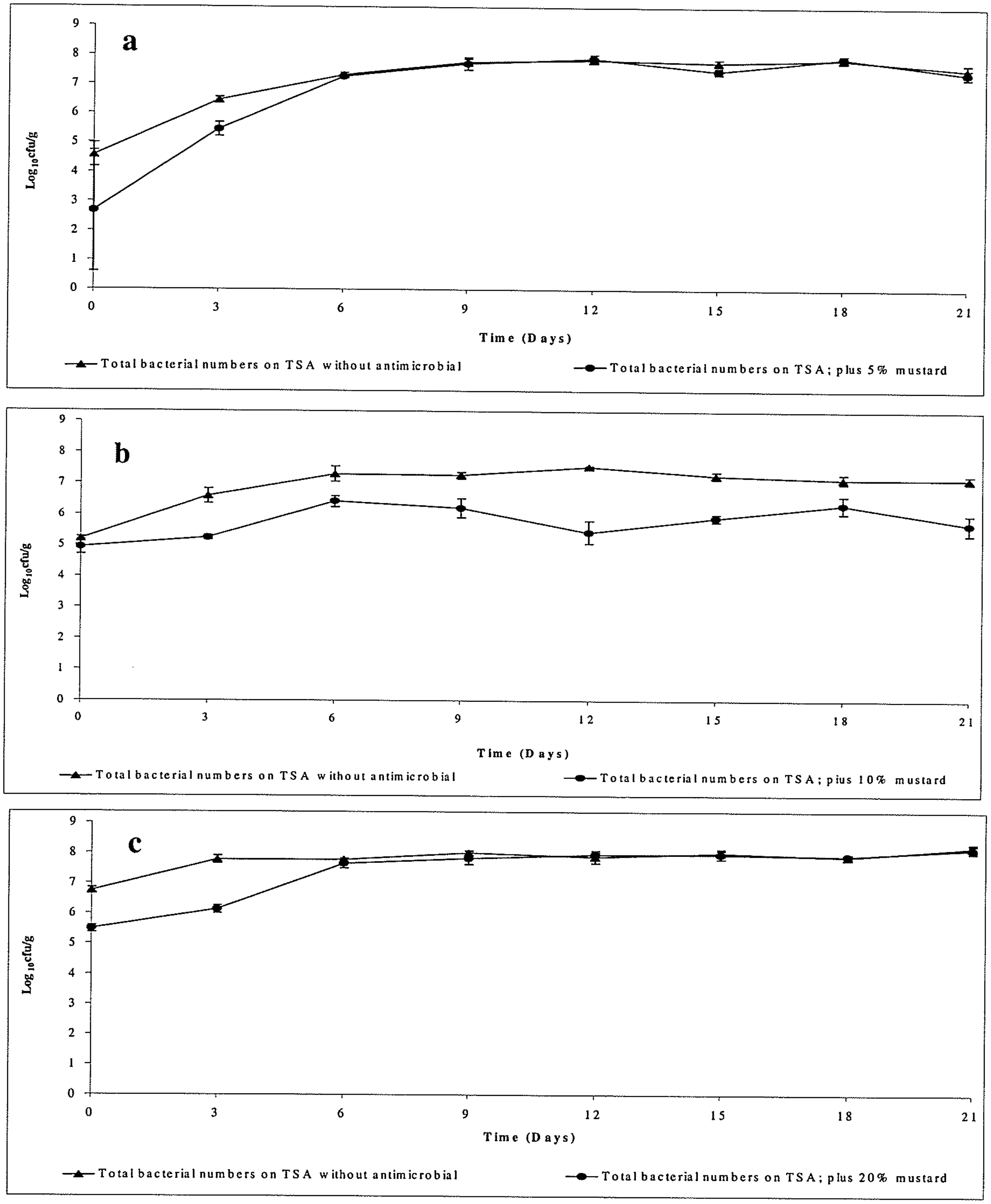

Figure 4.16. Antimicrobial effects of mustard powder 5(a), 10 (b), and 20% (c) natural microflora in vacuum packed ground beef patties stored at 4°C and plated on TSA. Six replicates were used to generate the standard deviation bars.

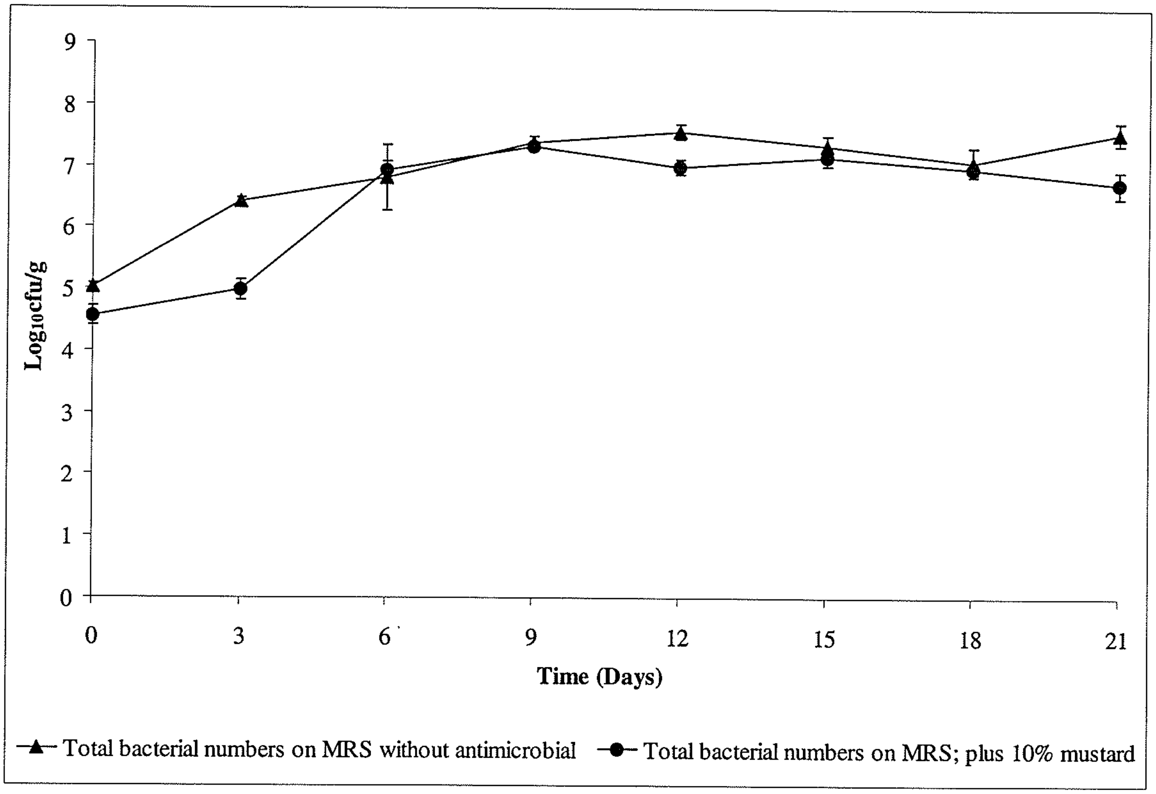

Figure 4.17. Antimicrobial effects of mustard powder at 10% on the natural microflora in vacuum packed ground beef patties stored at 4°C and plated on MRS. Six replicates were used to generate the standard deviation bars.

4.6.2. Natural microflora and inoculated levels of 3 $\log_{10}$ cfu/g of *E. coli* O157:H7

The antimicrobial effects of mustard powder at 5,10, and 20% on the natural microflora and inoculated levels of 3 log cfu/g of *E. coli* O157: H7 in ground beef patties stored at 4°C are presented in Fig. 4.18. At these levels, mustard powder (5,10, and 20%) was able to reduce 3 $\log_{10}$ cfu/g of *E. coli* O157:H7 to undetectable levels in 18, 12 and 3 d, respectively, without substantially affecting the numbers of bacteria naturally present in the ground beef patties.

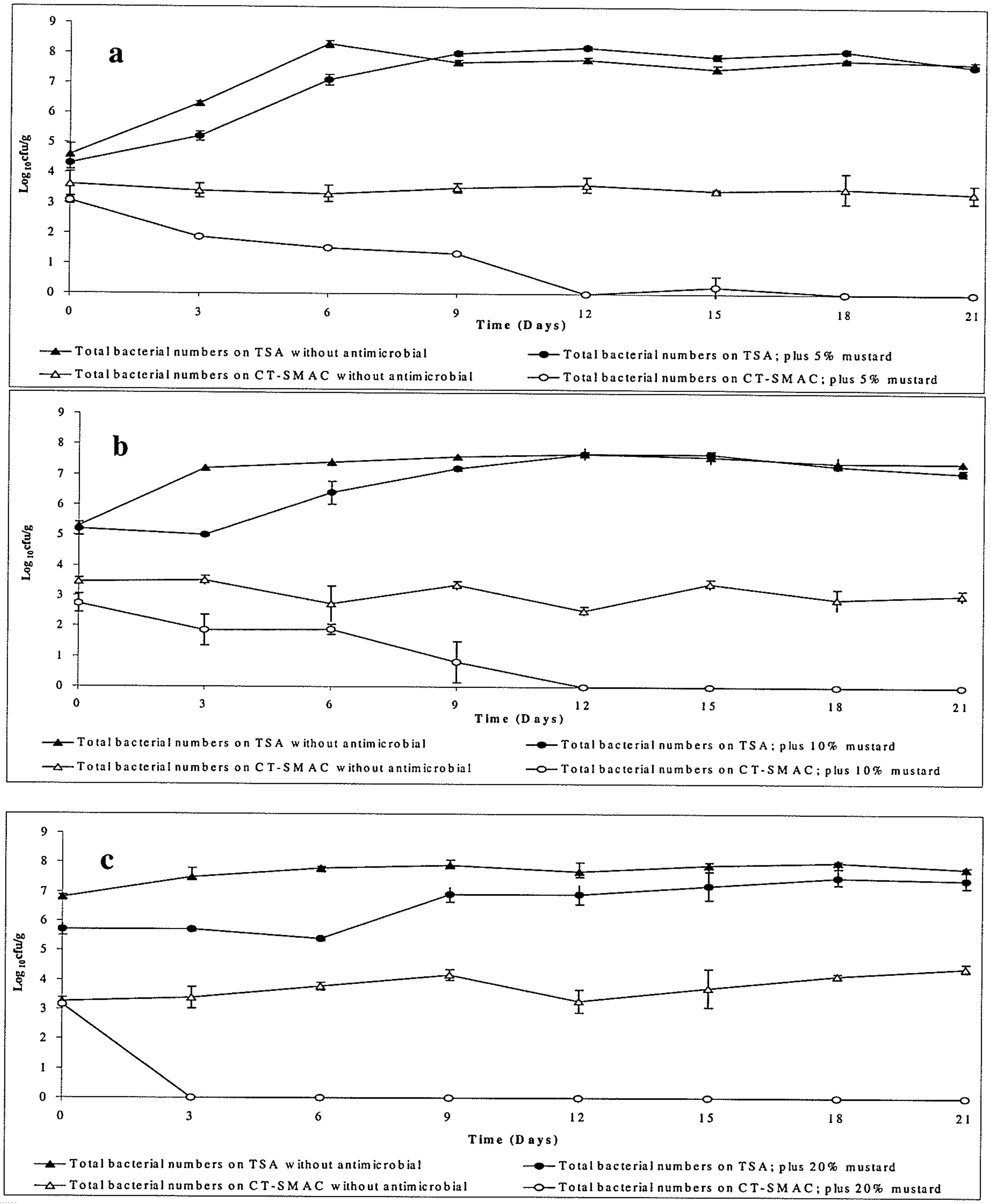

Figure 4.18. Antimicrobial effects of mustard powder 5 (a), 10 (b), and 20% (c) on natural microflora and inoculated levels of 3 $\log_{10}$ cfu/g *E. coli* O157:H7 in vacuum packed ground beef patties stored at 4°C and plated on TSA and CT-SMAC. Six replicates were used to generate standard deviation bars.

