EFFECT OF DIFFERENT MARINADE TREATMENTS ON SURVIVAL AND MORPHOLOGY OF PATHOGENS IN BEEF JERKY

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The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

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Dedicated to my Parents

Sh. Krishan Gopal Khurana
And
Smt. Raj Kumari Khurana

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EFFECT OF DIFFERENT MARINADE TREATMENTS ON SURVIVAL AND MORPHOLOGY OF PATHOGENS IN BEEF JERKY

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ABSTRACT

In the early fall of 2003, the Food Safety and Inspection Service (FSIS 2004) found that producers of meat and poultry jerky were not adequately processing the meat to achieve the lethality required to kill or reduce the number of microorganisms. In this project, ground beef jerky was prepared with four different treatments i.e. traditional marinade (TM), modified marinade (MM), acetic acid-traditional marinade (AATM), Tween 20-traditional marinade (TWTM), along with a control.

The jerky strips were individually inoculated with four different bacterial strains i.e. *E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes* and *S. aureus* and 30 minutes were allowed for bacterial attachment. The strips were then individually vacuum packaged and stored at ambient temperature for analysis at 7 day intervals up to 28 days. The parameters studied were pH, water activity and enumeration of microbial count.

The pH ranged from 5.78 for control to 4.45 for MM. The water activity ranged from 0.923 for control to 0.533 for MM. For microbial count, in case of *E. coli* O157:H7, a decline of 2.59 log, 2.96 log and 2.79 log was observed for MM, AATM and TWTM respectively. Even TM was able to manifest a reduction of 2.89 log units. The bacterial count reduction was significantly different ($P < 0.05$) between MM and other three treatments i.e. TM, AATM and TWTM at the starting point of Day 1. The same pattern was observed during the middle of observation period i.e. Day 14. However, by the end

of Day 28 all four treatments i.e. TM, MM, AATM and TWTM were significantly different from control but not from each other. For *S.* Typhimurium, a decline of 3.47 log, 3.72 log and 3.74 log was observed for MM, AATM and TWTM respectively. However, TM was the most effective of all four treatments resulting in a reduction of 4.17 log units. The bacterial count reduction was significantly different ($P < 0.05$) between MM and other three treatments i.e. TM, AATM and TWTM at the starting point of Day 1. It continued until Day 7, however by the end of Day 28, all four treatments i.e. TM, MM, AATM and TWTM were significantly different from control but not from each other.

In the case of *S. aureus*, a decline of 2.31 log, 1.85 log and 2.44 log was observed for MM, AATM and TWTM respectively. TM resulted in a reduction of 2.05 log units. The bacterial count reduction was not significantly different between any of the treatments in the beginning i.e. Day 1. However, by the end of Day 14, MM, AATM and TWTM were significantly different ($P < 0.05$) from TM. By the end of Day 28, MM was significantly different ($P < 0.05$) from all the other treatments i.e. TM, AATM and TWTM, with TM and AATM overlapping with each other, TWTM overlapping with MM and AATM.

For *L. monocytogenes*, a decline of 2.77 log, 2.76 log and 2.73 log was observed for MM, AATM and TWTM respectively. TM resulted in a reduction of 2.22 log units. The bacterial count reduction was not significantly different between any of the treatments until Day 21. However, by the end of Day 28 MM was significantly different ($P < 0.05$) from TM but overlapping with both AATM and TWTM.

Therefore, the results confirmed efficacy of all the treatments in controlling post-processing contamination of ground beef jerky which was evident by low microbial count

in treated jerky samples compared to control. However, the effect of four different marinade treatments used i.e. TM, MM, AATM and TWTM varied greatly with respect to four bacterial strains used. AATM was the most effective treatment against *E. coli* O157:H7, TWTM against *S. aureus*, MM against *L. monocytogenes* and TM against *S.* Typhimurium.

Scanning electron microscopy was performed to confirm the results of microbiological evaluation by visual observation of pathogens on the surface of beef jerky strips. In general, the cells from all the four bacterial populations treated with the marinade treatments appeared wrinkled and, unlike the control, some fibrils connecting the cells to each other or attaching to the membrane filter began to appear. These fibrils were believed to be from cytoplasmic leakage. It is also possible that exoploysaccharide material on the outer membrane of the cells were untangled and released due to marinade treatments (Slavik and others 1994).

The response of *E. coli* O157:H7, *S.* Typhimurium, *S. aureus* and *L. monocytogenes* to marinade-induced stress as observed from Transmission Electron Microscopy (TEM) micrographs was in agreement with the results of microbiological evaluation, and enhanced the understanding of behavior of bacterial cells under marinade-stress conditions and assisted in analyzing the data obtained via quantitative techniques.

CHAPTER 1

INTRODUCTION

1.1 Background

Beef jerky is a product that is easy to prepare, lightweight, nutrient rich and shelf stable. Thus, a significant amount of this product is consumed in the United States (Harrison and others 1997; Calicioglu and others 2002). In the early fall of 2003, the Food Safety and Inspection Service (FSIS 2004) discovered that producers of meat and poultry jerky were not processing jerky sufficiently to accomplish the lethality required to destroy or restrict the numbers of microorganisms. This indicates that the safety of beef jerky for human consumption may be lower than desired.

The affiliation of jerky products with foodborne disease outbreaks (CDC 1995; Keene and others 1997; Eidson and others 2000) has raised questions about their safety. A recent report (Levine and others 2001) designated that from 1990 to 1999 cumulative prevalence of *Salmonella* and *L. monocytogenes* in jerky produced in federally inspected plants was 0.31 and 0.52%, respectively. Despite the apparent low incidence, because the tolerance for *Listeria* on ready-to-eat (RTE) foods, such as jerky, is zero, safety improvements are needed.

It is noticeable in the outbreak reports that some of the pathogen contamination occurred post-processing through cross-contamination of dried product with raw product by way of knives, work surfaces or through worker handling (Calicioglu and others 2003). Therefore, control of pathogenic contamination post-processing is especially crucial in foods like jerky that are consumed without further cooking. Instead of

concentrating on post - processing treatments for lethality of pathogens, most of the efforts have been targeted on adjusting the thermal process used to make jerky (Calicioglu and others 2003). FSIS (2004) identified points in commercial jerky processing where producers need to modify current practices.

First, because jerky may not be adequately heat treated to meet the lethality performance standards, use of moist cooking is an option. If the lethality compliance guidelines issued by FSIS are used for jerky produced by small and very small plants, a relative humidity above 90% should be maintained throughout the cooking or thermal heating process by using a sealed oven or steam injection (FSIS 2004). However, adding humidity to the processing of jerky is unusual for most jerky producers. Also, some jerky producers may not be able to control humidity well in the smokehouse.

Secondly, FSIS (2004) suggested cooking of meat to 71.1°C before drying. However, heating the meat to 71.1°C prior to drying did not significantly reduce pathogens in the uncured jerky (Harrison and others 1997). Also, preheating meat and/or drying jerky at high temperatures for extended periods may result in a product that differs from traditional jerky and reduces the acceptability (Calicioglu and others 2002).

Several studies have investigated the effectiveness of drying treatments and parameters (time, temperature) of the jerky process for inactivating *Salmonella* and other pathogens (Holley 1985 a, b; Harrison 1996; Harrison and others 2001; Keene and others 1997; Albright 2000). Nevertheless, the survival of pathogens, including *Salmonella* on jerky products inoculated after drying has not been studied. Also, the use of chemical intervention strategies has not been studied adequately (Albright 2000). Such

interventions can be a viable option to avoid severe heat treatments and may render residual antimicrobial effects during product storage (Calicioglu and others 2002). These chemicals may include organic acids (e.g., acetic acid), ethanol, lactates and food-grade surfactants (e.g., polysorbates). The utilization of organic acids for accomplishing decontamination of raw beef has been successfully confirmed previously (Ariyapitipun and others 2000; Mustapha and others 2002) and a corresponding effect was predicted for ground and formed beef jerky.

Interventions before and after marinating the strips of raw meat have been shown to suppress the growth of pathogens greater than that achieved by heating and drying alone and may reduce contamination levels acquired postprocess (Calicioglu and others 2003). Previous studies have also determined that jerky made from ground beef may pose a greater risk of foodborne illness than that from whole meat strips (Harrison and Harrison 1996; Harrison and others 1997).

Therefore, it is more suitable to use ground and formed jerky as a model as it is commercially produced by small and large processing companies and provides a representative model with better laboratory control than whole muscle jerky.

An important goal of this project was to address the need for alternative methods of processing jerky products which would provide maximum safety. Microbial challenge studies with pathogenic organisms are recognized as sound methodology for verification of these methods but cannot be accomplished in commercial establishments due to risk of environmental contamination. It was, therefore, an aim of this project to develop and test a processing method using different marinade treatments in a commercially suitable

model of ground and formed beef jerky and verify the effectiveness of different treatments by conducting microbial challenge studies and electron microscopic analysis.

1.2 Objectives

Three objectives were established for this research:

1. To evaluate the efficacy of different processing methods (marinade treatments) on pathogen reduction in ground and formed beef jerky by doing microbial count.
2. To substantiate microbiological evaluation by visual observation of pathogens on the surface of beef jerky by utilizing Scanning Electron Microscopy (SEM).
3. To analyze the damage caused by different marinade treatments on the pathogens by observing the morphology of pathogens by employing Transmission Electron Microscopy (TEM).

CHAPTER 2

LITERATURE REVIEW

2.1 Beef Jerky

Jerky is one of the oldest meat products that are preserved by salting and drying. It is comparatively easy to process, has a typical flavor, and requires no refrigeration during commercial distribution due to its low water activity (a_w). Also, jerky is nutritious (high in protein and low in fat), shelf-stable (0.75:1.00 moisture protein ratio), and therefore is in high demand as a snack food in many countries (Yang and others 2009). Various jerky's can be made using numerous recipes and meats from different species such as beef (Calicioglu and others 2003), pork (Han and others 2007), poultry (Pegg and others 2006) and game animals.

The name jerky is derived from the word "Ch'arki", a name evolved from the Quechuan language of the Incas (which literally translates into "dried meat"). What the Native American tribes called "pemmican" was jerky meat added to either crushed dried fruit or animal fat. The Native Americans taught these settlers the cutting of meat and preparing it into long strips and subsequently communicated with them the entire jerky making process and an assortment of seasonings to make different recipes (www.jerky.com).

2.1.1 Equipment

The basic requirement for making jerky is the elimination of moisture from meat. There are different ways to accomplish this. Meat can be dried in electric dehydrators, in

smokers, in ovens or with air drying. The selection of method depends on available space both in and outdoors, quality concerns and equipment costs (Bell 1996).

2.1.1.1 Electric Dehydrator

Several electric food dehydrators, diversified in size, style and design are available in the market and all of them work on the same principle, creating dry air which causes removal of moisture from food, with the round shape being the most commonly used electric dehydrator. Both the heat source and fan are located at the bottom with trays stacked on top with a lid covering the top tray (Bell 1996). Dry air either flows up through the trays, or the heated air is forced up through an opening located around the outside of the trays, which flows across each tray in a horizontal manner. Other dehydrator shapes available for use are square or rectangle, which correspond to microwave ovens, with a front door and removable trays. This style of dehydrator has the heat source and fan at the back or on one of the sides (Bell 1996).

2.1.1.2 Smokers

Smokers are designed from metal with a heat source, either electricity or gas located on the bottom. Chips, shavings, chunks of wood or sawdust smolder over the heat source, producing smoke. The jerky strips are placed on a rack and smoke swirls up and around all surfaces of the meat or fish strips placed above it (Bell 1996).

The most frequently used woods for producing smoke include hickory, alder, mesquite, sugar maple, birch, willow, apple, cherry, peach, pecan and beech, which are

available in different sized chunks, chips, or sawdust and shavings. Additional items that can be used include, coffee husks, corn cobs, sugar cane pulp, husks of coconut, rice straw, grape vines, nut hulls, fruit pits and green willow branches (Bell 1996).

2.1.1.3 Oven Drying

Gas, electric and convection ovens are the most commonly used devices for producing jerky. Ovens heat up the surrounding air, but unlike the convection ovens, they do not circulate it. As a result, jerky dried in an oven varies a great deal from one type of oven to another and exhibits more of a cooked flavor compared to the jerky dried in a dehydrator. Also, if the temperature of the oven is too high, jerky becomes tough, has a burnt flavor, a mealy texture and is more brittle than when dried in a dehydrator (Bell 1996).

2.1.2 Making Jerky

The process of making jerky can be as simple as sprinkling salt and pepper on meat or fish strips and then drying them over a smoky fire. The most popular way to add flavor to meat is to marinate it in a seasoned liquid called a marinade (Bell 1996). Salt in one form or other is the most popular jerky ingredient, as it draws water and blood from meat, which effectuates partial drying. Other functions include inhibition of the growth of microbes that cause spoilage, as a preservative by lengthening the shelf life and addition of flavor (Bell 1996). There are a variety of commercial spice mixes, commonly available from grocery stores, dehydrator manufacturers, spice shops, mail order and the internet,

which include honey glaze, teriyaki, mountain, peppered, Hawaiian, Western, jalapeno, etc. Any of these can be used for beef, buffalo, elk, chicken, turkey, fish and venison (Bell 1996).

2.1.2.1 Strip Jerky

Regardless of the kind of meat, lean cuts such as flank, round and loin should be the first choice because they have less bone and fat to discard. Easily cleanable flat cutting surfaces along with a sharp knife are the other basic requirements; the knife is required to remove any excess fat, membranes and connective tissue. Eliminating as much fat as possible helps in reducing a gamey or wild flavor and also precludes the development of rancidity (Bell 1996).

An electric slicer is used for cutting the strips, especially with frozen meat. Slicers help in producing uniform sized strips of meat. Cutting the meat across the grain is the common practice, as it produces a jerky which is easier to break apart and chew. The meat should be thawed naturally in the refrigerator, as it has been shown to have a better flavor and texture than meat thawed in a microwave (Bell 1996).

2.1.2.2 Ground Jerky

There has been an extraordinary rise observed in the popularity of jerky made from ground meat in the last few years. Commercially produced ground jerkies are usually extruded, and are referred to as textured jerky. Ground and formed jerky is easy to make, tastes great and has numerous advantages; it is cheaper than making jerky out of

the strips, dries faster and is easier to chew. Ground beef, venison, turkey and chicken are the commonly used meats. Another plus for making this type of jerky is that no marinade is wasted; it's all absorbed during the marination process (Bell 1996).

After the meat is ground and mixed with all the desired seasonings, it can be uniformly shaped into 0.3 to 0.6 cm thick strips or round sticks and a dehydrator or a smoker can be used for drying jerky. One pound of meat makes 10 to 12 jerky strips that are 1.9 cm wide and 12.7 to 15.2 cms long. There are many handy products that extrude ground meat into uniform shapes and sizes e.g. "jerky gun" or "jerky shooter", which resemble caulk guns (Bell 1996).

The gun barrel is filled with the ground meat mixture and the trigger is pulled back. This allows the wet meat mixture to be extruded in uniformly - shaped strips. These guns have extruder nozzles of different shapes and sizes to produce strips or sticks.

2.1.3 Storage

The low water activity of beef jerky does not allow the growth of most bacteria, yeast and many molds. Also, properly dried jerky with good packaging has a shelf life of at least half a year at room temperature. The thick pieces of jerky can be vacuum packaged and stored in a refrigerator or freezer. Jerky containing fat should not be stored at room temperature, as it can turn rancid (Bell 1996).

2.2 Pathogens

2.2.1 *E coli* O157:H7

Escherichia coli was established as a foodborne pathogen in the United States in 1971 when some imported cheeses turned up in 14 states that were found to be contaminated with an enteroinvasive strain that caused illness in nearly 400 individuals. Prior to 1971, there were at least five foodborne outbreaks that were reported in other countries, with the earliest occurrence in 1947 in England. However, evidence suggests that it was recognized as a human pathogen that was a cause of infant diarrhea as early as the 1700s (Neill and others 2001). Since the outbreaks involving meats in the United States in 1982 and 1993, the status of this bacterium as a foodborne pathogen is unquestioned.

2.2.1.1 The Recognized Virulence Groups

Five different virulence groups of *E. coli* are recognized based on their disease syndromes, characteristics, and also on their effect on certain cell cultures and serological groupings: enteroaggregative (EAggEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteropathogenic (EPEC), and enterotoxigenic (ETEC) (Jay 2005). Of these five groups, EHEC is the main serovar of concern in humans.

2.2.1.2 Enterohemorrhagic *E. coli* (EHEC)

The strains of this group are both similar and dissimilar to EPEC strains. They are similar to EPEC in their possession of the chromosomal gene *eae*A (or one that is

similar) and in the production of attachment-effacement lesions. In contrast to EPEC, EHEC strains affect only the large intestine (in piglet models) and produce large quantities of Shiga-like toxins (SLT, Stx) (Jay 2005).

EHEC strains produce a 60-MDa plasmid that encodes fimbriae, which help in mediating their attachment to culture cells, and they do not invade HEp-2 or INT407 cell lines, although some of the strains have the ability to invade some human epithelial cell lines (Oelschlaeger and others 1994).

2.2.1.2.1 The Toxins

The toxins of EHEC strains of *E. coli* O157:H7 are referred to as Shiga-like toxins (verotoxin, verocytotoxin) and the two prototypes as SLT-I and SLT-II. However, new terminology has been applied, and SLT-I is now referred to as Stx1 and the former SLT-II is designated as Stx2 (Calderwood and others 1996). Stx1 differs from Stx (Shiga-toxin) by three nucleotides and one amino acid, and is neutralized by antibodies to Stx. Stx1 and Stx2 are differentiated by a lack of cross-neutralization by homologous polyclonal antisera, and by a lack of DNA-DNA cross hybridization of their genes under conditions of high stringency (Calderwood and others 1996). All Stxs consist of a single enzymatically active 'A' subunit and multiple 'B' subunits. The cells sensitive to Stx possess globotrioaosylceramide (Gb_3), which acts as a receptor to the toxin, and sodium butyrate appears to play a role in sensitizing cells to Stxs (Louise and others 1995).

Once toxins bind to Gb_3, internalization follows, which allows their transport to the trans-Golgi network. Once inside the host cells, the 'A' subunit binds to and releases

an adenine residue from the 28S ribosomal RNA (rRNA) of the 60S ribosomal subunit and this inhibits protein synthesis. The 'B' subunits then form pentamers in association with a single 'A' subunit and thus, they are responsible for the binding of the toxin to the neutral glycolipid receptors (O'Brien and others 1992). Although serotype O157:H7 is the known prototype for this group, Stxs are produced by other serotypes also. Stx2 appears to be more significant in the etiology of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) than Stx1, the reason for which is unknown (O'Brien and others 1992).

2.2.1.2.2 Human Disease Syndromes/Prevalence

The first O157:H7 strain was recovered in 1975 from a patient suffering with bloody diarrhea and Stx-producing strains of *E. coli* were subsequently identified in 1977 in the United States (O'Brien and others 1977) and Canada (Konowalchuk and others 1977). Following its first isolation in 1975, the next recorded isolation of *E. coli* O157:H7 was done in 1978, when it was recovered from diarrheal stools in Canada. Both Hemolytic Uremic Syndrome (HUS) and Hemorrhagic Colitis (HC) are caused by Stx-producing strains of *E. coli* O157:H7. It has been estimated that from 2 to 7% of infections caused by *E. coli* O157:H7 lead to the development of HUS (Griffin and Tauxe 1991). HUS manifests itself as hemolytic anemia, thrombocytopenia and acute renal failure.

The first clinical description of HUS was done in 1955. A German study was done focusing on the duration of the shedding of *E. coli* O157:H7 in 53 children. Out of

the total 53, 28 children who had HC diarrhea shed the organism between 2 and 62 days (median of 13), whereas the 25 who developed HUS shed organisms for 5-124 days (median of 21) (Karch and others 1995). HUS is associated more with strains that produce Stx2 alone than with those that produce Stx1, or Stx1 and Stx2 (Ostroff and others 1989).

The first case of Hemorrhagic colitis as a foodborne disease was seen in 1982 in Oregon and Michigan. In both instances, victims had consumed sandwiches at a fast-food restaurant that contained undercooked ground beef (Riley and others 1983). Of the 43 patients, all of them experienced bloody diarrhea and severe abdominal cramps, with 63% of them experiencing nausea, 49% vomiting, but only 7% fever. The bloody red stool is the telltale symptom for this syndrome, which reflects involvement of the etiological agent in the colon. Fever is rare as seen from the above example, and an infectious dose is believed to be as low as 10 cfu.

2.2.1.3 Prevention

The prevention/avoidance of foodborne illness by *E. coli* O157:H7 can be achieved by these following practices:

- Adequate refrigeration of foods.
- Practicing good personal hygiene.
- Adequate cooking or heat processing.

However, because of the risk involved with young children, special precautions have to be observed. Because these organisms are heat-sensitive, there should not be any cases when foods are properly cooked (Jay 2005).

In the case of ground beef, it is recommended that it should be cooked to at least 160°F (71.1°C), or the core temperature be brought to a minimum of 155°F (68.3°C) for at least 15 seconds and that the juices are clear. Because of the unevenness of hamburger patties, cooking at 155 – 160°F (68.3 – 71.1°C) is recommended, which provides a measure of safety. Once cooked, hamburgers, as well as other meats, should not be held between 40° and 140°F for more than 3 – 4 hours (Jay 2005).

2.2.2 *Salmonella*

The genus *Salmonella* are the most important members among the Gram-negative rods that cause foodborne gastroenteritis. They are small, Gram-negative, non-spore forming rods which are indistinguishable from *E. coli* under the microscope or on an ordinary nutrient media. They are ubiquitous in nature, with humans and animals being their primary reservoirs. *Salmonella* food poisoning is the result of ingesting foods containing appropriate strains of this genus in significant numbers (Jay 2005).

There have been significant changes adopted for the taxonomy of *Salmonella*. Even though food microbiologists, scientists and epidemiologists treat approximately 2,400 *Salmonella* serovars as though each was a species, all salmonellae have been placed in two species, namely *S. enterica* and *S. bongori*, with over 2,000 serovars being divided

into five subspecies or groups, most of which are classified under *S. enterica*, which is the type species (Le Minor and Popoff 1987).

All the major groups of *Salmonella* correspond to one of the following subspecies: group II (*S. enterica* subsp. *salmae*); group IIIa (*S. enterica* subsp. *arizonae*); group IIIb (*S. enterica* subsp. *diarizonae*); group IV (*S. enterica* subsp. *houtenae*); and group VI (*S. enterica* subsp. *indica*). The former group V organisms have been elevated in their status to *S. bongori* (Reeves and others 1989). These changes were made on the basis of DNA-DNA hybridization and multilocus enzyme electrophoretic characterizations of the salmonellae. Therefore, the long standing practice of treating *salmonella* serovars as species is no longer valid. For example, *S.* Typhimurium should be *S. enterica* serovar Typhimurium.

For epidemiological purposes, the salmonella have been placed into three groups:

1. Human pathogens: These include *S.*Typhi, *S.* Paratyphi A, *S.* Paratyphi C. This group comprises of agents of typhoid and the paratyphoid fevers, which are the most severe of all the diseases caused by salmonellae. Typhoid fever has the longest incubation time, which produces the highest body temperature, and has the highest mortality rate.
2. Host-adapted serovars (some of which are human pathogens and may be contracted from foods): Species included are *S.* Gallinarum (poultry), *S.* Dublin (cattle), *S.* Abortus-equi (horses), *S.* Abortus-ovis (sheep) and *S.* Cholerasuis (swine).

3. Unadapted serovars (no host preference): These are pathogenic for humans and other animals and they include most foodborne serovars.

2.2.2.1 Distribution

The intestinal tract of animals is the primary habitat for *Salmonella* spp. which includes birds, reptiles, farm animals, humans and occasionally insects. Even though their primary habitat is the intestinal tract, *Salmonella* can also be found in other parts of the body from time to time. As intestinal forms, the organisms are excreted in feces from which they may be transmitted by insects and other living creatures to a large number of places. They may also be found in water, especially polluted water. When polluted water and foods that have been contaminated by insects or by other means are consumed by humans and other animals, these organisms are once again shed through fecal matter with a continuation of the cycle (Jay 2005).

2.2.2.2 Growth and Destruction of Salmonellae

The growth pattern exhibited by these organisms is typical of other Gram-negative bacteria in that they are able to grow on a large number of culture media and produce visible colonies well within 24 hours at an incubation temperature of 37°C. They are generally able to ferment disaccharides such as lactose and sucrose, and some of the monosaccharides such as glucose are fermented with the production of gas.

The optimum pH for growth is around neutrality and values above 9.0 and below 4.0 are bactericidal. The minimum pH recorded for growth for some species is 4.05;

however, depending on the acid used to lower the pH, the minimum value may be as high as 5.5 (Chung and Goepfert 1970). The best growth for *Salmonella* is observed at a pH between 6.6 and 8.2 and the lowest temperatures at which growth has been observed are 5.3°C for *S.* Heidelberg and 6.2°C for *S.* Typhimurium (Matches and Liston 1968). The upper limit for growth temperature has been reported to be around 45°C. Inhibition of growth has been observed at water activity (a_w) values below 0.94 in the media with neutral pH; however, higher a_w values are required as the pH starts to decrease towards minimum growth value. Unlike the staphylococci, the salmonellae are not able to tolerate high salt concentrations and a brine solution of 9% has been reported to be bactericidal (Jay 2005).

2.2.2.3 The *Salmonella* Food-Poisoning Syndrome

Salmonella food-poisoning is caused by the ingestion of foods which contain a significantly large number of non-host-specific species or serotypes of the genus *Salmonella*. All the known *S. enterica* serovars carry pathogenicity islands 1 and 2 (SPI-1, SPI-2), which have been shown to be acquired by way of horizontal transfer either by the plasmids or phages (Baumler and others 1998). In the serovar *S.* Typhimurium, at least 60 genes are required for virulence (Groisman and Ochman 1997) and the two SPIs have been shown to contain at least 42 of these genes.

The virulent strains of *S. enterica* initiate their infection process in the nonphagocytic cells by attaching themselves to the intestinal mucosal cells by the means of fimbrial adhesions, which are encoded by a gene on *salmonella* pathogenicity island 1

SPI-1 (Van der Velden and others 1998). This is followed by the penetration of the intestinal mucosal cells, mainly at the lymphoid follicles of Peyer's patches. The initial site of infection for the virulent strains of *S. enterica* is the ileum portion of the small intestine.

In the case of *S.* Typhimurium, fimbriae are used that selectively allows them to adhere to M cells and although they can enter any intestinal epithelial cell type, M cells are the preferred site of attachment. The neutrophils start to migrate across the epithelial cells and cytokines (e.g. interlukin-8) are produced. Once they are inside the epithelial cells, they remain inside the membrane-bound vacuoles during their entire intracellular stage (Richter-Dahlfors and Finlay 1997).

Following the multiplication, the neutrophils ultimately burst and the pathogen is spread. The entry of salmonella cells into macrophages is also accompanied by the same mechanism of membrane ruffling and macropinocytosis (Richter-Dahlfors and Finlay 1997). Once inside the macrophages, they are found inside the membrane-bound phagosomes, which become enlarged.

Following the ingestion of food contaminated with *Salmonella* cells, the symptoms usually start to develop in 12-14 hours, although shorter and longer times have been observed. The symptoms comprise of nausea, vomiting, abdominal pain (not as severe as with staphylococcal food poisoning), headache, chills and diarrhea. These symptoms are accompanied by prostration, muscular weakness, faintness, moderate fever, restlessness and drowsiness (Jay 2005). The symptoms normally persist for 2-3 days and the average mortality rate is 4.1%. Even though these organisms generally

disappear frequently from the intestinal tract, up to 5% of patients can become carriers of these organisms even after recovery from this disease. The minimum number of cells required for salmonellosis is in the range of 10^7-10^9/gm (Jay 2005).

2.2.2.4 Incidence and Vehicle Foods

The fact that *S.* Enteritidis is highly associated with the consumption of raw or undercooked eggs in the United States, the Centers for Disease Control and Prevention has recommended the following guidelines (CDC, 2000):

1. Avoidance of raw or undercooked eggs, especially by the young, elderly and immunocompromised.
2. Using pasteurized egg products, in case the eggs are cooked improperly.
3. Cooking raw eggs at ≥ 63°C for at least 15 seconds or until both yolk and white become firm.
4. Cooking the dishes containing raw eggs up to 71.1°C.
5. Storage of raw eggs at ≤ 7.2°C at all times.

The exact reason for the increased incidence of *S.* Enteritidis outbreaks in association with the eggs and poultry products is not yet clear. The suggested possible routes for transmission of *S.* Enteritidis to the eggs are as follows (Keller and others 1995):

1. Transovarial
2. Translocation from the peritoneum to yolk sac or oviduct.
3. Penetration of shell by organisms as eggs pass through the cloaca.

4. Egg washing
5. Food handlers

2.2.2.5 Prevention and Control of Salmonellosis

The main reservoir for *Salmonella* is the intestinal tract of both humans and animals. Of greater importance is the animal fecal matter, as it can contaminate the hides of animals. The maintenance of *Salmonella* species within an animal population is mediated by means of nonsymptomatic animal infections and their prevalence in animal feeds. Another important source of salmonellae in human infections is the secondary contamination (Jay 2005).

Because of the worldwide distribution of Salmonella , the optimum control of foodborne salmonellosis can only be achieved by getting the animals and humans free from these organisms. This looks like a difficult task to accomplish, but it is not impossible because only 35 of the 2,400 known serovars account for 90% of the human isolates and approximately 80% of nonhuman isolates (Martin and Ewing 1969).

The human carriers are thought to play a role in pathogenesis at the consumer level, but the importance of their role is not yet clear. The primary factors involved in outbreaks are the improper preparation and handling of foods in homes and food service establishments (Jay 2005).

Of critical importance are the hatchery eggs, because if they get contaminated then hatchlings may also become infected at this early stage. As a result of this,

salmonellae can rapidly penetrate freshly laid fertile eggs, become entrapped in the membrane and get ingested by an embryo as it emerges from the egg (Jay 2005).

2.2.3 *Staphylococcus*

The study on staphylococcal food-poisoning syndrome began in 1894 by J. Denys which was continued by M.A. Barber in 1914, who produced the symptoms of disease in himself by consuming milk that had been contaminated with a culture of *S. aureus* (Jay 2005). However, the conclusive evidence for capacity of *S. aureus* to produce food poisoning was proved by Dack and others (1930), who showed that the symptoms could be produced by feeding culture filtrates of *S. aureus*.

Staphylococcal gastroenteritis is mediated by the enterotoxin producing species and strains, and enterotoxin production is believed to be associated with the strains that produce coagulase and thermonuclease (TNase), however many of the species of genus *Staphylococcus* producing neither coagulase nor TNase have been shown to produce enterotoxins (Jay 2005).

The genus *Staphylococcus* includes more than 30 species. Of the 18 species and subspecies of concern, only 6 are reported to be coagulase positive and all of them produce thermonuclease (TNase). Ten of the coagulase-negative species are reported to produce enterotoxins, and they do not produce nuclease, those producing it, produce it in a thermolabile form (Jay 2005).

There is no consistency exhibited by the coagulase-negative, enterotoxin producing strains in their production of hemolysins or their fermentation of mannitol. This has led to

underestimation of the prevalence of enterotoxin producing strains, because for a long time it was thought that coagulase and TNase-positive strains are the only staphylococci that require any further investigations in staphylococcus infected foods (Jay 2005).

2.2.3.1 Habitat and Distribution

The *Staphylococci* are predominantly host-adapted species with about half of the known species inhabiting only humans (e.g. *S. cohnii* subsp. *cohnii*) or humans and other animals (e.g. *S. aureus*). They tend to be heavily populated at openings to the body surfaces such as the anterior nares, axillae and the inguinal and perineal areas. In the moist areas their numbers may reach around 10^3 - 10^6, whereas in dry areas it is limited to 10 - 10^3 (Kloos and Bannerman 1994).

Two of the most important sources for their transmission in foods are the nasal carriers and individuals having boils and carbuncles, who are allowed to handle foods. Even though the coagulase-negative species are noted to adapt to nonhuman hosts, their entry into human foods cannot be precluded. Once they reach inside the susceptible foods, they may produce enterotoxins (Jay 2005).

The general expectancy about staphylococci is that they are bound to be present, at least in low numbers, in any or all food products that are of animal origin or that are handled directly by humans, unless some kind of heat-processing step has been applied to effect their destruction (Jay 2005).

2.2.3.2 Growth Requirements

Typical of other Gram-positive bacteria, staphylococci also require some organic compounds as a part of their nutrition. They have a requirement for amino acids as nitrogen source and thiamine and nicotinic acid among the B vitamins. They require uracil for anaerobic growth (Jay 2005). Even though it is a mesophile, some strains of *S. aureus* have been reported to grow at temperatures as low as 6.7°C. Angelotti and others (1961) observed three food-poisoning strains growing in custard at 45.6°C, which declined in growth at 46.7 - 48.9°C. In general, the observed growth range is 7 - 48°C, and enterotoxin production takes place between 10 - 46°C, with the optimum production occurring between 40 - 45°C as reported by Smith and others (1983).

Staphylococcus grows well in 7 - 10% of salt concentration, and some strains are even reported to grow up to 20%. In addition to this, *S. aureus* also has a high degree of tolerance to compounds such as tellurite, mercuric chloride, neomycin, polymyxin and sodium azide, all of which are used as selective agents in culture media. The differentiating feature of *S. aureus* with respect to other species is its greater resistance to acriflavine (Jay 2005).

S. aureus grows over a range of pH 4.0 - 9.8, but the optimum reported range is 6 - 7. With respect to water activity (a_w), staphylococcus is peculiar in being able to grow at values lower than those for any other nonhalophilic bacteria. Growth has been demonstrated at a_w values as low as 0.83 under conditions optimum in other respect, however, the generally recognized minimum a_w value is 0.86 (Jay 2005).