4.6.3. Natural microflora and inoculated levels of 6 $\log_{10}$ cfu/g of *E. coli* O157:H7

The antimicrobial effects of mustard powder at 5,10, and 20% on the natural microflora and inoculated levels of 6 $\log_{10}$ cfu/g of *E. coli* O157:H7 in ground beef patties stored at 4°C is presented in Fig. 4.19.

At the higher inoculation level, the effect of 5% mustard was small on both bacterial populations (Fig. 4.19a). However, the ground beef patties with 10% mustard showed a greater than 2 $\log_{10}$ reduction in *E. coli* O157:H7 after 21 d. The total bacterial population was significantly lower ($p<0.05$) on days 3 to 9 and also at days 18 to 21 (Fig. 4.19b).

Increasing the mustard concentration to 20% in ground beef patties led to a decrease of 5.6 $\log_{10}$ cfu/g of *E. coli* O157:H7 after 21 d. The total bacterial population was lower from day 0 to 15 in mustard-treated samples, but on days 18 to 21 no differences were noticeable (Fig. 4.19c).

Mustard concentrations of 5, 10, and 20% were able to reduce *E. coli* O157:H7 by 0.5, 2.3 and 5.6 $\log_{10}$ cfu/g of *E. coli* O157:H7 at 21 d, respectively, while having minimal effects on the natural microflora. Thus, mustard-containing AIT was able to kill *E. coli* O157:H7 in ground beef without affecting the numbers of background microflora when used at high concentrations (20% w/w). Lesser concentrations (about 10% w/w) of mustard powder when used as an ingredient in ground beef could be effective in eliminating *E. coli* O157:H7 present at natural levels in the meat.

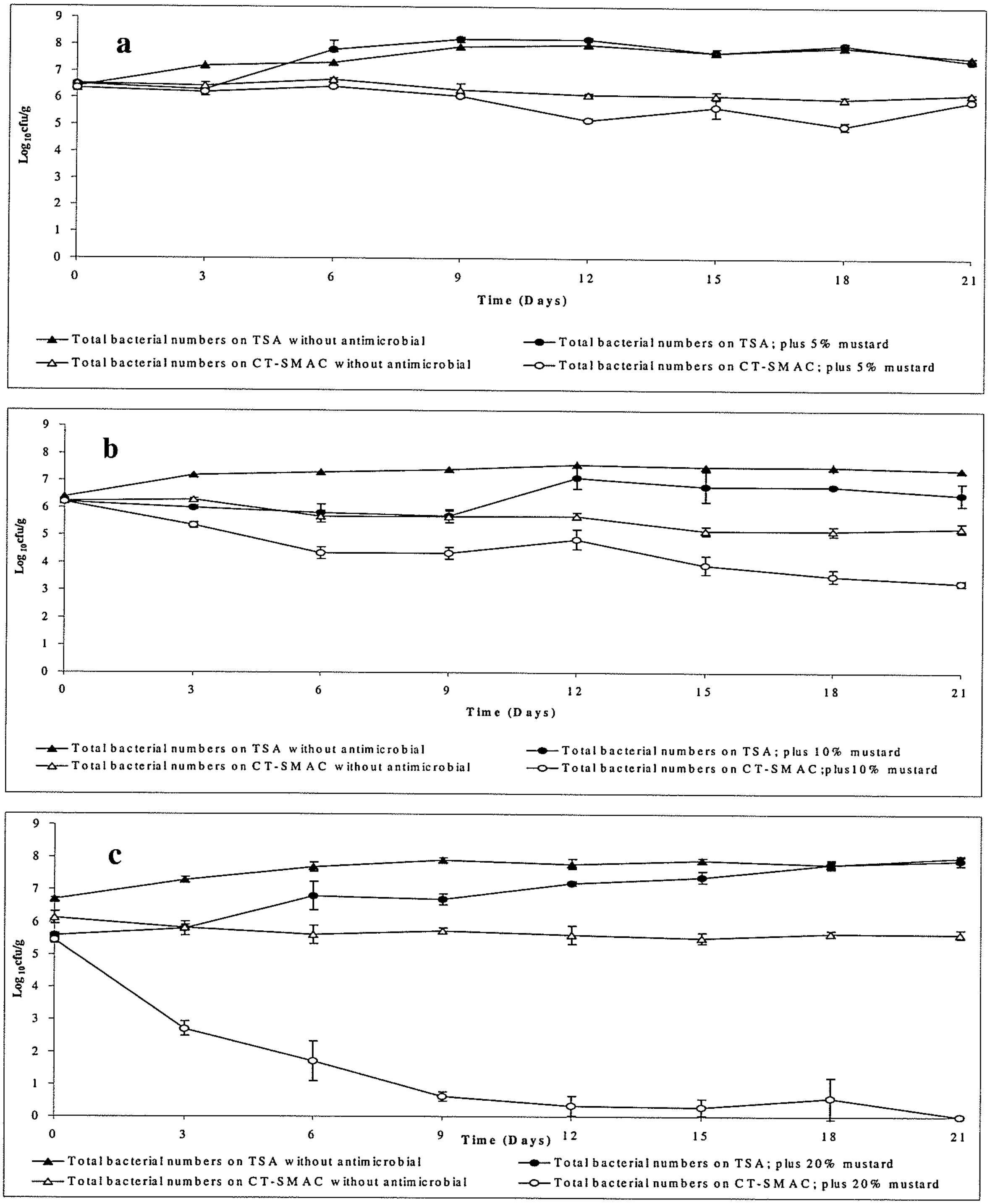

Figure 4.19. Antimicrobial effects of mustard powder 5 (a), 10 (b), and 20% (c) on natural microflora and inoculated levels of 6 $\log_{10}$ cfu/g *E. coli* O157:H7 in vacuum packed ground beef patties stored at 4°C and plated on TSA and CT-SMAC. Six replicates were used to generate the standard deviation bars.

4.7. Antimicrobial effects of 5% mustard powder on low levels of *E. coli* O157:H7 in ground beef patties using Immunomagnetic Separation

Mustard powder at a concentration of 5% did not eliminate very low levels of inoculated *E. coli* O157:H7 (14 to 40 cfu/g) in ground beef patties at 6 d when held at 4°C. *E. coli* O157:H7 growth was found on CT-SMAC plates when *E. coli* O157:H7 was recovered using immunomagnetic separation (IMS).

4.8. Sensory analysis of cooked ground beef patties containing 5 and 10% mustard

Sensory tests were conducted using seventy five panelists (30 males and 45 females). The ages of the panelists were as follows: 22 were 18 – 24 years, 26 were 25 – 34 years, 13 were 35 – 44 years, 7 were 45 - 54 years and 7 were 55 years of age and above. When asked how often they generally ate hamburgers, 37 panelists answered at least once a week, 29 answered at least once a month and 9 answered a few times a year. The sensory acceptability of cooked ground beef containing 0, 5 or 10% mustard powder is presented in Fig. 4.20. Panelists could not tell whether patties contained 5 or 10% mustard, but were able to distinguish the untreated control from both 5 and 10% mustard containing meat. Overall the panelists did not find the 5 and 10% mustard-containing meat objectionable since the acceptability score for each was greater than 5 (neither like nor dislike).

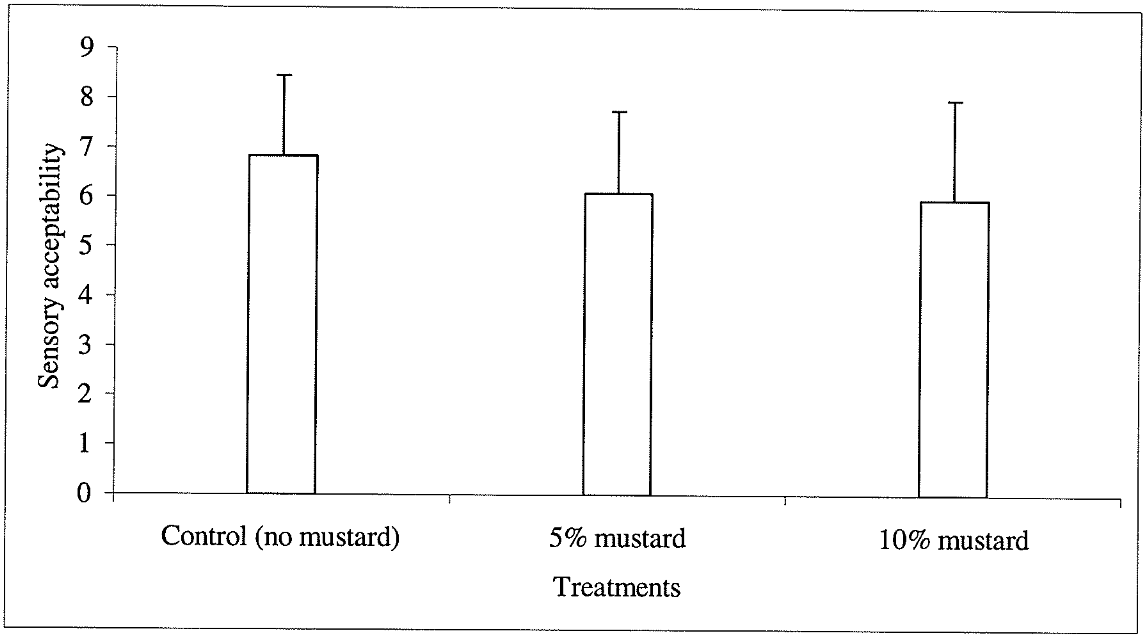

Figure 4.20. Sensory acceptability of cooked ground beef containing 0, 5, and 10% mustard powder.