2.2.3.3 Staphylococcal Enterotoxins

Thirteen staphylococcal enterotoxins (SEs) had been identified by 2001. SEA has been recovered more frequently from food-poisoning outbreaks compared to other toxins, with SED being the second most common. SEE is associated with the fewest number of outbreaks. All the enterotoxins of staphylococci are simple proteins, which upon hydrolysis yield about 18 amino acids, with aspartic acid, glutamic acid, lysine and tyrosine being the predominant ones. Based on their amino acid sequence, SEA, SED, SEE and SEI are categorized into one group, while SEB, the SECs and SEG are grouped into another (Munson and others 1998).

2.2.3.3.1 Mode of Action

All the enterotoxins produced by *Staphylococcus*, along with the toxic shock syndrome toxin (TSST), are bacterial superantigens (pyrogenic toxin superantigens - PTSags) relative to *in vivo* antigen recognition in contrast to conventional antigens (Jay 2005). The enterotoxins bind directly to T cell receptor β chains. Once bound to major histocompatability complex (MHC) class II molecules, the enterotoxins stimulate helper T cells to produce cytokines such as the interleukins (IL), gamma-interferon and tumor necrosis factor (Jay 2005). Among the cytokines produced, there is an over-abundance of IL-2 (Johnson and others 1992), which is the phenomenon responsible for manifestation of the symptoms of staphylococcal gastroenteritis, including vomiting and diarrhea.

2.2.3.4 The Gastroenteritis Syndrome

The symptoms of staphylococcal food poisoning start to appear within 4 hours, after the ingestion of contaminated food. However, a range of 1 - 6 hours has been reported. The symptoms include nausea, vomiting, abdominal cramps (very severe and painful), diarrhea, sweating, headache, prostration and last from 24 to 48 hours. There is no mortality involved and the treatment usually consists of bed rest and lots of fluid intake to restore the fluid balance in the body (Jay 2005). The minimum infectious dose of enterotoxin needed for causing illness is about 20 ng (Evenson and others 1988).

2.2.3.5 Prevention

Susceptible foods can be kept free of *S. aureus* enterotoxins and other food - poisoning hazards, if they are kept either at or below 4.4°C or above 60°C until consumed (Jay 2005). The most frequently involved factors contributing to *S. aureus* food - poisoning syndromes (Bryan 1974) are:

- Inadequate refrigeration
- Preparing foods in advance
- Poor personal hygiene
- Inadequate cooking or heat processing
- Holding foods in danger zone (4.4 - 60°C)

So, by taking care of the above listed factors, the prevention of staphylococcus food - poisoning can be accomplished.

2.2.4 *Listeria*

Listeria belongs to a group of Gram - positive, non-spore-forming and acid-fast rods, that were once known as "*Listerella*". Six different types of species have been recognized on the basis of the possession of antigens, which give rise to 17 serovars (Jay 2005). The main pathogenic species, which is *L. monocytogenes* is represented by 13 serovars, some of which are shared by *L. innocua* and *L. seeligeri*. Even though *Listeria innocua* is represented by only 2 serovars (6a/6b), it is referred to as the nonpathogenic variant of *L. monocytogenes* (Jay 2005). The genus listeria is widely distributed in nature and can be detected from decaying vegetation, soils, animal feces, sewage, silage and water. In general, listeria can be expected to exist in the environment favorable for lactic acid bacteria, *Brochothrix* and some coryneform bacteria. Their association with certain dairy products and silage is also well documented (Jay 2005).

Any fresh food product of animal or plant origin can harbor *L. monocytogenes* in variable numbers. This organism can be found in raw milk, soft cheeses, fresh and frozen meat, poultry and seafood as well as on fruits and vegetable products (Jay 2005). Its predominance in milk and other dairy products has received a lot of attention because of early outbreaks.

2.2.4.1 Growth

The nutritional requirement of listeria is typical of any other Gram-positive bacteria. It grows well in common media, such as brain heart infusion (BHI), tryptic soy and tryptose broth. *Listeria* exhibits a requirement for B vitamins, biotin, riboflavin,

thiamine and thioctic acid along with the amino acids cysteine, glutamine, isoleucine, leucine and valine (Jay 2005).

Listeria is reported to grow best in the pH range 6 - 8, however the minimum pH permitting its growth and survival is still a subject of research. All of the studies have been conducted on *L. monocytogenes* and correlation of these findings with other species can only be assumed (Jay 2005).

2.2.4.2 Virulence Properties

Out of the six recognized species of *Listeria, L. monocytogenes* is the major pathogen of interest for humans. Even though *L. ivanovii* is able to multiply in the mouse model, it is much less severe than *L. monocytogenes*, and even a concentration of 10^6 cells did not cause any infection in the mice as observed by Hof and Hefner (1988). Other listerial species such as *L. innocua, L. welshimeri* and *L. seeligeri* are nonpathogens. The most profound virulence factor associated with *L. monocytogenes* is listeriolysin O (LLO) (Jay 2005).

2.2.4.2.1 Listeriolysin O

The exhibition of beta - hemolysis on blood agar plates and production of acid from rhamnose are the hallmarks of the pathogenic/virulent strains of *L. monocytogenes* (Jay 2005). With respect to hemolysis, there is a lot of evidence, which confirms that all the virulent strains of listeria produce a unique substance known as LLO, which is responsible for beta - hemolysis on erythrocytes and the killing of phagocytic cells that

envelop them (Jay 2005). Listeriolysin O has been purified and demonstrated to have a molecular weight of 60,000 Da and is comprised of 504 amino acids (Geoffroy and others 1987; Mengaud and others 1988). It is reported to develop during the exponential growth phase of listeria as seen by Geoffroy and others (1989), with maximum levels produced after 8-10 hours of growth.

2.2.4.2.2 Intracellular Invasion

Immediately after the acquisition of *L. monocytogenes* through the oral route, it seems to colonize in the intestinal tract by mechanisms that are not yet understood properly. From the intestinal tract, the bacterial cells permeate other tissues, including the placenta in pregnant women, and therefore enter the blood stream, from where they reach other vulnerable body cells (Jay 2005). As an intrinsic pathogen, listeria, first enters the vulnerable cells and then starts to replicate within those cells. In case of phagocytes, the influx occurs in two steps: first, a direct entry into the phagosomes and then from the phagosomes into the cytoplasm of phagocytes (Jay 2005).

L. monocytogenes outlasts the protective mechanisms of macrophages by avoiding the phagolysosomal membranes and migrating into the cytosol (cytoplasm), a process assisted by LLO (listeriolysin). After it reaches the cytoplasm, the surface protein ActA (encoded by *actA*) supports in the formation of actin tails, which propel the bacterial cells towards the cytoplasmic membrane (Jay 2005). With the help of LLO and two other bacterial phospholipases, namely, the phosphatidylinositol-specific phospholipase C (encoded by *plcA*) and the broad-range phospholipase C (encoded by

plcB), the bacterial cells are liberated and this whole process is replicated upon the entry of bacterial cell into the adjacent host cells (Jay 2005).

2.2.4.2.3 Monocytosis - Producing Activity

One of the riveting aspects of *L. monocytogenes* is the presence of a lipid-containing component within the cell envelope, which is reported to share one property with the lipopolysaccharide (LPS), an integral component of Gram - negative bacteria (Jay 2005). This component of *L. monocytogenes* is designated as lipoteichoic acid (LTA). It has been demonstrated long ago that phenol-water extracts of listerial cells are capable of generating the production of monocytes, and as a result of this monocyte-generating activity, listeria received the species name of *monocytogenes* (Jay 2005).

2.2.4.3 Symptoms

As such there are no specific symptoms defined for human listeriosis, because the pathway of this disease depends on the immunological state of the individual host (Jay 2005). Non-pregnant, non-immunosuppressed healthy individuals are extremely resistant to *L. monocytogenes*, and there is slight evidence to prove that such individuals even get infected by listeriosis. However, there are certain conditions of the body, which act as the predisposing factors to adult listeriosis e.g., acquired immuno deficiency syndrome (AIDS), alcoholism, diabetes Type I, cardiovascular disease, renal transplant and steroid therapy (Jay 2005). Meningitis and sepsis are the commonly spotted symptoms in vulnerable adults. Other complications related with the adult syndrome are cervical and

generalized lymphadenopathy, which corresponds with mononucleosis. Infected pregnant females usually do not exhibit any symptoms, but if they do, they are mild and influenza like. However, the consequences of concern are miscarriage, premature birth or stillbirth as the fetus gets infected congenitally (Jay 2005).

2.2.4.4 Regulatory Status of *L. monocytogenes* in Foods

There have been juristic limits set up by many countries on the number of organisms tolerable in foods, particularly ready-to-eat products; however, some of them have proposed certain guidelines that do not have any legal judgment (Jay 2005). The United States has a virtually intolerant policy or zero tolerance, according to which *L. monocytogenes* has been denominated as an "extraneous". This entails that any ready-to-eat food containing listeria is regarded as adulterated and will be susceptible to recall (Jay 2005).

The stipulation of the European community (EC) on milk and milk products necessitates zero tolerance for soft cheeses. The International Commission on Microbiological Specification for Foods (ICMSF) concurs that if listeria counts do not transcend 100 / gram of food at the time of consumption, the food is fit for healthy individuals (Jay 2005).

2.3 Electron Microscopy

2.3.1 Scanning Electron Microscopy

The original model of the scanning electron microscope (SEM) was fabricated by Knoll and von Ardenne in Germany in 1930. However, the resolution of this prototype was of mediocre quality (Bozzola 1999). The commercial SEM was made available in 1963 after a series of refinements accomplished by Zworykin at the RCA laboratories in the United States. The contemporary equipment has the resolution of 2.0 nm, with magnifications up to 200,000X (Bozzola 1999).

SEM is utilized by biologists to analyze the *three-dimensional* characteristics of individual cells, as compared to the Transmission Electron Microscopy, which is utilized to view thin slices of biological specimens (Bozzola 1999). SEM possesses electromagnetic lenses that are employed to reproduce a demagnified, focused spot of electrons that is run down over the surface of an electrically conductive specimen (Bozzola 1999).

These marching electrons then collide with the specimen giving rise to various signals including low energy secondary electrons from the topmost layer of the specimen. Some of these secondary electrons get amassed, processed and finally transformed as a series of pixels (picture elements) on the monitor (Bozzola 1999). For every point of strike of the electron beam on the specimen surface, a comparable pixel is displayed on the monitor used for viewing, with brightness of the pixel being directly proportional to the number of secondary electrons produced from the specimen surface (Bozzola 1999). The electron beam glances over the specimen rapidly, giving rise to numerous, infinite

points, which coalesce to form a perpetual image comprised of several shades of gray (Bozzola 1999). The shades are analogous to an average black and white photograph, in which light and dark areas correspond to the depth.

2.3.1.1 Electron Optical and Beam Control Systems

A system comprising of electromagnetic lenses, deflection coils and stigmators is necessitated for controlling and polishing the electron beam, after it leaves the electron gun and before it strikes the specimen (Bozzola 1999). Virtually all SEMs employ a V-shaped tungsten filament, which is heated to effectuate the thermionic emission of electrons, which speed up in the direction of the anode due to the application of negative high voltage. An initial focalized spot of electrons of roughly 50 μm in diameter is formed, which requires a series of two to three condenser lenses for doing consecutive demagnification down to 2 nm (Bozzola 1999).

2.3.1.2 Specimen Preparation for Scanning Electron Microscopy

A typical protocol for preparing specimens for SEM necessitates rinsing the surfaces to remove debris, stabilization in an aldehyde fixative, followed by a secondary fixation with osmium tetroxide, rinsing in distilled water, dehydrating, mounting of the sample on a metal stub, and coating the specimen with a thin, electrically conductive layer (Bozzola 1999).

Table 1: Commonly used Buffers and Fixatives in SEM (Bozzola 1999)

Specimen	Fixative	Buffer System
Prokaryotes	glutaraldehyde	cacodylate, phosphate
	osmium tetroxide	veronal - acetate
	FAA (10% formalin, 85% ethanol, 5% acetic acid	none
Fungi	glutaraldehyde / OsO_4 followed by OsO_4	cacodylate, phosphate
	glutaraldehyde followed by aqueous uranyl acetate	cacodylate
Protozoa	glutaraldehyde / formaldehyde	cacodylate
	glutaraldehyde followed by OsO_4	cacodylate, phosphate
Higher Plants	glutaraldehyde followed by OsO_4	phosphate buffer
	FAA alone or followed by OsO_4 in buffer	phosphate
Animals	glutaraldehyde or glutaraldehyde / formaldehyde followed by OsO_4	cacodylate or phosphate
	OsO_4	cacodylate or phosphate

2.3.2 Transmission Electron Microscopy

The transmission electron microscope (TEM) possesses magnification and resolution that is at least thousand times more than a light microscope. It is a device basically used to reveal the ultrastructure of plant and animal cells as well as viruses (Bozzola 1999). It is a complicated viewing system rendered with electromagnetic lenses which manipulate the imaging electrons for yielding exceedingly fine structural details which are used for recording on a photographic film. The electrons go through the specimens, hence the information is termed as a transmitted image (Bozzola 1999).

2.3.2.1 Basic Systems of Transmission Electron Microscope

TEM is comprised of a number of assorted systems that are incorporated to form a single operational unit competent of aligning and imaging exceedingly thin specimens (Bozzola 1999). The illuminating system comprises of the electron gun and condenser lenses which produce and manipulate the amount of radiation impinging on the specimen. A specimen manipulation system consisting of the specimen stage, specimen holders and associated hardware required for positioning of the thin specimens outside and inside the microscope. The imaging system is composed of an objective, intermediate and projector lenses which are required to constitute, focalize and magnify the image on the viewing screen as well as the camera utilized for recording the image. A vacuum system is essential for dispatching intrusive air molecules in the column of the electron microscope (Bozzola 1999).

2.3.2.2 Specimen Preparation for Transmission Electron Microscopy

Almost each step of specimen preparation has an impact on the quality of the ultimate electron micrographs. Hence, it is crucial to process the tissues accurately and also interpret the ongoing process (Bozzola 1999). There are eight major steps for preparing tissues for TEM: primary fixation, washing, secondary fixation, dehydration, infiltration with transitional solvents, infiltration with resin, embedding, and curing. The process commences with a living hydrous tissue and stops with an anhydrous tissue fixed within a plastic resin matrix (Bozzola 1999).

Table 2: Common steps in tissue preparation for TEM (Bozzola 1999)

Activity	Chemical	Time Involved
Primary Fixation	tissue is fixed with 2 - 4% glutaraldehyde in buffer	1 - 2 hours
Washing	buffer (three changes at 4°C) one of which may be overnight	1 - 12 hours
Secondary Fixation	osmium tetroxide (1 - 2%; usually buffered)	1 - 2 hours
Dehydration	30% ethanol 50% ethanol 70% ethanol 95% ethanol (2 changes) absolute ethanol (2 changes)	5 minutes 5 - 15 minutes 5 - 15 minutes 5 - 15 minutes 20 minutes each
Transitional Solvent	propylene oxide (3 changes)	10 minutes each
Infiltration of Resin	propylene oxide: resin mixtures; gradually increasing concentration of resin	overnight - 3 days
Embedding	pure resin mixture	2 - 4 hours
Curing (at 60°C)		1 - 3 days

2.3.3 Ultramicrotomy

Ultramicrotomy entails cutting of specimen blocks into excessively thin slices, or sections, for analyzing in TEM. The thickness of sections should range between 30 to 60 nm because the 50 to 125 kV electrons of standard electron microscope cannot pass through a biological material thicker than 150 nm (Bozzola 1999). It is a very rigorous technique necessitating a lot of practice and patience for observing the details about specimen preparation and embedding, preparation of knives and specimen support grids (Bozzola 1999). The liquid plastic utilized for infiltrating the specimens renders support to the tissue as the knife passes through the specimen. The specialized instrument utilized for cutting sections of the specimen is referred to as an ultramicrotome. They process the

specimen in meticulous, repeatable steps by employing either a mechanized or thermal advancement mechanism (Bozzola 1999). The cut sections are pulled together on a copper grid and stained for contrast employing salts of heavy metal prior to viewing in TEM (Bozzola 1999).

2.3.3.1 Thick Sectioning

Thick sectioning is performed to do a preview of the specimen (Bozzola 1999). A roughly trimmed block is mounted into the ultramicrotome and 0.5 to 2.0 µm sections are cut, which are stained with a solution of 1% toluidine blue dissolved in 1% aqueous sodium borate before examining under a light microscope.

2.3.3.2 Fine Trimming

Following an analysis by light microscopy and location of an area of interest, the plastic blocks necessitate a retrim to leave out the extraneous area (Bozzola 1999). These final cuts are made with the help of a sharp, clean razor blade so that the block sides parallel to the knife edge are smooth.

2.3.3.3 Types of Ultramicrotome Knives

The metal knives utilized for histology are too blunt for cutting ultrathin sections, therefore a gem-quality diamond was proposed for performing the microtomy of these specimens (Bozzola 1999).

2.3.3.4 Grids

Specimen grids are the analogs of glass slides utilized in light microscopy. They are extremely fine mesh supports on which the sections are positioned for viewing under TEM (Bozzola 1999). The standard diameter of a grid is 3.05 mm, and most of them have one side that is brighter than the other. Grids are highly delicate, thin and arduous to handle, with only a fine-pointed jeweler's forceps employed to pick them up (Bozzola 1999). They are usually positioned onto a filter paper or lens paper and then retrieved from these surfaces, as the flat surfaces like glass and plastic can present a problem.

The grids are stored in a special grid storage box for long-term storage. These boxes consist of about 20 to 100 numbered slots which help in ascertaining the precise location of all the specimens until required for viewing (Bozzola 1999).

CHAPTER 3

MATERIALS AND METHODS

3.1 Beef

All the beef used in this research was procured from the Meat Laboratory at the University of Missouri in the form of beef sirloin, as the initial test batches of beef jerky were made from beef sirloin left from previous research. All the extraneous fat and connective tissue was removed with the help of a sharp knife and trimmed sirloin was used for making ground and formed beef jerky.

Beef sirloin was tested for any microbial contamination before using it for making jerky by employing the standard plate count (SPC) method.

3.2 Bacterial Cultures

The following bacterial strains from the culture collection of the food microbiology laboratory of the Food Science program in the Division of Food System and Bioengineering in the University of Missouri were used for this research:

1. *E. coli* O157:H7 - ATCC - 35150
2. *S.* Typhimurium
3. *S. aureus* - ATCC - 25923
4. *L. monocytogenes*

3.3 Chemicals and Reagents

- Chemicals purchased from M.P. Biomedicals, LLC (Solon, OH) were: Tryptic Soy Broth.
- Chemicals purchased from Difco Laboratories (Detroit, MI) were: Yeast Extract.
- Chemicals purchased from Becton Dickinson (Sparks, MD) were: Bacto™ Peptone, Difco™ MacConkey Sorbitol Agar, Difco™ Oxford Medium Base, Difco™ Modified Oxford Antimicrobial Supplement, Difco™ XLD Agar, Difco™ Baird Parker Agar Base, Difco™ Tryptic Soy Agar, and Difco™ EY (Egg Yolk) Tellurite Enrichment.
- Spices and Seasonings purchased from Walmart (Columbia, MO) were: Kikkoman Soy Sauce, Heinz Worcestershire Sauce, McCormick Black Pepper, McCormick Garlic Powder, Great Value Onion Powder, Morton Salt and Heinz Vinegar (5% Acetic Acid).
- Chemicals purchased from Fisher Scientific (Fair Lawn, NJ) were: Glacial Acetic Acid.
- Chemicals purchased from Acros Organics (Morris Plains, NJ) were: Tween 20, and L - Lactic Acid, Sodium Salt, 60% wt.

3.4 Growth Curve

The growth curve for all four bacterial strains i.e. *E. coli* O157:H7, *S.* Typhimurium, *S. aureus*, and *L. monocytogenes* was run to ascertain the challenge dose required for inoculating the beef jerky strips. Optical Density (O.D.) of all four bacterial cultures were

recorded at various time points (10, 15 and 20 hours) and then correlated with plate count (CFU). The curves showing determination of O.D. can be found in Appendix 1.

3.4.1 Media and Broth

1% Peptone: 44 test tubes (Fisher Scientific Co, LLC) with screw caps (9 mL each) of peptone were prepared following the manufacturer's instructions.

Tryptic Soy and Yeast Broth: Four different flasks (100 mL each) of tryptic soy broth labeled with *E. coli* O157:H7, *S.* Typhimurium, *S. aureus*, and *L. monocytogenes* were prepared following the manufacturer's instructions.

Tryptic Soy and Yeast Agar: 350 mL of tryptic soy agar was prepared following the manufacturer's instructions.

3.4.2 Methodology

The autoclaved tryptic soy agar was poured into 12 petri plates (Fisher Scientific Co, LLC), three plates each for *E. coli* O157:H7, *S.* Typhimurium, *S. aureus*, and *L. monocytogenes*. After cooling, the solidified plates were placed in a refrigerator for further use. The four different tryptic soy flasks labeled with the bacterial names were inoculated with 1.5% of the stock culture of *E. coli* O157:H7, *S.* Typhimurium, *S. aureus*, and *L. monocytogenes*.

The Optical Density (O.D.) of all four flasks was measured at 600 nm on a Beckman DU® 640 UV/Visible Spectrophotometer (Beckman Instruments Inc., Fullerton, CA) by withdrawing 1 mL of solution from each flask and transferring it to a cuvette.

The parameters were set for the visible test and the four cuvettes along with a blank (distilled water) were put in the cuvette holder with the blank in position 1. The O.D. was recorded after clicking the auto sample option on the spectrophotometer. This was referred to as the 0 hour reading. The flasks were then placed in a G24 Environmental Incubator Shaker (New Brunswick Scientific, Co, Inc. Edison, NJ) at 37°C.

The subsequent recording of O.D. was done at 10, 15 and 20 hours, respectively. At each of these time points, 1mL of solution from all four flasks was transferred to a 9 mL peptone water test tube, thus giving a dilution of 10^{-1}. Subsequent dilutions up to 10^{-10} were prepared and dilutions 10^{-8}, 10^{-9} and 10^{-10} were placed on the tryptic soy agar plates prepared in advance. The dilutions were spread with the help of a spreader and petri plates incubated in STABIL - THERM® Dry Type Bacteriological Incubator (Electric Company, Blue Island, IL) at 37°C for 24 hours.

The results of the O.D. for all four bacteria were correlated with the plate count the next day and a growth time of 15 hours was determined to be optimum for preparing a challenge dose of 10^8 CFU (colony forming units) for inoculating the beef jerky strips.

3.5 Preparation of Inoculum

The inoculum for all four bacteria i.e. *E. coli* O157:H7, *S.* Typhimurium, *S. aureus*, and *L. monocytogenes* was prepared for inoculating the beef jerky strips to be used for determining pH and water activity values, for microbiological evaluation and performing the electron microscopy studies, SEM (Scanning Electron Microscopy) and TEM (Transmission Electron Microscopy).

3.5.1 Media and Broth

1% Peptone: 1000 mL of 1% peptone was prepared following the manufacturer's instructions to be used for washing the pellets and final suspension of the four bacterial cultures.

Tryptic Soy and Yeast Broth: Four different flasks of tryptic soy broth (200 mL each) for *E. coli* O157:H7, *S.* Typhimurium, *S. aureus,* and *L. monocytogenes* were prepared following the manufacturer's instructions.

3.5.2 Methodology

The four different tryptic soy flasks labeled with the bacterial names were inoculated with 1.5% of the stock culture of *E. coli* O157:H7, *S.* Typhimurium, *S. aureus,* and *L. monocytogenes*. The flasks were then placed in a G24 Environmental Incubator Shaker (New Brunswick Scientific, Co, Inc. Edison, NJ) at 37°C for 15 hours.

After 15 hours, the contents of the four flasks were transferred to 50 mL centrifuge tubes labeled with the respective bacterial names. The centrifuge tubes were placed in a Beckman Coulter Avanti^{TM} J-25 centrifuge, rotor I.D. JA-17 (Beckman Coulter Inc., Fullerton, CA) and the temperature set to 4°C to offset the heat generated during centrifugation. The centrifuge was run at a speed of 6400 x g for 10 minutes.

The used tryptic soy broth was decanted carefully and pellets of all four bacteria were collected in the centrifuge tubes. The pellets were dissolved in 1% peptone water and vortexed on the Vortex Genie Touch Mixer (Model: SI-0136, Scientific Industries, Inc., Bohemia, NY) until mixed thoroughly. The centrifuge tubes were placed back in the

centrifuge and run at a speed of 6400 x g for 5 minutes. This constituted the first washing. The bacterial cells were washed again for 5 minutes and the pellets of all four bacteria were resuspended in a final volume of 50 mL and stored in a refrigerator to be used for inoculating beef jerky strips. The concentration of all four bacterial cultures was 10^8 CFU/mL.

3.6 Jerky Making

Two hundred strips of ground and formed jerky were made from beef sirloin based on the following requirement:

Five (4 treatments + 1 control) x 5 (time points of experiment starting from day 0 to day 28 at 1 week apart) x 2 (1 strip for pH/a_w and 1 strip for microbiological evaluation) x 4 (different bacterial cultures) = 5 x 5 x 2 x 4 = 200.

3.6.1 Preparation of Marinade Treatments

The marinade treatments were prepared according to the recipe of Andress and Harrison (1999) and Calicioglu and others (2003):

Traditional Marinade (TM) for 1 Kilogram Meat (Andress and Harrison, 1999)

Table 3: Composition of Traditional Marinade

1. 60 mL Soy Sauce
2. 15 mL Worcestershire Sauce
3. 0.6 grams Black Pepper
4. 1.25 grams Garlic Powder
5. 1.5 grams Onion Powder
6. 4.35 grams Hickory-Smoked Salt

Modified Marinade (MM) for 1 Kilogram Meat (Andress and Harrison, 1999)

Table 4: Composition of Modified Marinade

1. 120 mL Soy Sauce
2. 30 mL Worcestershire Sauce
3. 0.6 grams Black Pepper
4. 1.25 grams Garlic Powder
5. 1.5 grams Onion Powder
6. 4.35 grams Hickory-Smoked Salt
7. 3.6 mL Food-Grade Sodium-L-Lactate (2% of 60%)
8. 16 mL Glacial Acetic Acid

Acetic Acid Traditional Marinade (AATM): A two-step process involving dipping in household vinegar (5% acetic acid) followed by marination with a traditional marinade (Calicioglu and others 2003). Tween 20 Traditional Marinade (TWTM): A

three-step process involving sequential dipping in 1% Tween 20 (polyoxyehtylene-20-sorbitan monolaurate) solution and 5% acetic acid followed by marination with a traditional marinade (Calicioglu and others 2003). Therefore, based on these recipes, the following marinades were prepared for five batches of 1200 grams of beef:

Control: No treatment.

Table 5: Marinade Treatments for Ground and Formed Beef Jerky Strips used for Determination of pH, Water Activity and Microbiological Evaluation

Traditional Marinade (TM)	**Modified Marinade (MM)**	**Acetic Acid Traditional Marinade (AATM)**	**Tween 20 Traditional Marinade (TWTM)**	**Ingredients**
72 mL	144 mL	72 mL	72 mL	Soy Sauce
18 mL	36 mL	18 mL	18 mL	Worcestershire Sauce
0.72 grams	0.72 grams	0.72 grams	0.72 grams	Black Pepper
1.5 grams	1.5 grams	1.5 grams	1.5 grams	Garlic Powder
1.8 grams	1.8 grams	1.8 grams	1.8 grams	Onion Powder
5.22 grams	5.22 grams	5.22 grams	5.22 grams	Salt
	4.32 mL of (0.5 mL sodium lactate + 24.5 mL distilled water)			2% Sodium Lactate
	19.2 mL			Glacial Acetic Acid
		120 mL (1:10)	60 mL (1:20)	5% Acetic Acid
			120 mL of (1.2 mL tween 20 + 118.8 mL distilled water)	1% Tween 20

3.6.2 Preparation of Jerky Strips

All five batches of 1200 grams beef were diced on a cutting board with the help of a chef's knife and placed in five different bowls. Each of the diced batch was ground in a Cuisin Art® Pro Custom11™ Food Processor (Model: DLC-8S TX, Pro Custom, East Windsor, NJ) for 3-4 minutes. All the ingredients of respective marinade treatments were added one by one while grinding the beef.

The ground mixture of beef was transferred into a jerky gun (LEM Products Inc., Harrison, OH) and beef jerky strips were extruded through the nozzle (2.9 cm x 0.6 cm) attached to the jerky cannon onto trays placed on a rack by pushing the beef mixture with the help of a piston. Each of the trays were weighed and the weight recorded to be used later. Ten jerky strips were extruded on each tray, hence two trays were placed on each rack and two such racks for each batch making it 40 jerky strips from each batch of 1200 grams beef.

The racks were positioned in a WIMCO (Whirlwind Isothermic Muffled Convection Oven) (Model: 27-2DOX, Keating of Chicago, Inc., Chicago, IL). The oven was operated for 1 hour and 40 minutes at a set temperature of 60°C. The whole process was monitored with intermittent checking of the jerky strips after every 15 minutes for degree of dryness. Following the completion of the cooking process, the trays were set on a countertop and allowed to cool for approximately 5 minutes. The jerky strips were then put on a tray with aluminum foil for inoculating with four different bacteria cultures (*E. coli* O157:H7, *S.* Typhimurium, *S. aureus*, and *L. monocytogenes*). Fifty of the two hundred beef jerky strips made were inoculated with each of the bacterial cultures. A

basting brush was used to inoculate *E. coli* O157:H7 and *S. aureus*, whereas a bent glass rod was used to inoculate *S.* Typhimurium and *L. monocytogenes*. This was done because in the initial test batches of beef jerky inoculation, there was a low recovery observed for *S.* Typhimurium and *L. monocytogenes* using a basting brush. Each beef jerky strip was inoculated on both sides with 0.5 mL of the bacterial cultures at a concentration of 10^8CFU/mL and left for 15 minutes a side to allow the attachment of bacterial cells on the beef surface.

The inoculated beef jerky strips were put into Prime Source 8x10 3MIL STD. Barrier Nylon/PE Vacuum Pouches (Koch Supplies, Kansas City, MO). One jerky strip was put in each vacuum pouch. All the 200 vacuum pouches were vacuum sealed by employing a Multi Sepp Haggenmüller KG Vakuum-Verpackungs-Maschinen (Model: AG 800, Germany). All the vacuum sealed pouches were transferred to a cardboard box and stacked in the food microbiology laboratory in the Department of Food Science, University of Missouri at a temperature of 22°C to be used further for determining pH and water activity and performing the microbiological evaluation.

3.7 Microbiological Evaluation

The vacuum sealed bags stored for microbial analysis were opened on their designated days beginning Day 1 through Day 28 with a gap of one week in between.

3.7.1 Media and Broth

1% Peptone: Eighty five test tubes (Fisher Scientific Co, LLC) with screw caps (9 mL each) of 1% peptone were prepared following the manufacturer's instructions to be used for making dilutions from the stomached beef jerky strips. Another batch of 2000 mL of 1% peptone water was prepared to be used for stomaching the beef jerky strips.

Sorbitol - MacConkey Agar: It is a selective medium utilized for isolating and differentiating *E. coli* O157:H7 from other *E. coli* strains and non-lactose fermenters. Forty five petri plates were prepared according to the manufacturer's instructions.

XLD (Xylose Lysine Deoxycholate) Agar: It is a selective growth medium used for isolating *Salmonella* and *Shigella* species. Forty five petri plates were prepared according to the manufacturer's instructions.

Baird Parker Agar: It is a selective growth medium used for isolating gram-positive *Staphylococcus* species. Forty five petri plates were prepared according to the manufacturer's instructions.

Modified Oxford Agar: It is a selective growth medium used for isolating *Listeria* species. Forty five petri plates were prepared according to the manufacturer's instructions.

3.7.2 Methodology

After solidification, the petri plates were stored in a refrigerator for further use. The vacuum sealed bag was opened on Day 1 under a laminar hood using a pair of scissors. Approximately a 1 gram piece was taken from a beef jerky strip and aseptically

transferred to a stomaching bag (Fisher Brand®, Fisher Scientific). Ninety nine mL of autoclaved 1% peptone water was added to the stomaching bag, which constituted a dilution of 10^{-2}. The stomaching bag was inserted into a Stomacher® 400 Circulator Seward Stomacher Listed Lab Blender (England).

The beef jerky sample was stomached for 2 minutes at a speed of 250 rpm and 1 mL of this stomached sample was transferred to another 9 mL test tube, containing 1% peptone water using a 1 mL pipette (Fisher Scientific Co, LLC., Hanover Park, IL). This constituted a dilution of 10^{-3}. Further serial decimal dilutions were made up to 10^{-7} for beef jerky slices inoculated with *E. coli* O157:H7, *S. aureus*, *L. monocytogenes* and *S.* Typhimurium. One mL aliquots from dilutions 10^{-5}, 10^{-6} and 10^{-7} for *E. coli* O157:H7 were spread-plated on Sorbitol - MacConkey Agar petri plates. The petri plates were then placed in an inverted position in STABIL - THERM® Dry Type Bacteriological Incubator (Electric Company, Blue Island, IL) at 37°C for overnight incubation.