Chapter 5

DISCUSSION

AIT has been reported as having antimicrobial activity in both liquid and gaseous forms. Recent reports have shown that the vapor form of AIT has increased antimicrobial activity over the liquid form (Lin et al., 2000a). Since AIT is volatile in nature, additional methods were being investigated to control the release rate of AIT from the liquid to the vapor form. One possible way of controlling the release rate of AIT is by dissolving it in fats and oils. AIT is able to be dissolved in corn oil, lard, shortening, beef tallow, and butter due to its lipophilic properties. The volatilization of AIT from these fats and oil can be controlled by varying the storage temperature (-18, 4, and 22°C). In our experiments (Fig. 4.1), AIT vapor concentration in the headspace was seen to increase when the storage temperature was increased, with the exception of beef tallow where changes were noted, but they were not large. There was also no difference observed in the levels of AIT released into the headspace when dissolved in solid fats (lard, shortening, beef tallow, and butter) compared to oil (corn oil) at 4°C. Differences in AIT volatility from the fats were found at 22°C, but this is an abusive storage temperature for meat. At normal storage temperature, the liquid (oil) or solid (fat) phase showed no effect on AIT's ability to change from the liquid to vapor phase. The release rate of AIT from fats and oil reached equilibrium after 10 min and was maintained up to 50 min. Lim and Tung (1997) reported that when AIT was mixed with canola oil its volatilization was increased as temperatures were increased. It was shown that the vapor form of AIT in the headspace could be controlled by changing the concentration of AIT in canola oil. The vapor form of AIT could also be controlled by altering the concentration of cis-oleic acids (C18:1 9c) and capric acids (C10:0) (Sekiyama et al., 1994; Lim and Tung, 1997). Hasegawa et al. (1999) reported that AIT was able to reduce viability of *V. parahaemolyticus* in fatty tuna when compared to lean tuna because of the fatty acids found naturally in tuna meat such as cis-vaccenic acid (C18:1 7c), palmitic acid (C16:0), and docosahexaenoic acid (C22:6). Fatty acids were believed to complex with AIT and allow for increased antimicrobial effects in the higher fat tuna. In our experiments (Table 4.1), cis-oleic acid and palmitic acid were found in all fats and oils studied while capric acid was found in the smallest quantities in lard (0.12%), beef tallow (0.07%), and butter (3.60%). The lower volatilization of AIT from beef fat at 22°C may have been due to the higher levels of cis-oleic acid present which may have complexed with the AIT molecules. Lard also had a larger amount of cis-oleic acid (39.8%), but unlike beef tallow it released a higher amount of AIT both at 4 and 22°C in the headspace. From linear regression study (Table 4.2), it is concluded that headspace AIT concentration, which related to the volatility of AIT is significantly affected by fatty acids compositions of lipid. It is possible to control headspace AIT concentration and volatility by altering fatty acid composition.

AIT can be more effectively used in food applications if the packaging material chosen can maintain the AIT in a vapor form at inhibitory levels throughout storage and evaporate upon pack opening leaving no objectionable residues. In these tests AIT was mixed with corn oil because it was easier to dissolve. The Ziplock® freezer bags used retained the least amount of AIT at all three temperatures studied (4, -18, 22°C) while

Nylon/EVOH/Polyethylene maintained the highest amounts at 4 and 22°C. AIT permeated through Ziplock® bags faster than the other plastic films used because Ziplock® bags are made from LDPE, which does not retain AIT. The AIT odor was detectable outside the bag during the 41 d of storage at 4 and 22°C. In contrast, Lin et al. (2000a) reported that Ziplock® freezer bags placed insde a Glad-Lock Zipper and Rubbermaid storage container were able to maintain 88 µl/l of AIT for 2 d. Sekiyama et al. (1995) reported that most of the plastic films they used did not deform with up to 100 ppm of AIT, but softening and deformation were reported at 3,000 ppm with the majority of plastics other than polyethylene (50 µm thick), polypropylene (70 µm thick), nylon (20 µm thick), polyacetal and teflon. The above authors also reported that AIT permeation was higher through ethylene vinylacetate copolymer (EVA) compared to polyethylene (PE) or nylon monolayer films. Lim and Tung (1997) analyzed PVDC/PVC copolymers (10 µm thick) for their AIT vapor barrier properties and concluded that these films did not retain AIT and that penetration increased with higher temperature. In another study done by Lim et al. (1999) it was reported that the polymer nylon prevented AIT from film penetration. Other researchers have used different types of plastic materials to maintain AIT levels such as: OPP/EVOH/LDPE (oriented polypropylene/ethylene vinyl alcohol/low density polyethylene), polyethylene terephthalate/aluminum/cast polypropyrene, OPP20/EVOH/LDPE45/LLDPE30, and polyvinylidene chloride/nylon/polyethylene (Isshiki et al., 1992; Kim et al., 2002; Nielsen and Rios 2000; Ogawa et al., 2000). In our study (Fig. 4.2), it was observed that the Nylon/EVOH/Polyethylene film (75 µm thick) retained more AIT than the

Polyester/PVDC EVA copolymer (62 μm thick) and this result is consistent with those in the literature showing that individual components such as nylon/EVOH/polyethylene retained more AIT compared to PVDC and EVA (Lim et al., 1999; Sekiyama et al., 1995).

Since the Nylon /EVOH/Polyethylene bags retained the highest amount of AIT, these bags were used to evaluate the antimicrobial activity of both commercially prepared AIT liquid and AIT from mustard powder in ground beef patties. The liquid commercial AIT in the headspace of the bag was seen to decrease over time when incorporated with the ground beef patties (Figs. 4.3-4.6). The AIT vapor was found to be present in the highest concentration (893 μg/ml) in the headspace at 10°C for 8 d compared to 540 μg/ml at 4°C for 21 d or 261 μg/ml at –18°C for 35 d. This being due to liquid AIT being able to vaporize faster at higher temperatures. At –18°C, low levels of AIT were found in the headspace because the crystallized beef fat may act to trap the AIT molecules better. Fatty acids such as oleic acid (C18:1 9c) may complex with AIT molecules and reduce its evaporation rate. Ward et al. (1998) reported that 20, 4 and 2 μl/l of AIT in the headspace of packaged roast beef deceased 21.2, 2.4, and 1.1% from the original amount, respectively, after 7 d storage at 12°C. In other work it was found that AIT vapors were absorbed by the plastic packaging film material and water vapor inside the bags, which resulted in depletion of the AIT in the headspace (Lim and Tung 1997; Sekiyama et al., 1995). Another reason for the depletion of AIT in the headspace is that AIT tends to react with thiol, suphydryl, free amino groups and disulfide groups of proteins in meat. In addition, spontaneous degradation resulting from increase in temperature, nucleophilic attack of water, as well as hydroxide ions were reported responsible for the depletion of AIT over time (Cejpek et al., 2000; Chen and Ho 1998; Delaquis and Sholberg, 1997; Kim et al., 2002; Ohta et al., 1995; Palop et al., 1995; Sekiyama et al., 1994; Ward et al. 1999). Maintenance of the AIT concentration in the vapor phase at a threshold level $\geq$ 500 μg/ml was an important contributor to the antimicrobial activity seen against *E. coli* in experiments conducted here (Fig. 4.3).

Based on a density of 1.0130 g/cm^3 and 94% purity, 0.5 ml of the commercial AIT preparation contained 0.49 mg AIT and 1.0 ml contained 0.97 mg. AIT at the lower volume (0.5 ml) in meat stored at 4°C did not affect the natural microflora, but increasing the AIT volume to 1 ml resulted in a greater reduction in colony counts. However, on day 21, no significant difference was noticed between the two levels of AIT (Fig. 4.7). The initial bacterial reduction was probably caused by the change in the initial microflora from Gram-negative to Gram-positive lactic acid bacteria, which normally occurs during refrigerated vacuum packed storage of fresh meat. The Gram-positive organisms are more resistant to AIT exposure and will become the dominant bacterial population during storage under these conditions. Increasing the temperature to 10°C resulted in greater bacterial reductions on days 1 and 2 (Fig. 4.8). This was caused by the liquid AIT more rapidly changing to vapor at the higher temperature. Therefore AIT at 10°C demonstrated a higher microbial reduction on days 1 and 2 compared to 4°C, where a larger reduction was only observed after day 6. As the exposure time increased further at 10°C, the antimicrobial activity of AIT decreased. This may have been a result of AIT interacting with the meat proteins as well as the faster penetration of AIT through the plastic film. AIT treatment of *E. coli*-inoculated beef patties stored at 10°C was ineffective and the experiment was terminated after 8 d. When the storage temperature of the patties was decreased to –18°C, AIT was unable to reduce the natural microflora because the average levels of AIT (0.5 ml or 1 ml) in the headspace were too low (158 and 237 μg/ml) compared with levels at 4°C (318 and 540 μg/ml) or 10°C (873 μg/ml). It was evident that AIT did not affect the natural microflora in ground beef patties stored at 4, 10 and –18°C.

Other researchers also found similar results where it was observed that AIT did not affect the natural lactic acid bacteria microflora. A study published by Palop et al. (1995) reported that *Lactobacillus agilis* strain R16 was capable of survival in 10 mM AIT. In 1997, Kyung and Fleming reported that 50 and 500 ppm of AIT were required to inhibit the growth of lactic acid bacteria in a broth medium. Another study done by Ward et al. (1998) showed that lactic acid bacteria held at 12°C for 7 d in roast beef were unaffected at 20 μl/l of AIT vapor. Shofran et al. (1998) reported that lactic acid bacteria were more resistant to AIT than Gram-negative bacteria and the minimum inhibitory concentration was reported to be 500 to 1000 ppm. Lin et al. (2000a) found that 88 μl/l AIT was able to reduce native bacteria on lettuce from 5 $\log_{10}$ to 2.2 $\log_{10}$ cfu/g in 4 d at 4°C. AIT was shown to have potential for reduction of Gram-negative contaminants without having a major effect upon Gram-positive bacteria in these studies.

AIT was able to reduce *E. coli* O157:H7 in ground beef patties, but did not eliminate them. AIT (0.5 ml) was able to reduce 3 $\log_{10}$ cfu/g to undetectable levels after 18 d storage at 4°C. With AIT (1 ml), 3 $\log_{10}$ cfu/g *E. coli* O157:H7 were reduced to undetectable levels in 12 d. Therefore increasing the AIT concentration resulted in a similar reduction but over a shorter time frame at 4°C (Fig. 4.10). At 10°C AIT was not seen to have a greater reduction of *E. coli* O157:H7 in ground beef compared to 4°C. Although AIT at 10°C was able to be vaporized more rapidly than at 4°C, it failed to inhibit bacterial growth in 8 d (Fig. 4.11). At –18°C, 0.5 ml of AIT was able to reduce 3 $\log_{10}$ cfu/g of *E. coli* to undetectable levels after 35 d of exposure. When the concentration was increased to 1 ml, AIT was able to reduce 3 $\log_{10}$ cfu/g to undetectable levels in 10 days (Fig. 4.12). Even though AIT did show a strong antimicrobial activity against *E. coli* O157:H7, an increase of the initial inoculum levels of *E. coli* O157:H7 to 6 $\log_{10}$ cfu/g prevented complete elimination of the bacteria at 4, 10, and –18°C. A larger reduction of *E. coli* O157:H7 was observed at 4°C using 1 ml of AIT. At 4°C AIT (1 ml) was able to change from liquid to vapor and cause a 3.4 $\log_{10}$ reduction when the initial level of *E. coli* was 6.2 $\log_{10}$ cfu/g (Fig. 4.13). Although at 10°C AIT was able change from the liquid to vapor phase more rapidly than at 4°C, its antimicrobial effects were limited and the experiment was terminated after 8 d due to spoilage of the meat (Fig. 4.14). At –18°C AIT was less volatile and may have been trapped by crystallized beef fat. At 261 μg/ml, AIT was able to reduce *E. coli* by 1 $\log_{10}$ cfu/g at the higher *E. coli* inoculum level (Fig. 4.15), while a greater than 3 $\log_{10}$ cfu/g reduction was found at the lower levels of inoculated *E. coli* (Fig. 4.12). When comparing both inoculated levels of 3 and 6 $\log_{10}$ cfu/g of *E. coli* O157:H7 and natural microflora on TSA plates, an initial bacterial reduction was only noticed at 4°C, while there was no difference at 10 and –18°C. The microbial reduction at 4°C was probably caused by an initial decrease in Gram-negative bacteria during vacuum package storage. As storage increased the naturally present Gram-positive lactic acid bacteria in meat grew. It was found that AIT did not substantially affect the natural microflora, but significantly reduced the viability of *E. coli* O157:H7 in ground beef patties. Vold et al. (2000) reported that the presence of natural microflora such as lactic acid bacteria can prevent the growth of *E. coli* O157:H7. Similarly, Bredholt et al. (1999) reported that addition of 4-5 $\log_{10}$ cfu/g lactic acid bacteria prevented the growth of *E. coli* O157:H7 in cooked ham stored at 10°C for four weeks. While there may have been some inhibitory effect of the lactic bacteria naturally present in meat used in our experiments, clearly, the *E. coli* lethality observed here was due to AIT.

Previously researchers found that AIT inhibited *E. coli* O157:H7 at different temperatures, concentrations, times and in various model systems (broth, agar, and food). Delaquis and Sholberg (1997) showed that 1500 µg/L of AIT in the gaseous stage at 4°C after 48 h was able to reduce 6 $\log_{10}$ cfu/cm^2 to undetectable levels in an agar model. Lin et al. (2000b) observed that AIT at 2500 µg/ml for 30 min decreased *E. coli* from 7 $\log_{10}$ cfu/ml to an undetectable level in the early exponential growth phase while only 2 h was needed when bacteria were in the stationary phase of growth. Ward et al. (1998) reported 20 µl/l AIT as vapor reduced *E. coli* from 3.03 to 2.26 $\log_{10}$ cfu/cm^2 in roast beef after 7 d storage at 12°C. Shofran et al. (1998) reported that 60 to 140 ppm of AIT at pH 5 to 7 were 6 to 21 times more bacteriostatic to *E. coli* 33625 compared to benzoate. Kyung and Fleming (1997) reported that the MIC value for AIT against *E. coli* B34 (ATCC 33625) was 50 ppm. Ogawa et al. (2000) found that 80 μg/ml of AIT along with 200 MPa pressure at 4°C was able to reduce *E. coli* O157:H7 from 5 $\log_{10}$ to 3 $\log_{10}$. Lin et al. (2000a) reported that 88 μl/l of AIT was able to reduce 4 and 8 $\log_{10}$ cfu/g to undetectable levels after 48h in lettuce. The same authors also reported that 133 μl/l of AIT reduced 3 $\log_{10}$ of *E. coli* O157:H7 in apple stem scars and 111 μl/l caused a 5 $\log_{10}$ reduction in tomatoes. Park et al. (2000) reported that 50 μl/l of AIT in the vapor form inhibited 2.2 $\log_{10}$ cfu/g in alfalfa seeds while 10 μl/l eliminated 7 $\log_{10}$ cfu/g in the vapor phase over agar at 37°C for 24 h. Isshiki et al. (1992) reported that 4 $\log_{10}$ cfu *E. coli* JCM-1649 were eliminated at 34 ng/ml AIT vapor after 2 d at 37°C. All of the above research supports our observations that AIT is capable of eliminating significant numbers of *E. coli* O157:H7 from ground beef patties.

The antimicrobial mechanism of AIT still not clearly understood. Previous studies have shown than AIT is more active against aerobic bacteria than facultative anaerobes. The inhibitory action of AIT toward aerobic micro-organisms results from the disruption of aerobic respiration and interference with the enzymes that are needed for aerobic metabolism. AIT affects facultative anaerobes by non-specifically attacking enzymes or by denaturing protein (Delaquis and Sholberg, 1997). Ward et al. (1998) reported that depletion of AIT in aqueous solution is the result of natural breakdown and nucleophilic attack of water. These authors also suggested that the bacterial cell could easily absorb the gaseous AIT more effectively and therefore more antimicrobial activity was observed where AIT was in the gaseous state compared to a liquid. It is also thought that AIT may attach to sulfhydryl groups and affect the growth and survival of sensitive bacteria since these are the active sites for many enzymes (Shofran et al., 1998). Further experiments are needed to fully understand the antimicrobial mechanism of AIT.

AIT from mustard also has been reported to have antimicrobial activity against Gram-negative pathogenic bacteria such as *E. coli* O157:H7 (Chinen et al., 2001; Isshiki et al., 1992; Kanemaru and Miyamoto 1990; Kojima and Ogawan, 1971; Mayerhauser, 2001; Nielsen and Rios, 2000; Ono et al., 1998; Pechacek et al., 1997; Rhee et al., 2002). AIT is produced in mustard when the cell walls are damaged or injured. During this time AIT precursors (glucosinolates) are hydrolyzed by naturally present myrosinase, a cell wall bound enzyme, in the presence of water. In our experiments when non-deheated mustard powder was formulated in ground beef, it was able to generate AIT upon contact with the moisture present in the meat. Increasing the mustard concentration from 5 to 10 and 20% in the ground beef resulted in increased levels of AIT (14.7, 19.0, and 57.2 μg/ml, respectively, Fig. 4.6) in the headspace of the bags containing the meat.

In the 5 and 20% mustard powder treatments, the natural microflora was decreased on day 0 but gradually increased to the control levels by day 6 (Fig. 4.16). Gram-negative bacteria were probably reduced in numbers initially as AIT was released from the mustard powder. As the exposure time increased, the Gram-positive lactic acid bacteria, which are resistant to AIT, would probably become the dominant group present in the natural microflora at 4°C. When 10% mustard was used, the number of bacteria recovered on TSA agar was lower than in either 5 or 20% mustard treatment. This result may simply reflect uneven distribution of the natural microflora in ground beef (Fig. 4.16).

Increasing the mustard powder concentration from 5 to 20% resulted in the rapid reduction of *E. coli* O157:H7 at both 3 and 6 $\log_{10}$ cfu/g (Figs. 4.18 and 4.19). A larger reduction was due to the higher levels of AIT formed from the glucosinolates present in the mustard powder. A greater reduction in viable *E. coli* O157:H7 was observed when mustard powder was used (compared to pure AIT). It was also of interest that the AIT levels were lower (27 μg/ml) in the headspace of mustard treatments compared to the headspace where 94% pure concentrated commercial solution (540 μg/ml) was used and stored 21 d at 4°C. Since mustard powder was directly mixed with the ground beef, as AIT was released from the mustard powder it came into direct physical contact with *E. coli* O157:H7 present in the ground beef. In the case of the 94% concentrated commercial AIT liquid, it was mixed with corn oil then added to a filter paper and placed on top of the ground beef patty. In this situation AIT had to be vaporized and penetrate the meat before it could inhibit the *E. coli*. Another possible reason for mustard having greater antimicrobial activity than the concentrated AIT solution is that mustard has many other components present, although in small amounts, than AIT such as 3-methylthiopropyl isothiocyanate, 3-butenyl isothiocyanate, butyl isothiocyanate, phenyl isothiocyanate, 2-phenylethyl isothiocyanate, hexyl isothiocyanate, 4-phenethyl isothiocyanate, and benzyl isothiocyanate which may play a synergistic role in inhibiting the *E. coli* O157:H7.

When *E. coli* O157:H7 was inoculated at a lower level (14 to 40 cfu/g) in the meat, (which is roughly representative of the commercial situation, Uhitil et al., 2001) mustard at 5% did not eliminate the bacteria within 6 d at 4°C. Therefore a higher level of mustard may be necessary to provide assurance that viable *E. coli* O157:H7 are absent from ground beef.

Earlier studies of the antimicrobial activity of mustard were done in broth cultures. Rhee et al. (2002) reported that three stains of *E. coli* O157:H7 at levels of 6 - 7 $\log_{10}$ cfu/g were reduced to <0.3 $\log_{10}$ cfu/g after 7 d when 10% mustard was used and treatments stored at 5°C. Kanemaru and Miyamoto (1990) showed that upon analyzing AIT and mustard at the same concentration levels, mustard had significantly higher antimicrobial activity against *E. coli* (non-O157:H7). Their study showed that 0.8% mustard had bacteriostatic effects. Mayerhauser (2001) found that deli-style mustard alone was able to reduce 6 $\log_{10}$ cfu/g of *E. coli* O157:H7 to undetectable levels at 5 and 25°C in 24h. Therefore mustard has significant potential to control viable *E. coli* O157:H7.

Although AIT from plant sources has been demonstrated to have strong antimicrobial activity against pathogenic bacteria, it has rarely been used in food systems. AIT at higher concentrations (>500 ug/ml) has been shown to generate eye watering, a burning sensation on the tongue, and cause nasal cavity irritation. The sensory evaluation of cooked ground beef with mustard showed that there were no significant differences between the overall sensory acceptability of cooked ground beef formulated with 5 or 10% mustard. It is likely that formulation of hamburgers with between 5 to 10% mustard powder will eliminate *E. coli* O157:H7 from ground beef contaminated at levels (10 cfu/g) likely to be found in commerce (Uhitil et al., 2001)

Chapter 6

CONCLUSIONS

This research examined the use of a volatile natural antimicrobial agent, allyl isothiocyanate (AIT), for the elimination of *E. coli* O157:H7 from ground beef patties. The conclusions that were drawn from this study were:

1. The vaporization of AIT can be controlled by changing the temperature of the stored product, in our case the AIT vaporization was shown to increase when the temperature was increased from freezer (-18°C) to refrigerator (4°C) and to abusive (10°C) storage conditions.

2. Unsaturated fatty acids present in fats and oils were believed to complex with the AIT molecule, and delayed the release of AIT into the headspace. Temperature was more important than the hydrophobic solute used for carrying AIT, from an antimicrobial perspective.

3. Of the three plastic films studied, it was determined that Nylon/EVOH/Polyethylene was the best film for use in retaining AIT within the package. This was attributed to the nylon and polyethylene components in the plastic material.

4. *E. coli* O157:H7 was effectively controlled in packaged ground beef by the addition of concentrated commercial AIT to a filter paper placed in packages of ground meat.

5. *E. coli* O157:H7 was also significantly reduced in ground beef patties when non-deheated mustard flour was added as an ingredient in raw hamburger at 5-10% (w/w). AIT from non–deheated mustard flour was more effectively lethal than the commercial preparation of concentrated AIT used.

6. Sensory studies showed that levels of 5 or 10% (w/w) mustard powder were acceptable in cooked ground beef patties and therefore could be used to avoid food safety problems with these products caused by *E. coli* O157:H7. The minimum threshold concentration between 5 - 10% (w/w) mustard required to ensure elimination of all *E. coli* O157:H7 when present at very low levels needs to be determined.

Chapter 7

RECOMMENDATIONS FOR FUTURE STUDIES

Investigate the fatty acids that can be combined to sustain release of AIT over longer periods of time. This may facilitate the preparation of microcapsules, which can permit delivery of effective (threshold) levels of AIT enabling longer safe shelf life of foods stored at refrigerator temperatures.