Similarly, 1 mL aliquots from dilutions 10^{-5}, 10^{-6} and 10^{-7} for *S.* Typhimurium were spread plated on XLD (Xylose Lysine Deoxycholate) Agar petri plates, One mL aliquots from dilutions 10^{-5}, 10^{-6} and 10^{-7} for *S. aureus* were spread plated on Baird Parker Agar petri plates and 1 mL aliquots from dilutions 10^{-5}, 10^{-6} and 10^{-7} for *L. monocytogenes* were spread plated on Modified Oxford Agar petri plates. The petri plates placed in an inverted position in STABIL - THERM® Dry Type Bacteriological Incubator (Electric Company, Blue Island, IL) at 37°C for overnight incubation. The petri plates were visually observed the next day and colonies were counted directly from the petri plates. The numbers were recorded in the laboratory notebook. The same procedure was

performed for the rest of the 19 beef jerky strips [5(4 treatments + 1 control) x 4 bacteria = 20]. The final results for all four treatments used against the bacterial cultures i.e. TM (traditional marinade), MM (modified marinade), AATM (acetic acid-traditional marinade) and TWTM (Tween 20-traditional marinade) along with the control were expressed as CFU/gm of beef jerky. This whole procedure was repeated every week until Day 28 starting from Day 1.

3.8 Determination of Water Activity

The water activity of beef jerky samples was determined by employing an Aqua Lab Water Activity Meter (Model: CX-2, Decan Devices Inc., Pullman, WA). The vacuum sealed bag was opened on Day 1 under a laminar hood using a pair of scissors. The water activity meter was calibrated by using the desiccator cup.

A piece was taken from a beef jerky strip, which was big enough to fill the area of the measuring cup and aseptically transferred to the measuring cup. The cup was inserted inside the water activity meter and the knob closed. The water activity value was displayed digitally on the screen along with the temperature accompanied by the sound of a beep.

The value was recorded. The same procedure was performed for the rest of the nineteen beef jerky strips [5(4 treatments + 1 control) x 4 bacteria = 20]. This whole procedure was repeated every week until Day 28 starting from Day 1.

3.9 pH Determination

The vacuum sealed bag was opened on Day 1 under a laminar hood using a pair of scissors. The pH was measured by using the following procedure: approximately 5 grams was taken from a beef jerky strip previously used for measuring water activity and aseptically transferred to a blending jar. Fifty mL of distilled water was added to the blending jar. The jar was then inserted in an Osterizer® blender (Service no: 4094, Mexico) and blended for 2 minutes. The slurry of beef jerky was filtered through a Whatman filter paper # 54 and the clear filtrate was used for measuring the pH (Koniecko 1985).

Fisher ACCUMENT® pH/ION meter (Model: 230A, Fisher Scientific Co, LLC., Hanover Park, IL) was calibrated using two buffers of pH 4.0 and 7.0, respectively. The pH measuring rod was inserted in the beaker and the pH was observed visually. The reading was recorded. The same procedure was performed for the rest of the nineteen beef jerky strips [5(4 treatments + 1 control) x 4 bacteria = 20]. This whole procedure was repeated every week until Day 28 starting from Day 1.

3.10 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) was performed on the beef jerky samples for corroborating the results of microbiological evaluation.

3.10.1 Jerky Making

Forty strips of ground and formed jerky were made from beef sirloin based on the following requirement:

Five (4 treatments + 1 control) x 2 (time points of experiment starting from day 0 to day 28 at 4 weeks apart) x 4 (different bacterial cultures) = 5 x 2 x 4 = 40.

3.10.1.1 Preparation of Marinade Treatments

The following marinades were prepared for five batches of 450 grams of beef:

Control: No treatment.

Table 6: Marinade Treatments for Ground and Formed Beef Jerky Strips used for SEM

Traditional Marinade (TM)	**Modified Marinade (MM)**	**Acetic Acid Traditional Marinade (AATM)**	**Tween 20 Traditional Marinade (TWTM)**	**Ingredients**
27 mL	54 mL	27 mL	27 mL	Soy Sauce
6.75 mL	13.5 mL	6.75 mL	6.75 mL	Worcestershire Sauce
0.27 grams	0.27 grams	0.27 grams	0.27 grams	Black Pepper
0.563 grams	0.563 grams	0.563 grams	0.563 grams	Garlic Powder
0.675 grams	0.675 grams	0.675 grams	0.675 grams	Onion Powder
1.96 grams	1.96 grams	1.96 grams	1.96 grams	Salt
	1.65 mL of (0.5 mL sodium lactate + 24.5 mL distilled water)			2% Sodium Lactate
	7.5 mL			Glacial Acetic Acid
		45 mL (1:10)	22.5 mL (1:20)	5% Acetic Acid
			45 mL of (1.2 mL tween 20 + 118.8 mL distilled water)	1% Tween 20

3.10.1.2 Preparation of Jerky Strips

Beef jerky strips were prepared following the same procedure as described in section 3.6.2.

3.10.2 Chemicals and Reagents

- Chemicals purchased from Electron Microscopy Sciences (Hatfield, PA) were: Cacodylic Acid - Sodium Salts, Glutaraldehyde, Paraformaldehyde.
- Chemicals purchased from Ted Pella, Inc. (Redding, CA) were: 4% Osmium Tetroxide.
- Chemicals purchased from Fisher Chemicals (Fair Lawn, NJ) were: Ethanol, Hydrochloric Acid and Sodium Hydroxide.

3.10.2.1 Methodology

0.4M Cacodylate Buffer Stock (500 mL): 42.8 g of cacodylic acid-sodium salts was measured in a weighing pan and transferred to a 1000 mL flask. Ultra pure water was added to make a total volume of 500 mL.

0.1M Cacodylate Buffer Working Solution: 120 mL of 0.4M cacodylate stock solution was added to 360 mL of ultrapure water in a 500 mL beaker to make a total of 480 mL of working buffer. The pH was checked and adjusted to 7.2 - 7.4 by adding HCl (Hydrochloric Acid) or NaOH (Sodium Hydroxide) solution.

2% Glutaraldehyde / 2% Paraformaldehyde in 0.1M Cacodylate Buffer: 9.6 mL of 50% glutaraldehyde was added to a 500 mL beaker. Thirty mL (3 vials) of 16% paraformaldehyde were added to this 50% glutaraldehyde. Sixty mL of 0.4M cacodylate buffer stock solution was added to this mixture and finally ultra pure water was added to bring the total volume to 240 mL. The pH was checked and adjusted to 7.2 - 7.4 by adding HCl (Hydrochloric Acid) or NaOH (Sodium Hydroxide) solution.

3.10.3 Specimen Preparation for SEM (Scanning Electron Microscopy)

The vacuum sealed bags were opened on Day 1 and because the samples were too large for SEM (Scanning Electron Microscopy) processing, samples with dimensions 2 mm long and 0.5 mm wide, were cut from the strips of beef jerky and placed into a freshly prepared primary fixative (2% Glutaraldehyde / 2% Paraformaldehyde in 0.1M Cacodylate Buffer) and microwaved at 120 Watts in a PELCO BioWave® Pro Standard System (Model: 36500, Ted Pella, Inc., Redding, CA) microwave oven with the following procedure: one minute hold, 80 seconds irradiation, three minutes hold, 40 seconds irradiation. This represented the primary fixation. The trimmed samples were then stored at 4°C until ready to finish processing.

The smaller trimmed samples were rinsed for 5 minutes three times with 0.1M Sodium Cacodylate Buffer. The next step was to perform the secondary fixation of the beef jerky samples by utilizing 2% buffered (same buffer as above) osmium tetroxide (diluted from 4% aqueous OsO_4). The fixed samples were microwaved at 120 Watts in a PELCO BioWave® Pro Standard System (Model: 36500, Ted Pella, Inc., Redding, CA) microwave oven with the following procedure: one minute hold, 80 seconds irradiation, three minutes hold, 40 seconds irradiation.

After the secondary fixation, the samples were quickly rinsed three times with 0.1M Sodium Cacodylate Buffer. This was followed by one five minute rinse. The rinsed samples were then given three deionized water rinses for five minutes each using ultra pure Milli-Q water dispenser. The samples then underwent a series of dehydration with ethanol starting at 20% and gradually increasing the concentration to 50, 70, 90%. The

final dehydration step comprised of three steps with 100% ethanol. Each dehydration step required the samples to be microwaved at 120 Watts in a PELCO BioWave® Pro Standard System (Model: 36500, Ted Pella, Inc., Redding, CA) microwave oven for 40 seconds irradiation.

3.10.4 Critical Point Drying and Sputter Coating

The dehydration of samples was followed by critical point drying in a Tousimis Auto-Samdri 815 automatic critical point dryer (Tousimis Research Corporation, Rockville, MD). The dried samples were mounted on a 25mm SEM stub using carbon adhesive tabs and coated with silver paint on the sides. Four such stubs were prepared for four different bacteria, with five samples on each stub. The samples were then sputter coated with Platinum at 20 mA for 120 seconds in Emitech K575 X Turbo Sputter Coater (Emitech LTD., Kent, England).

The coated samples were viewed in a Hitachi S4700 Field Emission Scanning Electron Microscope (FESEM) (Hitachi LTD., Tokyo, Japan) and images photographed straight off the monitor through the lens of a 35 mm roll film camera. The photographs were saved on the hard disk of an attached computer for future use. All the microwave steps for sample processing were performed under vacuum with the cold spot, whereas the rinses were executed outside of the microwave at room temperature and this whole procedure was repeated on Day 28.

3.11 Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy (TEM) was performed on the beef jerky samples for analyzing the underlying protective mechanisms of different marinade treatments against the pathogens in comparison to the control.

3.11.1 Jerky Making

Forty strips of ground and formed jerky were made from beef sirloin based on the following requirement:

Five (4 treatments + 1 control) x 2 (time points of experiment starting from day 0 to day 28 at 4 weeks apart) x 4 (different bacterial cultures) = 5 x 2 x 4 = 40.

3.11.1.1 Preparation of Marinade Treatments

The following marinades were prepared for five batches of 225 grams of beef:

Control: No treatment.

Table 7: Marinade Treatments for Ground and Formed Beef Jerky Strips used for TEM

Traditional Marinade (TM)	**Modified Marinade (MM)**	**Acetic Acid Traditional Marinade (AATM)**	**Tween 20 Traditional Marinade (TWTM)**	**Ingredients**
13.5 mL	27 mL	13.5 mL	13.5 mL	Soy Sauce
3.375 mL	6.75 mL	3.375 mL	3.375 mL	Worcestershire Sauce
0.135 grams	0.135 grams	0.135 grams	0.135 grams	Black Pepper
0.282 grams	0.282 grams	0.282 grams	0.282 grams	Garlic Powder
0.3375 grams	0.3375 grams	0.3375 grams	0.3375 grams	Onion Powder
0.98 grams	0.98 grams	0.98 grams	0.98 grams	Salt
	0.825 mL of (0.5 mL sodium lactate + 24.5 mL distilled water)			2% Sodium Lactate
	3.75 mL			Glacial Acetic Acid
		22.5 mL (1:10)	11.25 mL (1:20)	5% Acetic Acid
			22.5 mL of (1.2 mL tween 20 + 118.8 mL distilled water)	1% Tween 20

3.11.1.2 Preparation of Jerky Strips

Beef jerky strips were prepared following the same procedure as described in section 3.6.2.

3.11.2 Chemicals and Reagents

- Chemicals purchased from Electron Microscopy Sciences (Hatfield, PA) were: Cacodylic Acid - Sodium Salts, 2 - Mercaptoethanol, Glutaraldehyde, Paraformaldehyde and Lead Nitrate.
- Chemicals purchased from Ted Pella, Inc. (Redding, CA) were: 4% Osmium Tetroxide.
- Chemicals purchased from Fisher Scientific (Fair Lawn, NJ) were: Ethanol, Sucrose, Lead Acetate, Hydrochloric Acid and Sodium Hydroxide.
- Chemicals purchased from Acros Organics (Morris Plains, NJ) were: Lead Citrate, tri hydrate (96%).
- Chemicals purchased from Mallinckrodt Chemical Works (St. Louis, MO) were: Sodium Citrate.
- Chemicals purchased from SPI Supplies / Structure Probe, Inc. (West Chester, PA) were: SPI-Pon™ 812 Epoxy Embedding Kit (1500 grams) and Spurr Formula Kit (975 grams).

3.11.2.1 Methodology

0.4M Cacodylate Buffer Stock (500 mL): 42.8 g of cacodylic acid-sodium salts was measured in a weighing pan and transferred to a 1000 mL flask. Ultra pure water was added to make a total volume of 500 mL.

0.1M Cacodylate Buffer Working Solution: 120 mL of 0.4M Cacodylate stock solution was added to 360 mL of ultrapure water in a 500 mL beaker to make a total of

480 ml of working buffer. The pH was checked and adjusted to 7.2 - 7.4 by adding HCl (Hydrochloric Acid) or NaOH (Sodium Hydroxide) solution.

2% Glutaraldehyde / 2% Paraformaldehyde in 0.1M Cacodylate Buffer: 9.6 mL of 50% glutaraldehyde was added to a 500 mL beaker. Thirty mL (3 vials) of 16% paraformaldehyde were added to this 50% glutaraldehyde. Sixty mL of 0.4M cacodylate buffer stock solution was added to this mixture and finally ultra pure water was added to bring the total volume to 240 mL. The pH was checked and adjusted to 7.2 - 7.4 by adding HCl (Hydrochloric Acid) or NaOH (Sodium Hydroxide) solution.

0.1M Cacodylate Buffer with 0.01M 2-Mercaptoethanol and 0.13M Sucrose: For preparing a 250 mL solution, 62.5 mL of 0.4M Cacodylate buffer stock solution was added in a 500 mL beaker. To this buffer 11.12 grams of sucrose was added. Then 0.18 mL of 2-Mercaptoethanol was added and pH was checked and adjusted to 7.2 - 7.4 by adding HCl (Hydrochloric Acid) or NaOH (Sodium Hydroxide) solution. Finally, ultrapure water was added to bring the total volume to 250 mL to complete the buffer.

Epon - Spurr Recipe: The resin is composed of two separate components, i.e. Epon and Spurr. For Epon formulation, 25 mL of Epon 812 Epoxy Resin Monomer (Glyceringlycidether), 13 mL of DDSA Hardener (Dodecenyl Succinic Anhydride HY 964), 12 mL of NMA Hardener (Nadic Methyl Anhydride Generic form of HY 906) and 0.53 mL of DMP-30 Epoxy Accelerator (2,4,6-tris dimethylaminomethyl phenol; DY 064) were mixed for five minutes in a plastic disposable container.

For the Spurr formulation, 10 mL of ERL 4221 Epoxy Plasticizer (Vinylcyclohexene dioxide), 6 mL of DER 736 Epoxy plasticizer (Diglycidyl ether of

polypropylene glycol), 26 mL of NSA Epoxy Hardener (Nonenyl Succinic Anhydride) and 0.4 mL of DMAE Accelerator for Epoxy Resins (2-Dimethylaminoethanol) were mixed for five minutes in a plastic disposable container.

Both these resins were then mixed together in a 1:1 ratio. Acetone was used as both the dehydrating agent and the transition solvent. This mixture was then cured for 24 hours in an Imperial II Radiant Heat Oven (Model: 3502, Lab-Line Instruments Inc., Melrose Park, IL) at 60°C.

Sato's Triple Lead Stain: This is the stain used for staining the samples for viewing under the Transmission Electron Microscope. For making 50 mL of the staining solution, 0.5 gram of lead acetate, 0.5 gram of lead citrate, 0.5 gram of lead nitrate, 1 gram of sodium citrate were mixed together in a 100 mL beaker. Ultra pure Milli-Q water was added to increase the volume up to 41 mL. To this solution, 9 mL of a freshly prepared 4% sodium hydroxide was added and stored in a capped container.

3.11.3 Specimen Preparation for TEM (Transmission Electron Microscopy)

The vacuum sealed bags were opened on Day 1 and because the samples were too large for TEM (Transmission Electron Microscopy) processing, samples with dimensions 1 mm long and 0.5 mm wide were cut from the strips of beef jerky and placed into a freshly prepared primary fixative (2% Glutaraldehyde / 2% Paraformaldehyde in 0.1M Cacodylate Buffer) and microwaved at 120 Watts in a PELCO BioWave® Pro Standard System (Model: 36500, Ted Pella, Inc., Redding, CA) microwave oven with the

following procedure: one minute hold, 80 seconds irradiation, three minutes hold, 40 seconds irradiation. This represented the primary fixation.

The trimmed samples were then stored at 4°C until ready to finish processing. The smaller trimmed samples were rinsed for 20 minutes three times with 0.1M Sodium Cacodylate, 0.01M 2-Mercaptoethanol, 0.13M Sucrose Buffer. The next step was to perform the secondary fixation of the beef jerky samples by utilizing 1% buffered (2-ME buffer) osmium tetroxide (diluted from 4% aqueous OsO_4). The fixed samples were microwaved at 120 Watts in a PELCO BioWave® Pro Standard System (Model: 36500, Ted Pella, Inc., Redding, CA) microwave oven with the following procedure: one minute hold, 80 seconds irradiation, three minutes hold, 40 seconds irradiation.