Determine the mechanism through which AIT acts as an antimicrobial agent against Gram-positive as well as Gram-negative bacteria.

Investigate the antimicrobial effectiveness of mustard powder against other pathogenic bacteria such as *Listeria, Salmonella, Campylobacter,* and *Shigella.*

Examine the effectiveness of mustard powder as an antimicrobial agent in modified atmosphere packages of various sliced produce.

Characterize the other active components present in mustard powder besides AIT, which may contribute to its antimicrobial activity against pathogenic organisms.

Examine the synergistic effect of mustard powder with other spices that contain either AIT or different antimicrobial components, and determine their combined effectiveness against pathogenic bacteria.

Additional work is needed to exactly characterize the concentration of mustard flour between 5-10% (w/w) that can eliminate low levels of added *E. coli* O157:H7 from ground beef.

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APPENDICES

Appendix 1. AIT released in the headspace from corn oil, butter, shortening, lard, and beef tallow stored at –18, 4 and 22°C. Headspace samples were analyzed at 5 min intervals for 50 min.

	AIT (µg/ml)														
Time	Corn oil			Butter			Shortening			Lard			Beef tallow		
(min.)	-18°C	4°C	22°C	-18°C	4°C	22°C	-18°C	4°C	22°C	-18°C	4°C	22°C	-18°C	4°C	22°C
5	91.8	153.4	261.2	85.1	155.8	275.5	130.2	199.0	237.4	107.9	198.7	278.5	133.2	209.9	222.9
10	64.2	99.7	249.0	69.5	86.8	213.1	92.3	121.1	239.8	98.1	103.6	274.6	109.1	116.3	158.2
15	64.0	88.2	257.7	36.8	95.9	239.4	101.8	111.7	247.1	96.1	102.4	291.7	92.2	113.1	168.3
20	55.5	90.0	258.1	67.5	82.8	240.4	95.4	109.4	239.5	87.8	105.4	303.6	85.1	113.0	163.8
25	52.0	92.3	266.8	52.7	79.4	246.7	94.5	108.7	245.8	89.7	107.4	301.6	108.5	109.4	156.1
30	39.1	87.6	259.0	64.5	82.9	243.3	90.1	103.2	243.3	86.7	102.5	295.5	116.6	105.0	165.5
35	46.5	82.2	272.5	77.9	83.5	243.6	88.6	117.8	247.6	74.6	96.4	297.6	109.2	112.4	165.7
40	48.0	100.0	271.9	60.9	80.4	245.5	70.1	103.6	246.9	73.8	109.8	299.3	108.6	104.7	156.9
45	47.1	93.0	267.7	64.6	73.2	242.8	78.1	108.1	259.1	84.4	113.8	302.6	117.6	106.9	152.7
50	49.3	93.8	273.9	63.5	78.7	238.5	87.1	102.8	255.0	82.9	112.5	303.3	108.2	101.6	155.3

Appendix 2. Permeability of AIT dissolved in corn oil through Nylon/EVOH/Polyethylene, Polyester/PVDC/2 mil EVA Copolymer, and Ziplock® filmed bags stored at 4 and 22°C for 41 d

		Days AIT[1] (μg/ml)									
Plastic packaging materials	Temp. (°C)	0	1	2	5	7	12	16	23	30	41
Nylon/EVOH/PE	4	334.2±39.4	375.0±30.3	305.9±47.7	274.4±25.2	318.8±33.6	385.9±37.2	362.2±40.3	336.1±25.8	314.7±26.2	246.5±19.2
Nylon/EVOH/PE	22	334.2±56.2	648.0±45.8	432.9±36.7	395.3±23.7	337.2±12.5	448.9±45.2	416.5±34.1	335.3±24.3	357.0±37.3	247.4±34.2
Polyester/PVDC EVA Coploymer	4	246.1±48.8	392.9±52.3	347.0±38.3	288.9±30.3	232.0±25.3	372.2±26.6	333.8±59.1	278.3±15.2	234.7±33.2	214.0±28.2
Polyester/PVDC/ EVA Coploymer	22	246.1±39.4	387.4±110.2	376.2±76.5	295.3±56.9	211.5±37.9	257.9±35.8	248.7±67.3	232.5±59.9	187.5±36.7	137.9±54.2
Ziplock®	4	155.3±34.2	133.4±58.2	56.3±19.6	35.1±14.6	22.33±9.3	35.9±8.4	38.2±11.3	18.9±7.8	25.0±6.9	19.2±5.2
Ziplock®	22	155.3±34.2	55.8±15.6	25.1±8.9	25.6±4.5	21.6±5.7	25.5±12.8	49.6±14.5	28.1±11.6	17.4±9.4	14.0±12.9

[1] Each value represents the mean and standard deviation of three measurements

Appendix 3. AIT levels from 94% pure commercial liquid AIT released into the headspace of packaged ground beef patties stored at 4°C for 21 days

Treatments	Days AIT[1] (µg/ml)							
	0	3	6	9	12	15	18	21
AIT (0.5 ml)	662.8 ± 110.9^{bA}	439.4 ± 132.7^{bB}	172.4 ± 71.0^{bED}	414.0 ± 71.2^{aB}	330.2 ± 102.9^{bC}	223.9 ± 78.7^{bD}	181.4 ± 71.2^{bED}	120.7 ± 36.8^{bE}
AIT (1.0 ml)	783.1 ± 105.1^{aA}	$662.8 \pm 132.3.9^{aB}$	535.6 ± 135.6^{aCD}	420.2 ± 77.3^{aD}	569.0 ± 154.5^{aCB}	463.9 ± 97.5^{aCD}	470.8 ± 75.5^{aCD}	456.0 ± 109.7^{aCD}

[1] Each value represents the mean and standard deviation of six measurements.

Means with different lower case letters superscripts in each treatment of each day (column) are significantly different ($P<0.05$).

Means with different capital letter superscripts (A to E) in the same row are significantly different ($P<0.05$).

Appendix 4. AIT levels from 94% pure commercial liquid AIT released into the headspace of packaged ground beef patties stored at 10°C for 8 d

Treatment	Days AIT[1] (μg/ml)							
	0	1	2	3	4	4	6	8
AIT (1 ml)	1666.8.±338.3^{a}	1744.2±338.3^{a}	853.8±208.4^{b}	752.6±118.2^{cb}	560.7±90.6^{cb}	526.9±118.7^{d}	595.2±214.6^{cd}	444.4±191.0^{d}

[1] Each value represents the mean and standard deviation of six measurements.
Means with different lower case superscripts (a to d) in the same row are significantly different (P<0.05).

Appendix 5. AIT levels from 94% pure commercial liquid AIT released into the headspace of packaged ground beef patties stored at -18°C for 35 d

	Days AIT[1] (µg/ml)							
Treatments	0	5	10	15	20	25	30	35
AIT (0.5 ml)	515.6 ± 114.3^{aA}	151.4 ± 62.2^{bCB}	193.1 ± 88.1^{aB}	$188.0\pm57.2.^{bB}$	129.7 ± 29.04^{bCBD}	95.3 ± 45.6^{bCD}	71.5 ± 24.2^{bD}	76.7 ± 36.6^{aD}
AIT (1 ml)	549.0 ± 93.9^{aA}	346.5 ± 85.0^{aB}	168.6 ± 80.7^{aD}	277.1 ± 95.5^{aCB}	252.6 ± 72.7^{aC}	251.4 ± 58.1^{aC}	144.9 ± 66.1^{aD}	112.6 ± 37.2^{aD}

[1] Each value represents the mean and standard deviation of six measurements.
Means with different lower case letter superscripts (a or b) in each treatment of each day (column) are significantly different (P<0.05).
Means with different capital letter superscripts (A to E) in the same row are significantly different (P<0.05).

Appendix 6. AIT levels released into the package headspace from non-deheated mustard powder formulated in ground beef patties stored at 4°C for 21 d.

Treatments	Days AIT[1] (μg/ml) 0	3	6	9	12	15	18	21
Mustard 5%	14.7 ± 3.3^{bB}	12.2 ± 4.1^{bB}	31.6 ± 10.4^{aA}	20.2 ± 6.7^{bB}	28.4 ± 11.9^{aA}	19.7 ± 6.6^{aA}	20.2 ± 12.2^{aA}	19.6 ± 5.7^{aA}
Mustard 10%	19.0 ± 7.6^{bB}	11.6 ± 1.7^{bB}	12.3 ± 1.3^{cB}	32.7 ± 4.2^{aBA}	15.7 ± 6.7^{bB}	12.7 ± 2.25^{bBA}	11.5 ± 2.4^{bB}	15.6 ± 4.93^{aA}
Mustard 20%	57.2 ± 13.6^{aA}	28.9 ± 8.7^{aA}	23.9 ± 9.4^{bA}	32.4 ± 10.5^{aA}	16.2 ± 6.5^{bB}	18.3 ± 7.1^{abB}	16.7 ± 4.53^{abBA}	22.9 ± 13.5^{aA}

[1] Each value represents the mean and standard deviation of six measurements.

Means with different lower case letter superscripts (a to c) in each treatment of each day (column) are significantly different (P<0.05).

Means with different capital letter superscripts (A to E) in the same row are significantly different (P<0.05).

Appendix 7. Antimicrobial effects of AIT (0.5 and 1 ml) on natural microflora in vacuum packed (N_2 backflushed) ground beef patties stored at 4°C and plated on TSA

Treatments	Bacterial numbers^{1} ± standard deviation (Log$_{10}$ cfu/g)							
	Days							
	0	3	6	9	12	15	18	21
Total bacterial numbers on TSA without antimicrobial	5.9±0.24^{a}	6.8±0.15^{a}	7.3±0.13^{a}	7.5±0.23^{a}	7.8±0.30^{a}	7.7±0.40^{a}	7.7±0.38^{a}	7.9±0.08^{a}
Total bacterial numbers on TSA with 0.5 ml AIT	5.9±0.24^{a}	6.4±0. 48^{a}	6.3±0.86^{b}	6.6±0.73^{b}	7.5±0.14^{b}	7.4±0.17^{a}	7.5±0.24^{a}	7.5±0.18^{b}
Total bacterial numbers on TSA without antimicrobial	5.0±0.39^{a}	5.6 ±0.18^{a}	6.5 ±0.12^{a}	7.5±0.08^{a}	7.7±0.11^{a}	7.7±0.11^{a}	7.6±0.35^{a}	7.7±0.39^{a}
Total bacterial numbers on TSA with 1.0 ml AIT	5.0±0.39^{a}	5.1±0.22^{b}	4.5±0.57^{b}	5.0± 0.47^{b}	5.8±0.77^{b}	4.8±0.84^{b}	6.1±0.40^{b}	7.5±0.23^{a}

[1] Each value represents the logarithm of the mean of three trials. Each was done in duplicate, n=6.
Means with different lower case superscripts in each treatment of each day (column) are significantly different (P<0.05).

Appendix 8. Antimicrobial effects of AIT (1 ml) on natural microflora in vacuum packed (N_2 backflushed) ground beef patties stored at 10°C and plated on TSA

	Bacterial numbers[1] ± standard deviation (Log_{10} cfu/g)							
	Days							
Treatments	0	1	2	3	4	5	6	8
Total bacterial numbers on TSA without antimicrobial	6.8 ± 0.07^{a}	7.9 ± 0^{a}	8.1 ± 0.06^{a}	7.7 ± 0.16^{a}	7.9 ± 0.07^{a}	7.9 ± 0.07^{a}	7.8 ± 0.10^{a}	7.9 ± 0.15^{a}
Total bacterial numbers on TSA with 1 ml AIT	6.8 ± 0.07^{a}	6.7 ± 0.26^{b}	7.1 ± 0.74^{b}	7.6 ± 0.13^{a}	7.8 ± 0.16^{a}	7.8 ± 0.16^{a}	7.0 ± 0.27^{b}	7.4 ± 0.43^{b}

[1] Each value represents the logarithm of the mean of three trials. Each was done in duplicate, n=6.
Means with different lower case superscripts in each treatment of each day (column) are significantly different (P<0.05).

Appendix 9. Antimicrobial effects of AIT 0.5 and 1 ml on natural microflora in vacuum packed (N_2 backflushed) ground beef patties stored at -18°C and plated on TSA.

	Bacterial numbers[1] ± standard deviation (Log_{10} cfu/g)							
	Days							
Treatments	0	5	10	15	20	25	30	35
Total bacterial numbers on TSA without antimicrobial	3.8 ± 0.25^a	3.3 ± 0.27^a	3.9 ± 0.18^a	3.9 ± 0.19^a	3.8 ± 0.45^a	4.0 ± 0.24^a	3.6 ± 0.11^a	3.6 ± 0.09^a
Total bacterial numbers on TSA with 0.5 ml AIT	3.6 ± 0.40^a	4.0 ± 0^b	3.4 ± 0.28^b	3.5 ± 0.41^b	3.3 ± 0.23^b	3.6 ± 0.10^b	3.5 ± 0.28^a	3.4 ± 0.28^a
Total bacterial numbers on TSA without antimicrobial	5.5 ± 0.05^a	5.6 ± 0.07^a	6.1 ± 0.24^a	5.8 ± 0.33^a	6.6 ± 0.08^a	5.8 ± 0.40^a	6.8 ± 0.34^a	5.6 ± 0.22^a
Total bacterial numbers on TSA with 1 ml AIT	5.5 ± 0.05^a	5.5 ± 0.14^b	6.0 ± 0.09^a	6.0 ± 0.17^a	6.1 ± 0.05^b	5.8 ± 0.07^a	5.1 ± 0.21^b	5.4 ± 0.19^a

[1] Each value represents the logarithm of the mean of three trials. Each was done in duplicate, n=6.
Means with different lower case superscripts in each treatment of each day (column) are significantly different ($P<0.05$).

Appendix 10. Antimicrobial effects of AIT (0.5 and 1 ml) on natural microflora and inoculated levels (3 $\log_{10}$ cfu/g) of *E. coli* O157:H7 in vacuum packed (N_2 backflushed) ground beef patties stored at 4°C and plated on TSA and CT-SMAC

	Bacterial numbers[1] ± standard deviation (Log_{10} cfu/g)							
	Days							
Treatments	0	3	6	9	12	15	18	21
Total bacterial numbers on TSA without antimicrobial	3.6±0.19^{a}	4.5±0.12^{a}	5.7±0.24^{a}	5.7±0.23^{a}	6.5±0.32^{a}	7.1±0.23^{a}	7.0±0.17^{a}	7.3±0.08^{a}
Total bacterial numbers on TSA with 0.5 ml AIT	3.6±0.19^{a}	4.2±0.41^{a}	5.4±0.26^{b}	6.1±0.61^{a}	6.5±0.35^{a}	6.7±0.29^{b}	6.7±0.29^{b}	6.5±1.04^{a}
Total bacterial numbers on CT-SMAC without antimicrobial	3.3± 0.22^{a}	3.1±0.16^{a}	2.9±0.46^{a}	2.4±0.67^{a}	2.6±0.45^{a}	2.6±0.70a	2.1±0.19^{a}	2.1±0.12^{a}
Total bacterial numbers on CT-SMAC with AIT (0.5 ml)	3.3± 0.22^{a}	2.2±0.21^{b}	2.0±0^{b}	2.4±0.51^{a}	2.1±0.12^{b}	0.7±1.11^{b}	0±0^{b}	0±0^{b}
Total bacterial numbers on TSA without antimicrobial	4.6±0.34^{a}	6.3±0.06^{a}	8.3±0.11^{a}	7.7±0.06^{a}	7.8±0.08^{a}	7.5±0.11^{a}	7.8±0.05^{a}	7.7±0.06^{a}
Total bacterial numbers on TSA with 1 ml AIT	4.6±0.34^{a}	6.3±0.17^{a}	6.8±0.48^{b}	7.3±0.12^{b}	6.6±0.12^{b}	6.4±0.21^{b}	6.4±0.41^{b}	5.4±0.20^{b}
Total bacterial numbers on CT-SMAC without antimicrobial	3.6±0.40^{a}	3.4±0.23^{a}	3.3±0.28^{a}	3.5±0.16^{a}	3.6±0.24^{a}	3.5±0.04^{a}	3.5±0.50^{a}	3.4±0.29^{a}
Total bacterial numbers on CT-SMAC in 1 ml AIT	3.6±0.40^{a}	1.9±0.05^{b}	1.1±0.12^{b}	0.9±0.23^{b}	0±0^{b}	0.26±0.22^{b}	0±0^{b}	0±0^{b}

[1] Each value represents the logarithm of the mean of three trials. Each was done in duplicate, n=6.
Means with different lower case superscripts in each treatment of each day (column) are significantly different (P<0.05).

Appendix 11. Antimicrobial effects of AIT (1 ml) on natural microflora and inoculated levels 3 $\log_{10}$ cfu/g of *E. coli* O157:H7 in vacuum packed (N_2 backflushed) ground beef patties stored at 10°C and plated on TSA.

	Bacterial numbers[1] ± standard deviation (Log_{10} cfu/g)							
	Days							
Treatments	0	1	2	3	4	5	6	8
Total bacterial numbers on TSA without antimicrobial	6.8 ± 0.06^a	7.5 ± 0.13^a	7.6 ± 0.04^a	7.8 ± 0.02^a	7.6 ± 0.09^a	7.9 ± 0.15^a	7.8 ± 0.11^a	7.8 ± 0.10^a
Total bacterial numbers on TSA with 1 ml AIT	7.0 ± 0.12^b	7.3 ± 0.22^a	7.3 ± 0.20^b	7.5 ± 0.16^b	7.2 ± 0.17^b	7.4 ± 0.28^b	7.1 ± 0.39^a	6.8 ± 0.48^a
Total bacterial numbers on CT-SMAC without antimicrobial	3.1 ± 0.22^a	3.0 ± 0.36^a	2.8 ± 0.34^a	3.3 ± 0.13^a	3.13 ± 0.44^a	3.19 ± 0.23^a	3.73 ± 0.21^a	3.06 ± 0.31^a
Total bacterial numbers on CT-SMAC with 1 ml AIT	3.1 ± 0.22^a	3.1 ± 0.2^a	2.7 ± 0.38^a	2.5 ± 0.31^b	2.30 ± 0.33^b	2.23 ± 0.27^b	2.43 ± 0.29^b	2.06 ± 0.12^b

[1] Each value represents the logarithm of the mean of three trials. Each was done in duplicate, n=6.

Means with different lower case superscripts in each treatment of each day (column) are significantly different ($P<0.05$).

Appendix 12. Antimicrobial effects of AIT 0.5 and 1 ml on natural microflora and inoculated levels of 3 $\log_{10}$ *E. coli* O157:H7 in vacuum packed (N_2 backflushed) ground beef patties stored at -18°C and plated on TSA.

	Bacterial numbers[1] ± standard deviation (Log_{10} cfu/g)							
	Days							
Treatments	0	5	10	15	20	25	30	35
Total bacteria numbers on TSA without antimicrobial	6.1 ± 0.34^{b}	6.9 ± 0.11^{a}	7.0 ± 0.22^{a}	7.0 ± 0.11^{a}	7.2 ± 0.24^{a}	7.0 ± 0.18^{a}	7.3 ± 0.03^{a}	7.1 ± 0.13^{a}
Total bacterial numbers on TSA with 0.5 ml AIT	6.5 ± 0.18^{a}	6.5 ± 0.17^{b}	6.5 ± 0.15^{b}	6.8 ± 0.23^{a}	6.6 ± 0.48^{b}	6.6 ± 0.23^{b}	6.4 ± 0.17^{b}	6.5 ± 0.12^{b}
Total bacterial numbers on CT-SMAC without antimicrobial	3.5 ± 0.51^{a}	3.5 ± 0^{a}	3.3 ± 0.22^{a}	3.3 ± 0.34^{a}	2.7 ± 0.52^{a}	3.0 ± 0.67^{a}	3.0 ± 0.22^{a}	2.6 ± 0.45^{a}
Total bacteria numbers on CT-SMAC with 0.5 ml AIT	3.5 ± 0.55^{a}	3.0 ± 0^{b}	3.2 ± 0.25^{a}	3.1 ± 0.16^{a}	1.5 ± 1.18^{a}	1.2 ± 1.42^{b}	0.7 ± 1.03^{b}	0 ± 0^{b}
Total bacterial numbers on TSA without antimicrobial	6.2 ± 0.24^{a}	6.1 ± 0.26^{a}	6.2 ± 0.19^{a}	6.4 ± 0.19^{a}	6.2 ± 0.04^{a}	6.3 ± 0.08^{a}	6.2 ± 0.19^{a}	6.2 ± 0.10^{a}
Total bacteria numbers on TSA with 1 ml AIT	6.2 ± 0.24^{a}	6.1 ± 0.12^{a}	6.1 ± 0.14^{a}	6.0 ± 0.14^{b}	6.3 ± 0.19^{a}	6.3 ± 0.17^{a}	6.0 ± 0.16^{b}	5.9 ± 0.07^{b}
Total bacterial numbers on CT-SMAC without antimicrobial	3.2 ± 0.12^{a}	2.7 ± 0.10^{a}	2.4 ± 0.30^{a}	2.1 ± 0.12^{a}	2.9 ± 0.21^{a}	2.2 ± 0.12^{a}	2.0 ± 0^{a}	2.3 ± 0.35^{a}
Total bacteria numbers on CT-SMAC with 1 ml AIT	3.2 ± 0.12^{a}	2.2 ± 0.16^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}

[1] Each value represents the logarithm of the mean of three trials. Each was done in duplicate, n=6.
Means with different lower case superscripts in each treatment of each day (column) are significantly different (P<0.05).