After the secondary fixation, the samples were quickly rinsed three times with 0.1M Sodium Cacodylate, 0.01M 2-Mercaptoethanol, 0.13M Sucrose Buffer. This was followed by one five minute rinse. The rinsed samples were then given three ultra pure Milli-Q water rinses for five minutes each. The samples then underwent a series of dehydration with acetone starting at 20% and gradually increasing the concentration to 50, 70, 90%. The final dehydration step comprised of three steps with 100% acetone. Each dehydration step required the samples to be microwaved at 120 Watts in a PELCO BioWave® Pro Standard System (Model: 36500, Ted Pella, Inc., Redding, CA) microwave oven for 40 seconds irradiation.

3.11.4 Resin Infiltration and Staining

The dehydration of samples was followed by infiltration with Epon-Spurr's Resin using the following procedure: (Day 1) morning: 2 Acetone:1 Resin, evening: 1 Acetone:1 Resin and the samples were placed on a rocker (Model: 260350, Boekel Scientific, Feasterville, PA) for overnight. (Day 2) morning: 1 Acetone:2 Resin, evening: 1st pure resin exchange and the samples were placed on a rocker (Model: 260350, Boekel Scientific, Feasterville, PA) for overnight. (Day 3) morning: 2nd pure resin exchange, evening: 3rd pure resin exchange and samples were placed on a rocker (Model: 260350, Boekel Scientific, Feasterville, PA) for overnight.

Day 4, morning: 4th pure resin exchange and samples were then placed on a rocker (Model: 260350, Boekel Scientific, Feasterville, PA) at room temperature and embedded at the end of the day. Samples were then polymerized for 24 hours in an Imperial II Radiant Heat Oven (Model: 3502, Lab-Line Instruments Inc., Melrose Park, IL) at 60°C. The samples were microwaved at 250 Watts in a PELCO BioWave® Pro Standard System (Model: 36500, Ted Pella, Inc., Redding, CA) microwave oven for 3 minutes at each resin exchange step. Eighty five nanometers thin sections were collected from the embedded samples by utilizing a Leica Ultracut UCT ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany) with a 45 degree Diatome diamond knife. Sections were double stained with 5% uranyl acetate (UA) and Sato's triple lead stain (3 minutes Lead, 18 minutes Uranyl Acetate, 8 minutes Lead).

The stained sections were then viewed in JEOL 1400 Transmission Electron Microscope (TEM) (JEOL, LTD., Tokyo, Japan) and photographs were taken with a

Gatan Ultrascan (Model: 895, 2K x 2K) cooled CCD digital camera (Gatan, Inc., Pleasanton, CA). The photographs were saved on the hard disk of an attached computer for future use. All the microwave steps for sample processing were performed under vacuum with the cold spot, whereas the rinses were executed outside of the microwave at room temperature and this whole procedure was repeated on Day 28.

3.12 Statistical Analysis

The microbiological data were converted to log CFU/gm and data were analyzed by analysis of variance for main (fixed) effects (marinade treatment and storage time) and two way interactions between marinade treatment and storage time using the Statistical Analysis System (version 9.1, SAS Institute, Cary, NC). Least square means were separated using Fisher's least significance difference test (LSD) using the mixed model (MIXED) procedure of SAS. A significance level of 0.05 was used for all statistical analyses.

Similarly, pH and water activity data were also analyzed by analysis of variance for main (fixed) effects (marinade treatment and storage time) using the Statistical Analysis System (version 9.1, SAS Institute, Cary, NC). Least square means were separated using Fisher's least significance difference test (LSD) using the mixed model (MIXED) procedure of SAS. A significance level of 0.05 was used for all statistical analyses.

Analysis of Variance (ANOVA) tables for pH, water activity (a_w) and microbiological data can be found in Appendix 2.

CHAPTER 4

RESULTS

4.1 pH and Water Activity

The pH values ranged from 4.51 to 5.78 for *E. coli* O157:H7 (**Table 8**).

Table 8: pH of Ground and Formed Beef Jerky Strips Inoculated with *E. coli* O157:H7

Treatment	Day 1	Day 7	Day 14	Day 21	Day 28
Control	5.42^{b}	5.22^{b}	5.32^{b}	5.21^{c}	5.33^{b}
TM	5.51^{a}	5.60^{a}	5.46^{a}	5.78^{a}	5.72^{a}
MM	4.51^{e}	4.55^{d}	4.60^{e}	4.69^{e}	4.58^{e}
AATM	4.82^{d}	5.05^{c}	5.00^{d}	5.08^{d}	5.07^{d}
TWTM	5.05^{c}	5.24^{b}	5.19^{c}	5.32^{b}	5.10^{c}

Least Significant Difference (L.S.D.) for effect (trt*day) = Standard Error (S.E.) x $t_{5\%, df=40}$ = 0.01482 x 2.021 = 0.0299. If the difference of any two least square means ≥ L.S.D., then the two means are different.

The pH values were significantly different ($P < 0.05$) for all four treatments i.e. TM, MM, AATM and TWTM during the course of 28 Day period. However, on Day 7, TWTM and control were not significantly different.

The pH values ranged from 4.47 to 5.74 for *S.* Typhimurium (**Table 9**).

Table 9: pH of Ground and Formed Beef Jerky Strips Inoculated with *S.* Typhimurium

Treatment	Day 1	Day 7	Day 14	Day 21	Day 28
Control	5.51^{a}	5.28^{b}	5.32^{b}	5.28^{b}	5.42^{b}
TM	5.47^{b}	5.64^{a}	5.41^{a}	5.74^{a}	5.71^{a}
MM	4.47^{e}	4.56^{d}	4.56^{e}	4.65^{e}	4.52^{e}
AATM	4.80^{d}	5.09^{c}	5.10^{d}	5.21^{d}	5.19^{c}
TWTM	4.95^{c}	5.29^{b}	5.16^{c}	5.25^{c}	4.97^{d}

Least Significant Difference (L.S.D.) for effect (trt*day) = Standard Error (S.E.) x $t_{5\%, df=40}$ = 0.01431 x 2.021 = 0.0289. If the difference of any two least square means ≥ L.S.D., then the two means are different.

The pH values were significantly different ($P < 0.05$) for all four treatments i.e. TM, MM, AATM and TWTM during the course of 28 Day period. However, on Day 7, TWTM and control were not significantly different.

The pH values ranged from 4.46 to 5.69 for *S. aureus* (**Table 10**).

Table 10: pH of Ground and Formed Beef Jerky Strips Inoculated with *S. aureus*

Treatment	Day 1	Day 7	Day 14	Day 21	Day 28
Control	5.31^{b}	5.31^{b}	5.44^{a}	5.29^{b}	5.42^{b}
TM	5.52^{a}	5.67^{a}	5.34^{b}	5.69^{a}	5.68^{a}
MM	4.47^{e}	4.52^{e}	4.52^{e}	4.62^{e}	4.46^{e}
AATM	4.84^{d}	5.09^{d}	5.01^{d}	5.14^{d}	5.11^{c}
TWTM	4.98^{c}	5.22^{c}	5.11^{c}	5.21^{c}	5.02^{d}

Least Significant Difference (L.S.D.) for effect (trt*day) = Standard Error (S.E.) x $t_{5\%, df=40}$ = 0.01448 x 2.021 = 0.0292. If the difference of any two least square means ≥ L.S.D., then the two means are different.

The pH values were significantly different ($P < 0.05$) for all four treatments i.e. TM, MM, AATM and TWTM during the course of 28 Day period.

The pH values ranged from 4.45 to 5.75 for *L. monocytogenes* (**Table 11**).

Table 11: pH of Ground and Formed Beef Jerky Strips Inoculated with *L. monocytogenes*

Treatment	Day 1	Day 7	Day 14	Day 21	Day 28
Control	5.41^{a}	5.31^{b}	5.40^{a}	5.32^{b}	5.43^{b}
TM	5.39^{a}	5.75^{a}	5.33^{b}	5.65^{a}	5.60^{a}
MM	4.45^{d}	4.55^{e}	4.55^{e}	4.64^{d}	4.50^{e}
AATM	4.85^{c}	5.05^{d}	4.98^{d}	5.17^{c}	5.14^{c}
TWTM	5.02^{b}	5.20^{c}	5.09^{c}	5.16^{c}	5.00^{d}

Least Significant Difference (L.S.D.) for effect (trt*day) = Standard Error (S.E.) x $t_{5\%, df=40}$ = 0.01376 x 2.021 = 0.0278. If the difference of any two least square means ≥ L.S.D., then the two means are different.

The pH values were significantly different ($P < 0.05$) for all four treatments i.e. TM, MM, AATM and TWTM during the course of 28 Day period. However, on Day 1, TM and control were not significantly different. On Day 21, AATM and TWTM were not significantly different.

The water activity ranged from 0.596 to 0.923 for *E. coli* O157:H7 (**Table 12**).

Table 12: Water activity of Ground and Formed Beef Jerky Strips Inoculated with *E. coli* O157:H7

Treatment	Day 1	Day 7	Day 14	Day 21	Day 28
Control	0.923^{a}	0.901^{a}	0.895^{a}	0.897^{a}	0.903^{a}
TM	0.882^{b}	0.856^{b}	0.875^{b}	0.841^{b}	0.811^{c}
MM	0.673^{e}	0.644^{e}	0.651^{e}	0.665^{e}	0.596^{e}
AATM	0.790^{d}	0.768^{d}	0.816^{d}	0.724^{d}	0.786^{d}
TWTM	0.836^{c}	0.773^{c}	0.832^{c}	0.820^{c}	0.858^{b}

Least Significant Difference (L.S.D.) for effect (trt*day) = Standard Error (S.E.) x $t_{5\%, df=40}$ = 0.001914 x 2.021 = 0.003868. If the difference of any two least square means ≥ L.S.D., then the two means are different.

Water activity values were significantly different ($P < 0.05$) for all four treatments i.e. TM, MM, AATM and TWTM during the course of 28 Day period.

The water activity ranged from 0.663 to 0.915 for *S.* Typhimurium (**Table 13**).

Table 13: Water activity of Ground and Formed Beef Jerky Strips Inoculated with *S.* Typhimurium

Treatment	Day 1	Day 7	Day 14	Day 21	Day 28
Control	0.901^{a}	0.906^{a}	0.915^{a}	0.912^{a}	0.899^{a}
TM	0.883^{b}	0.873^{b}	0.838^{b}	0.820^{c}	0.852^{b}
MM	0.694^{e}	0.663^{e}	0.696^{d}	0.690^{e}	0.671^{e}
AATM	0.813^{d}	0.788^{d}	0.765^{c}	0.747^{d}	0.782^{d}
TWTM	0.853^{c}	0.805^{c}	0.835^{b}	0.865^{b}	0.827^{c}

Least Significant Difference (L.S.D.) for effect (trt*day) = Standard Error (S.E.) x $t_{5\%, df=40}$ = 0.002803 x 2.021 = 0.0056648. If the difference of any two least square means ≥ L.S.D., then the two means are different.

Water activity values were significantly different ($P < 0.05$) for all four treatments i.e. TM, MM, AATM and TWTM during the course of 28 Day period. However, on Day 14, TM and TWTM were not significantly different.

The water activity ranged from 0.533 to 0.912 for *S. aureus* (**Table 14**).

Table 14: Water activity of Ground and Formed Beef Jerky Strips Inoculated with *S. aureus*

Treatment	Day 1	Day 7	Day 14	Day 21	Day 28
Control	0.912^{a}	0.912^{a}	0.896^{a}	0.906^{a}	0.895^{a}
TM	0.863^{b}	0.864^{b}	0.863^{b}	0.815^{b}	0.821^{c}
MM	0.673^{e}	0.671^{e}	0.679^{e}	0.604^{e}	0.533^{e}
AATM	0.743^{d}	0.748^{d}	0.742^{d}	0.636^{d}	0.642^{d}
TWTM	0.846^{c}	0.852^{c}	0.823^{c}	0.806^{c}	0.845^{b}

Least Significant Difference (L.S.D.) for effect (trt*day) = Standard Error (S.E.) x $t_{5\%, df=40}$ = 0.001774 x 2.021 = 0.003585. If the difference of any two least square means ≥ L.S.D., then the two means are different.

Water activity values were significantly different ($P < 0.05$) for all four treatments i.e. TM, MM, AATM and TWTM during the course of 28 Day period.

The water activity ranged from 0.643 to 0.913 for *L. monocytogenes* (**Table 15**).

Table 15: Water activity of Ground and Formed Beef Jerky Strips Inoculated with *L. monocytogenes*

Treatment	Day 1	Day 7	Day 14	Day 21	Day 28
Control	0.913^{a}	0.906^{a}	0.891^{a}	0.905^{a}	0.893^{a}
TM	0.859^{b}	0.867^{b}	0.806^{c}	0.847^{b}	0.850^{b}
MM	0.664^{e}	0.674^{e}	0.657^{e}	0.660^{e}	0.643^{e}
AATM	0.817^{d}	0.762^{d}	0.752^{d}	0.762^{d}	0.760^{d}
TWTM	0.855^{c}	0.821^{c}	0.825^{b}	0.835^{c}	0.846^{c}

Least Significant Difference (L.S.D.) for effect (trt*day) = Standard Error (S.E.) x $t_{5\%, df=40}$ = 0.001701 x 2.021 = 0.003437. If the difference of any two least square means ≥ L.S.D., then the two means are different.

Water activity values were significantly different ($P < 0.05$) for all four treatments i.e. TM, MM, AATM and TWTM during the course of 28 Day period.

4.2 Microbiological Evaluation

In the case of *E. coli* O157:H7, a decline of 2.59 log, 2.96 log and 2.79 log was observed for MM, AATM and TWTM, respectively. Even TM was able to manifest a reduction of 2.89 log units. The bacterial count reduction was significantly different ($P < 0.05$) between MM and other three treatments i.e. TM, AATM and TWTM at the starting

point of Day 1. The same pattern was observed during the middle of observation period i.e. Day 14. However, by the end of Day 28 all four treatments i.e. TM, MM, AATM and TWTM were significantly different from control but not from each other (**Table 16**).

Table 16: Microbial count [log CFU] of Ground and Formed Beef Jerky Strips Inoculated with *E. coli* O157:H7

Treatment	Day 1	Day 7	Day 14	Day 21	Day 28	Difference in Log Reduction
Control	7.36^{a}	8.08^{a}	7.96^{a}	5.56^{a}	5.11^{a}	2.25
TM	7.33^{a}	8.12^{a}	6.91^{b}	5.52^{a}	4.44^{b}	2.89
MM	6.76^{b}	6.34^{b}	5.22^{c}	4.92^{b}	4.17^{b}	2.59
AATM	7.26^{a}	6.56^{b}	6.40^{b}	5.09^{a}	4.30^{b}	2.96
TWTM	7.12^{a}	6.49^{b}	6.42^{b}	5.23^{a}	4.33^{b}	2.79

Least Significant Difference (L.S.D.) for effect (trt*day) = Standard Error (S.E.) x $t_{5\%, df=50}$ = 0.2791 x 2.008 = 0.5604. If the difference of any two least square means ≥ L.S.D., then the two means are different.

In the case of *S.* Typhimurium, a decline of 3.47 log, 3.72 log and 3.74 log was observed for MM, AATM and TWTM respectively. However, TM was the most effective of all four treatments resulting in a reduction of 4.17 log units. The bacterial count reduction was significantly different ($P < 0.05$) between MM and other three treatments i.e. TM, AATM and TWTM at the starting point of Day 1. It continued until Day 7,

however by the end of Day 28, all four treatments i.e. TM, MM, AATM and TWTM were significantly different from control but not from each other (**Table 17**).

Table 17: Microbial count [log CFU] of Ground and Formed Beef Jerky Strips Inoculated with *S.* Typhimurium

Treatment	Day 1	Day 7	Day 14	Day 21	Day 28	Difference in Log Reduction
Control	7.24^{a}	7.14^{a}	4.21^{a}	4.11^{a}	3.96^{a}	3.28
TM	7.35^{a}	7.31^{a}	4.15^{a}	3.96^{a}	3.18^{b}	4.17
MM	6.50^{b}	6.30^{b}	3.96^{a}	3.76^{a}	3.03^{b}	3.47
AATM	6.91^{a}	6.72^{a}	3.98^{a}	3.91^{a}	3.19^{b}	3.72
TWTM	6.88^{a}	6.62^{b}	3.89^{a}	3.82^{a}	3.14^{b}	3.74

Least Significant Difference (L.S.D.) for effect (trt*day) = Standard Error (S.E.) x $t_{5\%, df=50}$ = 0.3197 x 2.008 = 0.6419. If the difference of any two least square means ≥ L.S.D., then the two means are different.

In the case of *S. aureus*, a decline of 2.31 log, 1.85 log and 2.44 log was observed for MM, AATM and TWTM respectively. TM resulted in a reduction of 2.05 log units. The bacterial count reduction was not significantly different between any of the treatments in the beginning i.e. Day 1. However, by the end of Day 14, MM, AATM and TWTM were significantly different ($P < 0.05$) from TM. By the end of Day 28, MM was significantly different ($P < 0.05$) from all the other treatments i.e. TM, AATM and TWTM, with TM

and AATM overlapping with each other, TWTM overlapping with MM and AATM (**Table 18**).

Table 18: Microbial count [log CFU] of Ground and Formed Beef Jerky Strips Inoculated with *S. aureus*

Treatment	Day 1	Day 7	Day 14	Day 21	Day 28	Difference in Log Reduction
Control	7.20^{a}	8.47^{a}	8.32^{a}	5.94^{b}	5.69^{a}	1.51
TM	7.36^{a}	8.57^{a}	8.62^{a}	6.74^{a}	5.31^{ab}	2.05
MM	6.94^{a}	7.24^{b}	7.12^{b}	5.73^{b}	4.63^{c}	2.31
AATM	7.06^{a}	7.33^{b}	7.38^{b}	5.83^{b}	5.21^{ab}	1.85
TWTM	7.14^{a}	7.56^{b}	7.27^{b}	5.78^{b}	4.70^{bc}	2.44

Least Significant Difference (L.S.D.) for effect (trt*day) = Standard Error (S.E.) x $t_{5\%, df=50}$ = 0.2987 x 2.008 = 0.5997. If the difference of any two least square means ≥ L.S.D., then the two means are different.

In the case of *L. monocytogenes*, a decline of 2.77 log, 2.76 log and 2.73 log was observed for MM, AATM and TWTM respectively. TM resulted in a reduction of 2.22 log units. The bacterial count reduction was not significantly different between any of the treatments until Day 21. However, by the end of Day 28 MM was significantly different ($P < 0.05$) from TM but overlapping with both AATM and TWTM (**Table 19**).

Table 19: Microbial count [log CFU] of Ground and Formed Beef Jerky Strips Inoculated with *L. monocytogenes*

Treatment	Day 1	Day 7	Day 14	Day 21	Day 28	Difference in Log Reduction
Control	7.08[a]	7.95[a]	7.77[a]	5.36[a]	5.12[a]	1.96
TM	7.10[a]	7.61[a]	7.49[a]	5.21[a]	4.88[ab]	2.22
MM	6.98[a]	7.34[a]	7.18[a]	4.89[a]	4.21[c]	2.77
AATM	7.03[a]	7.79[a]	7.50[a]	4.94[a]	4.27[bc]	2.76
TWTM	6.99[a]	7.40[a]	7.29[a]	4.86[a]	4.26[c]	2.73

Least Significant Difference (L.S.D.) for effect (trt*day) = Standard Error (S.E.) x $t_{5\%, df=50}$ = 0.3079 x 2.008 = 0.6182. If the difference of any two least square means ≥ L.S.D., then the two means are different.

4.3 Scanning Electron Microscopy (SEM)

In the case of *E. coli* O157:H7, the control cells did not show any difference during the 28 days of storage (**Figure 1a**) and (**Figure 2a**). The cells treated with TM show a decrease in number of bacterial cells, by the end of day 28 (**Figure 1b**) and (**Figure 2b**). The cells treated with MM appear wrinkled and fibrils connecting the cells to each other are clearly visible (**Figure 1c**), by the end of day 28, there is a reduction in number of bacterial cells (**Figure 2c**). The cells treated with AATM appear wrinkled (**Figure 1d**) and irregular shapes, exhibiting unusual elongated morphology by the end of day 28 (**Figure 2d**). The cells treated with TWTM show one or two fibrils (**Figure 1e**) and a decrease in number of bacterial cells by the end of day 28 (**Figure 2e**).

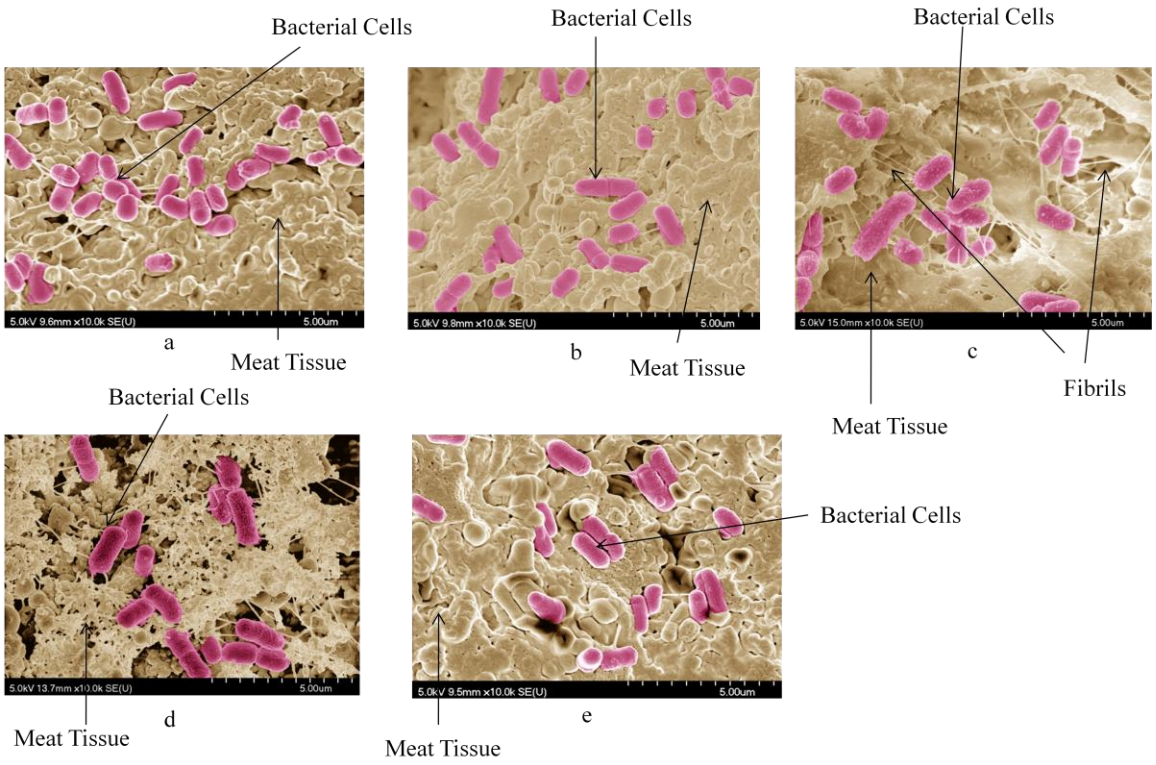


Figure 1: Scanning Electron Micrograph of Ground and Formed Beef Jerky Strip Inoculated with *E. coli* O157:H7, (**Treatments: a - Control**, **b - TM**, **c - MM**, **d - AATM**, **e - TWTM**) [Day 1]

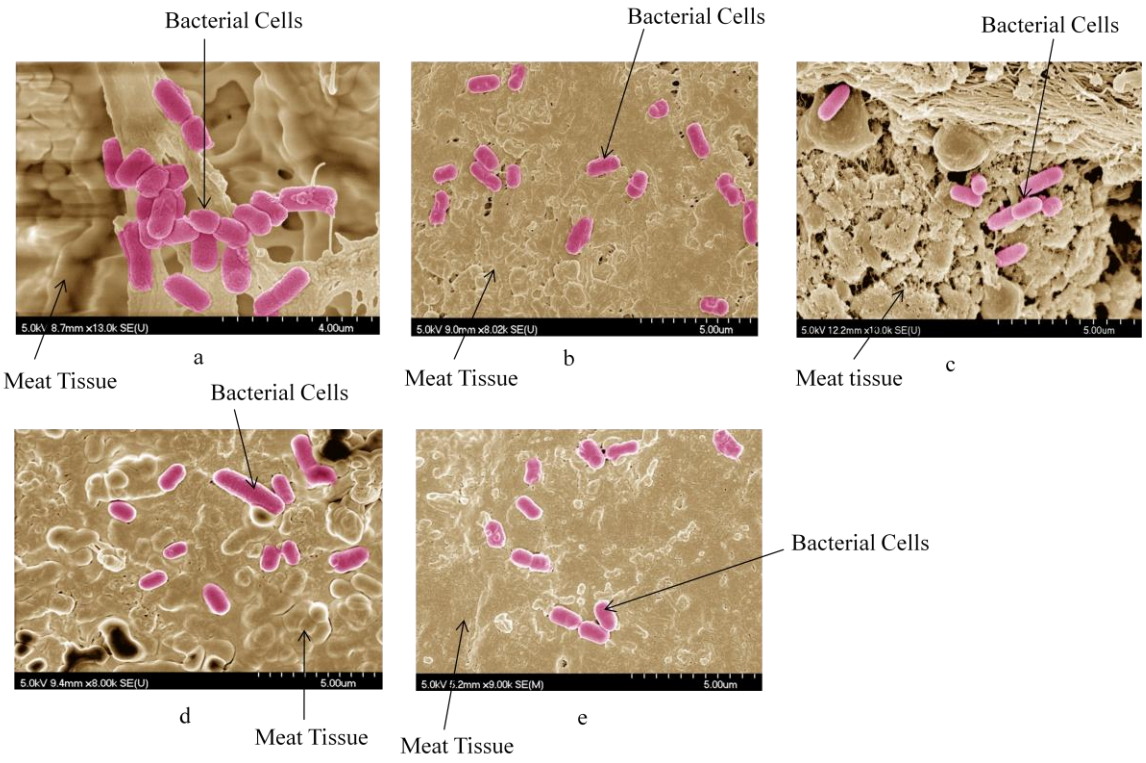


Figure 2: Scanning Electron Micrograph of Ground and Formed Beef Jerky Strip Inoculated with *E. coli* O157:H7, (**Treatments: a - Control**, **b - TM**, **c - MM**, **d - AATM**, **e - TWTM**) [Day 28]

In the case of *S.* Typhimurium, the control cells did not show any difference during the 28 days of storage (**Figure 3a**) and (**Figure 4a**). The cells treated with TM appear wrinkled (**Figure 3b**) and a decrease in number of bacterial cells by the end of day 28 (**Figure 4b**). The cells treated with MM appear wrinkled and fibrils connecting the cells to each other are clearly visible (**Figure 3c**), by the end of day 28, there is a reduction in number of bacterial cells (**Figure 4c**). The cells treated with AATM appear wrinkled and fibrils connecting the cells to each other are clearly visible (**Figure 3d**), by the end of day 28, there is a drastic reduction in number of bacterial cells (**Figure 4d**). The cells treated

with TWTM appear wrinkled both at the start and end of experiment (**Figure 3e**) and a decrease in number of bacterial cells by the end of day 28 (**Figure 4e**).

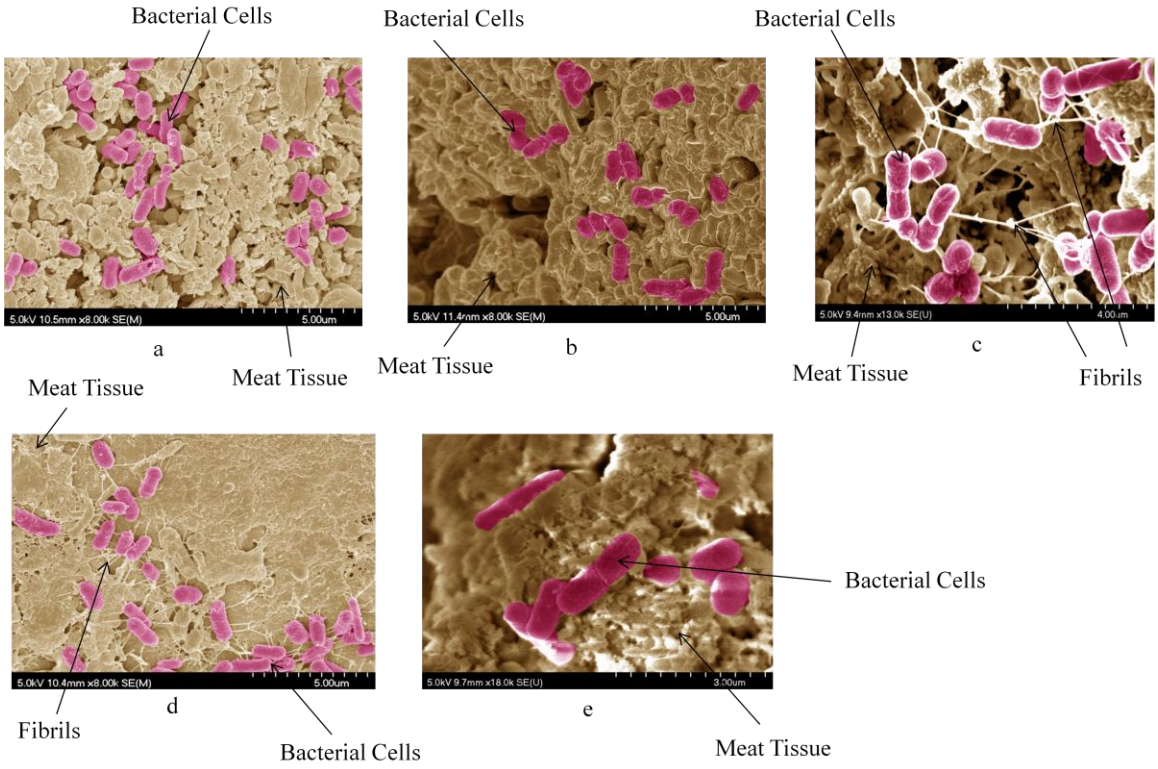


Figure 3: Scanning Electron Micrograph of Ground and Formed Beef Jerky Strip Inoculated with *S.* Typhimurium, (**Treatments: a - Control**, **b - TM**, **c - MM**, **d - AATM**, **e - TWTM**) [Day 1]

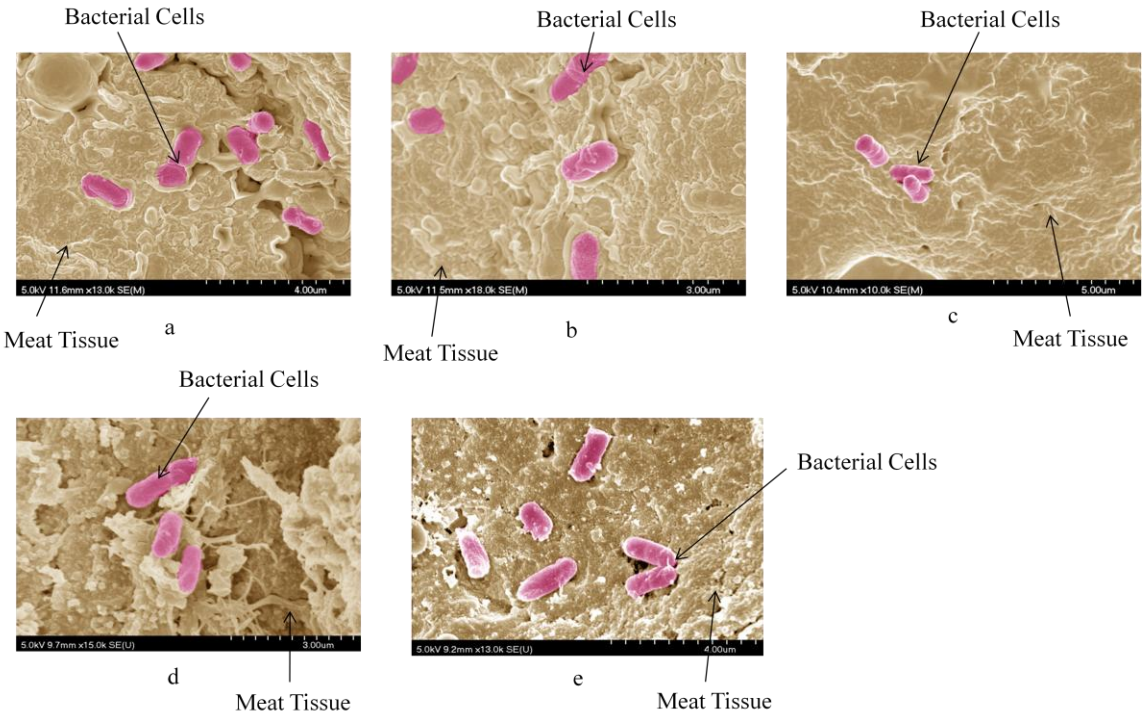


Figure 4: Scanning Electron Micrograph of Ground and Formed Beef Jerky Strip Inoculated with *S.* Typhimurium, (**Treatments: a - Control**, **b - TM**, **c - MM**, **d - AATM**, **e - TWTM**) [Day 28]

In the case of *S. aureus*, the control cells did not show any difference during the 28 days of storage (**Figure 5a**) and (**Figure 6a**). The cells treated with TM show a slight decrease in number of bacterial cells, by the end of day 28 (**Figure 5b**) and (**Figure 6b**). The cells treated with MM show some fibrils (**Figure 5c**) and a decrease in number of bacterial cells by the end of day 28 (**Figure 6c**). The cells treated with AATM show a decrease in number of bacterial cells by the end of day 28 (**Figure 5d**) and (**Figure 6d**). The cells treated with TWTM show a decrease in number of bacterial cells, by the end of day 28 (**Figure 5e**) and (**Figure 6e**).