Appendix 13. Antimicrobial effects of AIT (0.5 and 1 ml) on natural microflora and inoculated levels 6 $\log_{10}$ cfu/g) of *E. coli* O157:H7 in vacuum packed (N_2 backflushed) ground beef patties stored at 4°C and plated on TSA and CT-SMAC

	Bacterial numbers[1] ± standard deviation (Log_{10} cfu/g)							
	Days							
Treatments	0	3	6	9	12	15	18	21
Total bacterial numbers on TSA without antimicrobial	6.4 ± 0.14^{a}	6.4 ± 0.10^{a}	6.2 ± 0.10^{a}	6.4 ± 0.10^{a}	7.1 ± 0.20^{a}	7.2 ± 0.10^{a}	7.2 ± 0.08^{a}	7.2 ± 0.09^{a}
Total bacterial numbers on TSA with 0.5 ml AIT	6.4 ± 0.14^{a}	6.1 ± 0.11^{b}	5.8 ± 0.53^{a}	5.8 ± 0.46^{b}	6.1 ± 0.07^{b}	6.6 ± 0.45^{b}	6.6 ± 0.51^{b}	7.9 ± 0.15^{a}
Total bacterial numbers on CT-SMAC without antimicrobial	6.4 ± 0.05^{a}	6.2 ± 0.09^{a}	6.3 ± 0.18^{a}	5.7 ± 0.23^{a}	6.3 ± 0^{a}	5.3 ± 0.26^{a}	5.3 ± 0.22^{a}	5.2 ± 0.23^{a}
Total bacterial numbers on CT-SMAC with 0.5 ml AIT	6.4 ± 0.05^{a}	5.4 ± 0.12^{b}	6.0 ± 0.06^{b}	5.4±0.20a	5.3 ± 0.15^{b}	5.0 ± 0.35^{a}	4.9 ± 0.19^{b}	5.2 ± 0.12^{a}
Total bacterial numbers on TSA without antimicrobial	6.2 ± 0.03^{a}	6.3 ± 0.15^{a}	6.7 ± 0.16^{a}	7.4 ± 0.11^{a}	7.6 ± 0.08^{a}	7.5 ± 0.10^{a}	7.5 ± 0.15^{a}	7.4 ± 0.19^{a}
Total bacterial numbers on TSA with 1 ml AIT	6.2 ± 0.03^{a}	6.1 ± 0.05^{b}	6.2 ± 0.21^{b}	6.4 ± 0.54^{b}	6.5 ± 0.32^{b}	5.7 ± 0.51^{b}	6.1 ± 0.20^{b}	5.6 ± 0.26^{b}
Total bacterial numbers on CT-SMAC without antimicrobial	6.2 ± 0.06^{a}	6.2 ± 0.19^{a}	6.1 ± 0.08^{a}	5.8 ± 0.15^{a}	5.5 ± 0.14^{a}	5.6 ± 0.04^{a}	5.5 ± 0.23^{a}	5.6 ± 0.06^{a}
Total bacterial numbers on CT-SMAC with 1 ml AIT	6.2 ± 0.06^{a}	5.7 ± 0.25^{b}	5.7 ± 0.10^{b}	5.3 ± 0.58^{a}	4.9 ± 0.55^{b}	3.8 ± 0.69^{b}	3.4 ± 0.41^{b}	2.8 ± 0.28^{b}

[1] Each value represents the logarithm of the mean of three trials. Each was done in duplicate, n=6.
Means with different lower case superscripts in each treatment of each day (column) are significantly different (P<0.05).

Appendix 14. Antimicrobial effects of AIT (1 ml) on natural microflora and inoculated levels 6 $\log_{10}$ cfu/g of *E. coli* O157:H7 in vacuum packed (N_2 backflushed) ground beef patties stored at 10°C and plated on TSA and CT-SMAC

	Bacterial numbers[1] ± standard deviation (Log_{10} cfu/g)							
	Days							
Treatments	0	1	2	3	4	5	6	8
Total bacterial numbers on TSA without antimicrobial	7.1 ± 0.11^{a}	7.1 ± 0.26^{a}	7.6 ± 0.06^{a}	7.8 ± 0.06^{a}	7.9 ± 0.09^{a}	7.9 ± 0.10^{a}	7.7 ± 0.07^{a}	7.9 ± 0.06^{a}
Total bacterial numbers on TSA with 1 ml AIT	7.1 ± 0.11^{a}	7.1 ± 0.26^{a}	7.3 ± 0.36^{a}	7.4 ± 0.11^{b}	7.4 ± 0.17^{b}	7.6 ± 0.16^{b}	6.5 ± 0.32^{b}	6.1 ± 0.37^{b}
Total bacterial numbers on CT-SMAC without antimicrobial	6.1 ± 0.26^{a}	6.0 ± 0.81^{a}	5.9 ± 0.16^{a}	6.0 ± 0.22^{a}	5.9 ± 0.14^{a}	5.8 ± 0.16^{a}	5.2 ± 0.73^{a}	5.5 ± 0.21^{a}
Total bacterial numbers on CT-SMAC with 1 ml AIT	6.1 ± 0.26^{a}	5.6 ± 0.31^{a}	5.7 ± 0.16^{a}	5.8 ± 0.13^{a}	5.8 ± 0.06^{a}	5.2 ± 0.73^{b}	4.4 ± 0.61^{a}	5.0 ± 0.14^{b}

[1] Each value represents the logarithm of the mean of three trials. Each was done in duplicate, n=6.
Means with different lower case superscripts in each treatment of each day (column) are significantly different ($P<0.05$).

Appendix 15. Antimicrobial effects of AIT (0.5 and 1 ml) on natural microflora and inoculated levels 6 $\log_{10}$ cfu/g of *E. coli* O157:H7 in vacuum packed (N_2 backflushed) ground beef patties stored at -18°C and plated on TSA and CT-SMAC.

Treatments	Bacterial numbers[1] ± standard deviation (Log_{10} cfu/g)							
	Days							
	0	5	10	15	20	25	30	35
Total bacterial numbers on TSA without antimicrobial	7.3±0.17^{a}	8.2±0.26^{a}	7.4±0.14^{a}	7.5±0.12^{a}	7.6±0.16^{a}	8.0±0.28^{a}	7.3±0.07^{a}	7.5±0.18^{a}
Total bacterial numbers on TSA with 0.5 ml AIT	7.2±0.11^{a}	7.0±0.17^{b}	7.4±0.12^{a}	7.4±0.10^{a}	7.5±0.15^{a}	7.4±0.18^{a}	7.3±0.17^{a}	7.7±0.10^{a}
Total bacterial numbers on CT-SAMAC without antimicrobial	5.7±0.25^{a}	6.1±0.12^{a}	5.8±0.19^{a}	5.3±0.11^{a}	5.5±0.10^{a}	5.2±0.25^{a}	5.1±0.10^{a}	5.3±0.03^{a}
Total bacterial numbers on CT-SMAC with 0.5 ml AIT	5.7±0.25^{a}	5.5±0.21^{b}	5.4±0.10^{b}	5.0±0.11^{b}	5.2±0.12^{b}	4.8±0.17^{b}	4.7±0.17^{b}	4.5±0.27^{b}
Total bacterial numbers on TSA without antimicrobial	6.5±0.09^{a}	6.5±0.13^{a}	7.1±0.10^{a}	6.7±0.24^{a}	6.7±0.22^{a}	6..6±0.08^{a}	6.6±0.14^{a}	6.4±0.15^{a}
Total bacterial numbers on TSA with 1.0 ml AIT	6.5±0.09^{a}	6.1±0.18^{b}	6.5±0.22^{b}	6.3±0.25^{b}	6.4±0.11^{b}	6.5±0.09^{a}	6.5±0.14^{a}	6.1±0.42^{a}
Total bacterial numbers on CT-SAMAC without antimicrobial	6.1±0.21^{a}	5.9±0.20^{a}	5.6±0.12^{a}	5.6±0.30^{a}	4.4±0.19^{a}	5.2±0.30^{a}	5.6±0.30a	5.1±0.12^{a}
Total bacterial numbers on CT-SMAC with 1.0 ml AIT	6.0±0.06^{a}	5.4±0.18^{b}	5.2±0.14^{b}	5.4±0.24^{a}	5.0±0^{b}	5.2±0.12^{a}	5.1±0.25^{b}	4.6±0.37^{b}

[1] Each value represents the logarithm of the mean of three trials. Each was done in duplicate, n=6.
Means with different lower case superscripts in each treatment of each day (column) are significantly different (P<0.05).

Appendix 16. Antimicrobial effects of mustard powder 5, 10, and 20% on natural microflora in vacuum packed (N_2 backflushed) ground beef patties stored at 4°C and plated on TSA.

	Bacterial numbers[1] ± standard deviation (Log_{10} cfu/g)							
	Days							
Treatment	0	3	6	9	12	15	18	21
Total bacterial numbers on TSA without antimicrobial	4.6±0.40^{a}	6.5±0.11^{a}	7.3±0.09^{a}	7.8±0.11^{a}	7.9±0.06^{a}	7.8±0.11^{a}	7.9±0.06^{a}	7.5±0.17^{a}
Total bacterial numbers on TSA; plus 5% mustard	2.7±0.07^{b}	5.5±0.24^{b}	7.3±0.03^{a}	7.7±0.21^{a}	7.9±0.13^{a}	7.5±0.11^{a}	7.9±0.10^{a}	7.4±0.16^{a}
Total bacterial numbers on TSA without antimicrobial	5.2±0.09^{a}	6.6±0.22^{a}	7.3±0.25^{a}	7.3±0.10^{a}	7.5±0.03^{a}	7.3±0.12^{a}	7.2±0.15^{a}	7.1±0.12^{a}
Total bacterial numbers on TSA; plus 10% mustard	4.9±0.23^{b}	5.2±0.07^{b}	6.4±0.18^{b}	6.2±0.30^{b}	5.4±0.36^{b}	5.9±0.11^{b}	6.3±0.28^{b}	5.7±0.32^{b}
Total bacterial numbers on TSA without antimicrobial	6.8±0.08^{a}	7.8±0.12^{a}	7.8±0.05^{a}	8.0±0.08^{a}	7.9±0.20^{a}	8.0±0.09^{a}	7.9±0.09^{a}	8.2±0.13^{a}
Total bacterial numbers on TSA; plus 20% mustard	5.5±0.12^{b}	6.1±0.13^{b}	7.7±0.16^{a}	7.8±0.22^{a}	8.0±0.08^{a}	8.0±0.17^{a}	7.9±0.07^{a}	8.1±0.13^{a}

[1] Each value represents the logarithm of the mean of three trials. Each was done in duplicate, n=6.

Means with different lower case superscripts in each treatment of each day (column) are significantly different (P<0.05).

Appendix 17. Antimicrobial effects of mustard powder 10% on natural microflora in vacuum packed (N_2 backflushed) ground beef patties stored at 4°C and plated on MRS.

Treatment	Bacterial numbers[1] ± standard deviation (Log_{10} cfu/g)							
	Days							
	0	3	6	9	12	15	18	21
Total bacterial numbers on MRS without antimicrobial	5.0±0.07^{a}	6.4±0.06^{a}	6.8±0.53^{a}	7.4±0.10^{a}	7.6±0.11^{a}	7.3±0.16^{a}	7.1±0.24^{a}	7.5±0.18^{a}
Total bacterial numbers on MRS; plus 10% mustard	4.6±0.15^{b}	5.0±0.16^{b}	6.9±0.15^{a}	7.3±0.04^{a}	7.0±0.12^{b}	7.2±0.15^{a}	7.0±0.08^{a}	6.7±0.21^{b}

[1] Each value represents the logarithm of the mean of three trials. Each was done in duplicate, n=6.
Means with different lower case superscripts in each treatment of each day (column) are significantly different (P<0.05).

Appendix 18. Antimicrobial effects of mustard powder 5, 10, and 20% on natural microflora and inoculated levels 3 log$_{10}$ cfu/g of *E. coli* O157:H7 in vacuum packed (N_2 backflushed) ground beef patties stored at 4°C and plated on TSA and CT-SMAC.

	Bacterial numbers[1] ± standard deviation (Log$_{10}$ cfu/g)							
	Days							
Treatment	0	3	6	9	12	15	18	21
Total bacterial numbers on TSA without antimicrobial	4.6 ± 0.34^a	6.3 ± 0.06^a	8.3 ± 0.11^a	7.7 ± 0.06^b	7.8 ± 0.08^b	7.5 ± 0.11^b	7.8 ± 0.05^b	7.7 ± 0.06^a
Total bacterial numbers on TSA; plus 5%	4.3 ± 0.19^a	5.2 ± 0.15^b	7.1 ± 0.17^b	8.0 ± 0.07^a	8.2 ± 0.06^a	7.9 ± 0.10^a	8.1 ± 0.08^a	7.6 ± 0.07^a
Total bacterial numbers on CT-SMAC without antimicrobial	3.6 ± 0.40^a	3.4 ± 0.23^a	3.3 ± 0.28^a	3.5 ± 0.16^a	3.6 ± 0.24^a	3.5 ± 0.04^a	3.5 ± 0.50^a	3.4 ± 0.29^a
Total bacterial numbers on CT-SMAC; plus 5%	3.1 ± 0.11^b	1.9 ± 0.05^b	1.5 ± 0.01^b	1.3 ± 0.03^b	0 ± 0^b	0.2 ± 0.36^b	0 ± 0^b	0 ± 0^b
Total bacterial numbers on TSA without antimicrobial	5.3 ± 0.08^a	7.2 ± 0.08^a	7.4 ± 0.12^a	7.6 ± 0.06^a	7.7 ± 0.18^a	7.6 ± 0.20^a	7.4 ± 0.19^a	7.4 ± 0.11^a
Total bacterial numbers on TSA; plus 10%	5.2 ± 0.22^a	5.0 ± 0.05^b	6.4 ± 0.38^b	7.2 ± 0.06^b	7.7 ± 0.07^a	7.7 ± 0.09^a	7.3 ± 0.07^a	7.1 ± 0.10^a
Total bacterial numbers on CT-SMAC without antimicrobial	3.5 ± 0.13^a	3.5 ± 0.15^a	2.7 ± 0.58^a	3.7 ± 0.14^a	2.5 ± 0.1^a	3.4 ± 0.14^a	2.9 ± 0.34^a	3.04 ± 0.17^a
Total bacterial numbers on CT-SMAC; plus 10%	2.7 ± 0.29^b	1.9 ± 0.51^b	1.9 ± 0.17^b	0.8 ± 0.67^b	0 ± 0^b	0 ± 0^b	0 ± 0^b	0 ± 0^b
Total bacterial numbers on TSA without antimicrobial	6.8 ± 0.08^a	7.5 ± 0.29^a	7.8 ± 0.04^a	7.9 ± 0.20^a	7.7 ± 0.32^a	7.9 ± 0.11^a	8.0 ± 0.06^a	7.8 ± 0.06^a
Total bacterial numbers on TSA; plus 20%	5.7 ± 0.20^b	5.7 ± 0.07^b	5.4 ± 0.08^b	6.9 ± 0.24^b	6.9 ± 0.32^b	7.2 ± 0.47^b	7.5 ± 0.24^b	7.4 ± 0.26^b
Total bacterial numbers on CT-SMAC without antimicrobial	3.3 ± 0.14^a	3.4 ± 0.37^a	3.8 ± 0.14^a	4.2 ± 0.19^a	3.3 ± 0.39^a	3.7 ± 0.64^a	4.2 ± 0.09^a	4.4 ± 0.14^a
Total bacterial numbers on CT-SMAC; plus 20%	3.2 ± 0.14^a	0 ± 0^b	0 ± 0^b	0 ± 0^b	0 ± 0^b	0 ± 0^b	0 ± 0^b	0 ± 0^b

[1] Each value represents the logarithm of the mean of three trials. Each was done in duplicate, n=6.
Means with different lower case superscripts in each treatment of each day (column) are significantly different ($P<0.05$).

Appendix 19. Antimicrobial effects of mustard powder 5, 10, and 20% on natural microflora and inoculated levels of 6 $\log_{10}$ cfu/g *E. coli* O157:H7 in vacuum packed (N_2 backflushed) ground beef patties stored at 4°C and plated on TSA and CT-SMAC.

Treatment	Bacterial numbers[1] ± standard deviation (Log_{10} cfu/g)							
	Days							
	0	3	6	9	12	15	18	21
Total bacterial numbers on TSA without antimicrobial	6.4 ± 0.10^{b}	7.2 ± 0.12^{a}	7.3 ± 0.10^{b}	7.9 ± 0.11^{b}	8.0 ± 0.04^{a}	7.7 ± 0.07^{a}	7.9 ± 0.03^{a}	7.5 ± 0.10^{a}
Total bacterial numbers on TSA; plus 5%	6.5 ± 0.04^{a}	6.3 ± 0.06^{b}	7.8 ± 0.33^{a}	8.2 ± 0.07^{a}	8.2 ± 0.05^{b}	7.7 ± 0.16^{a}	8.0 ± 0.09^{a}	7.4 ± 0.12^{a}
Total bacterial numbers on CT-SMAC without antimicrobial	6.5 ± 0.04^{a}	6.4 ± 0.11^{a}	6.7 ± 0.08^{a}	6.3 ± 0.24^{a}	6.1 ± 0.07^{a}	6.1 ± 0.14^{a}	6.0 ± 0.11^{a}	6.1 ± 0.05^{a}
Total bacterial numbers on CT-SMAC; plus 5%	6.4 ± 0.11^{b}	6.2 ± 0.14^{b}	6.4 ± 0.06^{b}	6.1 ± 0.06^{b}	5.2 ± 0.07^{b}	5.7 ± 0.37^{b}	5.0 ± 0.14^{b}	5.9 ± 0.06^{b}
Total bacterial numbers on TSA without antimicrobial	6.4 ± 0.11^{a}	7.2 ± 0.09^{a}	7.3 ± 0.10^{a}	7.4 ± 0.05^{a}	7.6 ± 0.11^{a}	7.5 ± 0.07^{a}	7.5 ± 0.14^{a}	7.4 ± 0.08^{a}
Total bacterial numbers on TSA; plus 10%	6.2 ± 0.04^{b}	6.0 ± 0.07^{b}	5.8 ± 0.33^{b}	5.7 ± 0.23^{b}	7.1 ± 0.40^{b}	6.8 ± 0.56^{b}	6.8 ± 0.03^{b}	6.5±0.41b
Total bacterial numbers on CT-SMAC without antimicrobial	6.2 ± 0.10^{a}	6.3 ± 0.10^{a}	5.7 ± 0.21^{b}	5.7 ± 0.21^{a}	5.7 ± 0.13^{a}	5.2 ± 0.17^{a}	5.2 ± 0.17^{a}	5.3 ± 0.18^{a}
Total bacterial numbers on CT-SMAC; plus 10%	6.2 ± 0.04^{a}	5.3 ± 0.11^{b}	4.3 ± 0.21^{d}	4.3 ± 0.2^{b}	4.9 ± 0.37^{b}	3.9 ± 0.33^{b}	3.5 ± 0.22^{b}	3.3 ± 0.11^{b}
Total bacterial numbers on TSA without antimicrobial	6.7 ± 0.06^{a}	7.3 ± 0.10^{a}	7.7 ± 0.14^{a}	7.9 ± 0.07^{a}	7.8 ± 0.15^{a}	7.9 ± 0.09^{a}	7.8 ± 0.14^{a}	8.0 ± 0.07^{a}
Total bacterial numbers on TSA; plus 20%	5.6 ± 0.06^{b}	5.8 ± 0.22^{b}	6.8 ± 0.44^{b}	6.7 ± 0.17^{b}	7.2 ± 0.07^{b}	7.4 ± 0.18^{b}	7.8 ± 0.08^{a}	7.9 ± 0.12^{a}
Total bacterial numbers on CT-SMAC without antimicrobial	6.1 ± 0.19^{a}	5.8 ± 0.08^{a}	5.6 ± 0.28^{a}	5.7 ± 0.10^{a}	5.6 ± 0.29^{a}	5.5 ± 0.17^{a}	5.7 ± 0.10^{a}	5.6 ± 0.14^{a}
Total bacterial numbers on CT-SMAC; plus 20%	5.4 ± 0.09^{b}	2.7 ± 0.22^{b}	1.7 ± 0.61^{b}	0.6 ± 0.14^{b}	0.3 ± 0.31^{b}	0.3 ± 0.26^{b}	0.6 ± 0.65^{b}	0 ± 0^{b}

[1] Each value represents the logarithm of the mean of three trials. Each was done in duplicate, n=6.
Means with different lower case superscripts in each treatment of each day (column) are significantly different (P<0.05).

Appendix 20. Sensory ballot

Please answer the following questions by placing a check (√) where appropriate.

1. Female _______ Male _______

2. Age a. 18-24 _________
b. 25-34 _________
c. 35-44 _________
d. 45-54 _________
e. 55+ _________

3. How often do you generally eat hamburger?

a. at least once a week ________
b. at least once a month ________
c. a few times a year _________

Please rinse your mouth with water and eat unsalted crackers before starting. You may rinse again at any time during the test if you need to. There are 3 samples for you to evaluate. Taste each one on the coded samples in the sequence presented, from left to right. Please check the box that best describes your overall opinion of each sample.

Code _____	Code_______	Code_______
Like Extremely	Like Extremely	Like Extreme
Like Very Much	Like Very Much	Like Very Much
Like Moderately	Like Moderately	Like Moderately
Like Slightly	Like Slightly	Like Slightly
Neither Like Nor Dislike	Neither Like Nor Dislike	Nor Dislike Nor Dislike
Dislike Slightly	Dislike Slightly	Dislike Slightly
Dislike Moderately	Dislike Moderately	Dislike Moderately
Dislike Very Much	Dislike Very Much	Dislike Very Much
Dislike Extremely	Dislike Extremely	Dislike Extremely
Comments	Comments	Comments

Thank you for your participation

Appendix 21. Sensory acceptability of cooked ground beef patties containing 0, 5, or 10% mustard powder

Treatments	Sensory scores [1]
Control (no mustard)	6.84±1.62^a
5% mustard	6.09± 1.7^b
10% mustard	5.97±2.0^b

[1] Mean and standard deviation obtained from score of 75 panelists.
Mean values with different lower case superscript are significantly different ($P<0.05$)

Appendix 22. Fat (%) in ground beef determined by Soxhlet method

Treatments	Fat (%)
1	29.3 ± 0.7
2	22.5 ± 0.9
3	24.5 ±0.4
4	31.6 ±0.2
5	30.6 ±0.9
6	23.6 ±0.8
7	25.6 ±0.6
8	26.9 ±0.4

[1] Each value represents the mean and standard deviation of three measurements