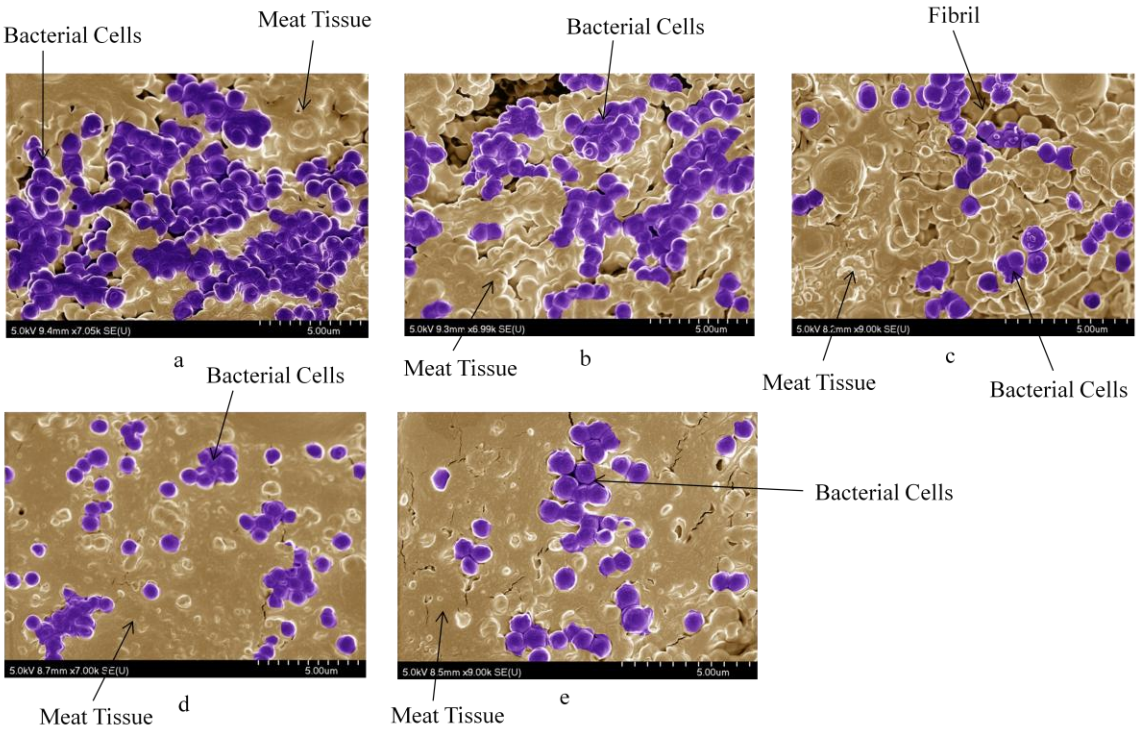


Figure 5: Scanning Electron Micrograph of Ground and Formed Beef Jerky Strip Inoculated with *S. aureus*, (**Treatments: a - Control**, **b - TM**, **c - MM**, **d - AATM**, **e - TWTM**) [Day 1]

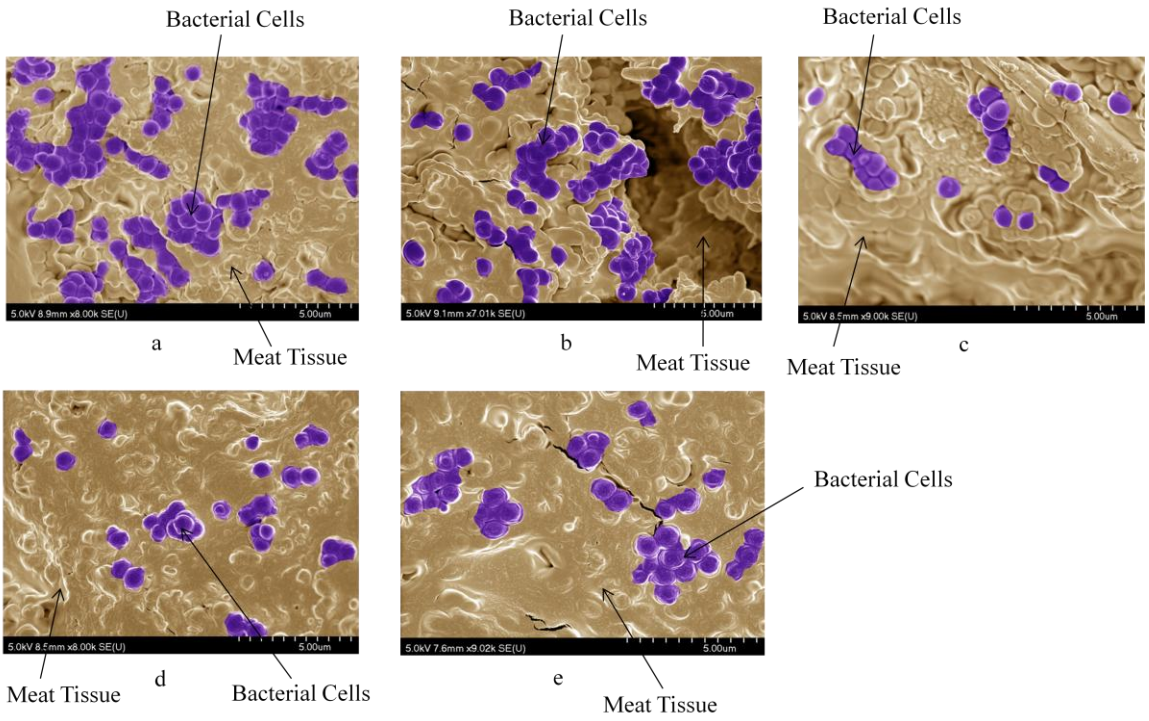


Figure 6: Scanning Electron Micrograph of Ground and Formed Beef Jerky Strip Inoculated with *S. aureus*, (**Treatments: a - Control**, **b - TM**, **c - MM**, **d - AATM**, **e - TWTM**) [Day 28]

In the case of *L. monocytogenes*, the control cells did not show any difference during the 28 days of storage (**Figure 7a**) and (**Figure 8a**). The cells treated with TM show some fibrils (**Figure 7b**) and a decrease in number of bacterial cells by the end of day 28 (**Figure 8b**). The cells treated with MM appear wrinkled and a few fibrils connecting the cells to each other are clearly visible (**Figure 7c**), by the end of day 28, there is a drastic reduction in number of bacterial cells (**Figure 8c**). The cells treated with AATM show some fibrils (**Figure 7d**) and a decrease in number of bacterial cells by the end of day 28 (**Figure 8d**). The cells treated with TWTM show some fibrils (**Figure 7e**) and a decrease in number of bacterial cells by the end of day 28 (**Figure 8e**).

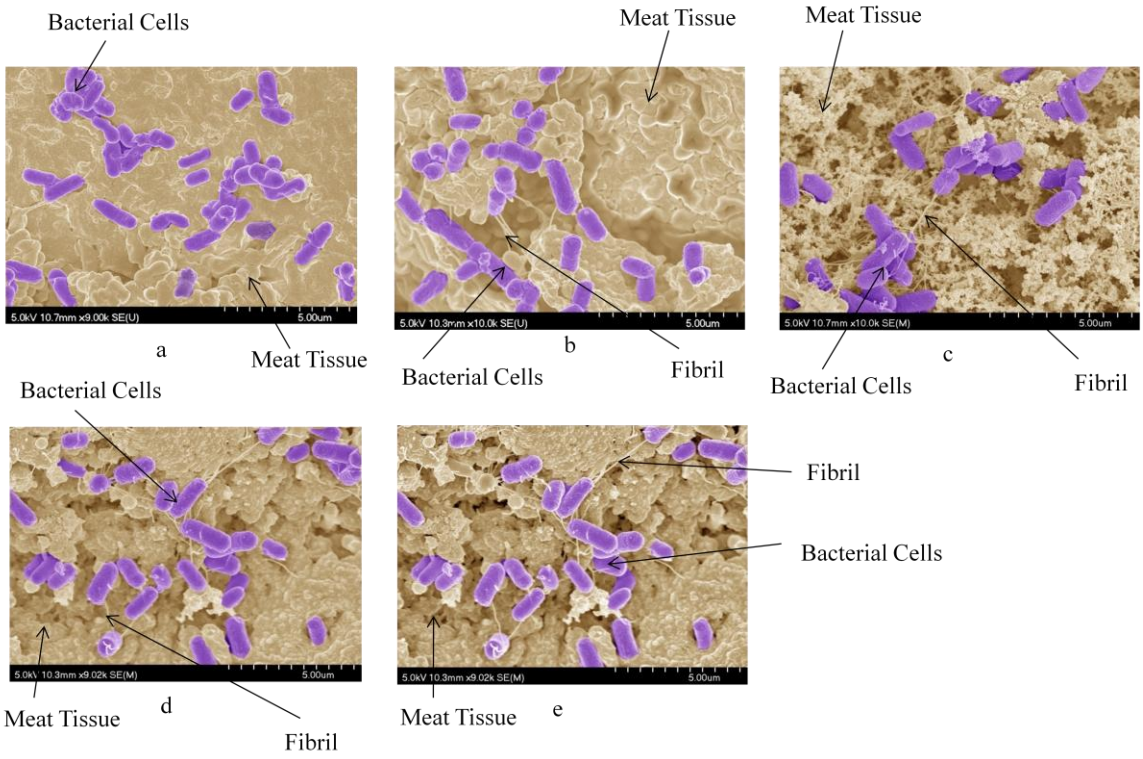


Figure 7: Scanning Electron Micrograph of Ground and Formed Beef Jerky Strip Inoculated with *L. monocytogenes*, (**Treatments: a - Control**, **b - TM**, **c - MM**, **d - AATM**, **e - TWTM**) [Day 1]

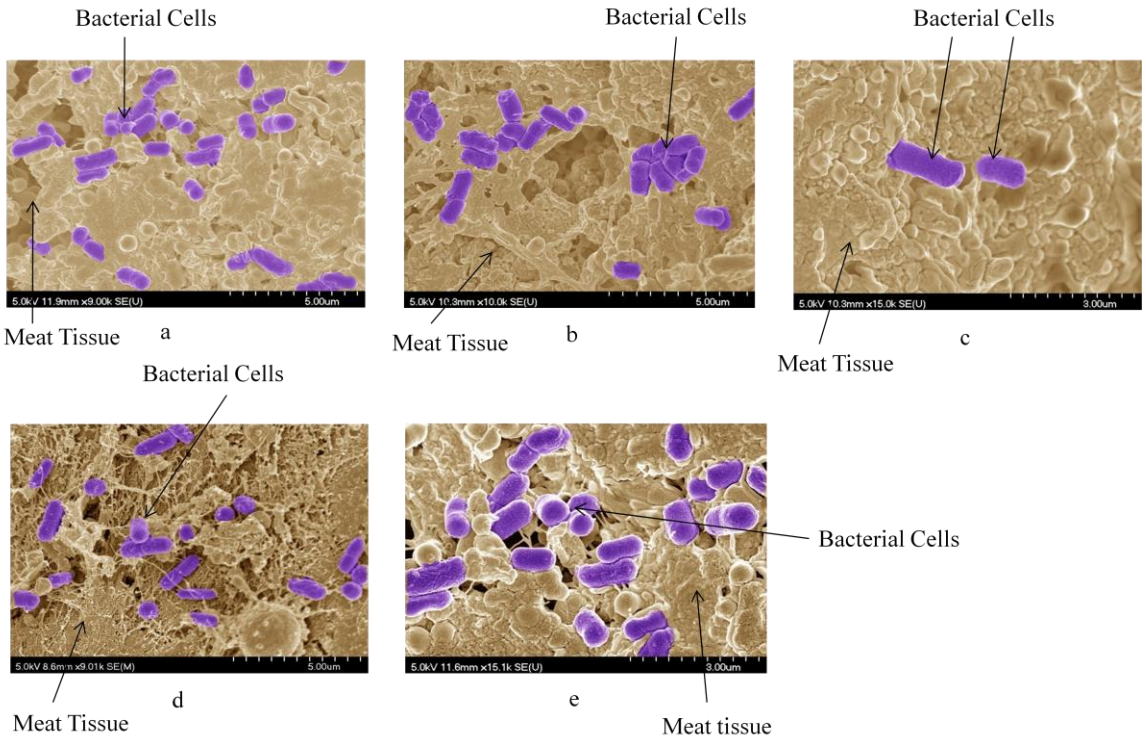


Figure 8: Scanning Electron Micrograph of Ground and Formed Beef Jerky Strip Inoculated with *L. monocytogenes*, (**Treatments: a - Control**, **b - TM**, **c - MM**, **d - AATM**, **e - TWTM**) [Day 28]

4.4 Transmission Electron Microscopy (TEM)

In the case of *E coli* O157:H7, even at the end of day 28, the control cells retained their uniform shapes with smooth continuous membranes and an evenly distributed cytoplasm within the intact cell membranes (**Figure 9a**) and (**Figure 10a**).

The cells treated with TM exhibit a little disorganized membrane structure in some cells on day 1 (**Figure 9b**). At the end of day 28, the cells clearly depict irregular shapes with cytoplasm-sparse areas within the cell. Membrane structure is disorganized with a partial disintegration of cell membrane (**Figure 10b**). The cells treated with MM display uniform shapes with smooth membrane structure in the beginning (**Figure 9c**), however,

at the end of day 28, the cells exhibit a little irregular shapes with some cytoplasm-sparse areas within the cell. Membrane structure is disorganized (**Figure 10c**). The cells treated with AATM show no observable irregularity on day 1 (**Figure 9d**), however, at the end of day 28, the cells clearly demonstrate irregular shapes with cytoplasm-sparse areas within the cell. Membrane structure is disorganized with a partial disintegration of cell membrane (**Figure 10d**).

The cells treated with TWTM with do not depict any noticeable anomaly initially (**Figure 9e**), however, at the end of day 28, the cells demonstrate irregular shapes with cytoplasm-sparse areas along with condensed cytoplasm within the cell. Membrane structure is disorganized with partial separation of outer and cytoplasmic membranes (**Figure 10e**).

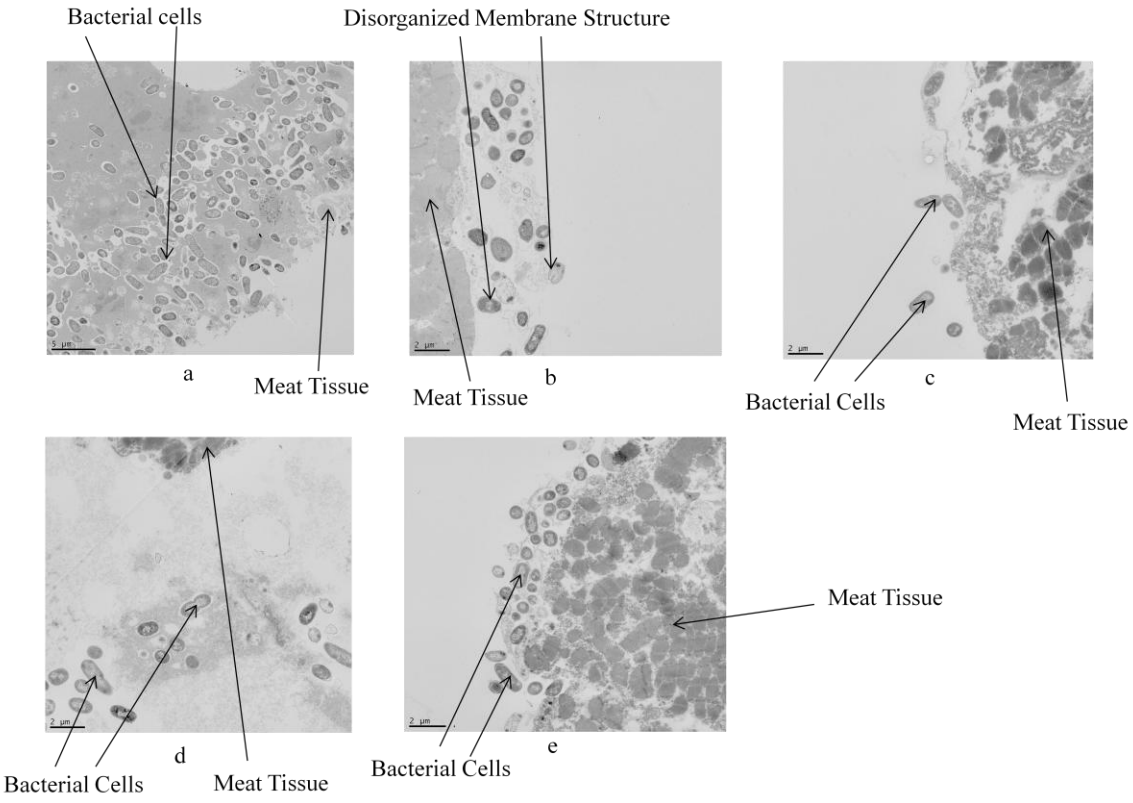


Figure 9: Transmission Electron Micrograph of Ground and Formed Beef Jerky Strip Inoculated with *E. coli* O157:H7, (**Treatments: a - Control**, **b - TM**, **c - MM**, **d - AATM**, **e - TWTM**) [Day 1]

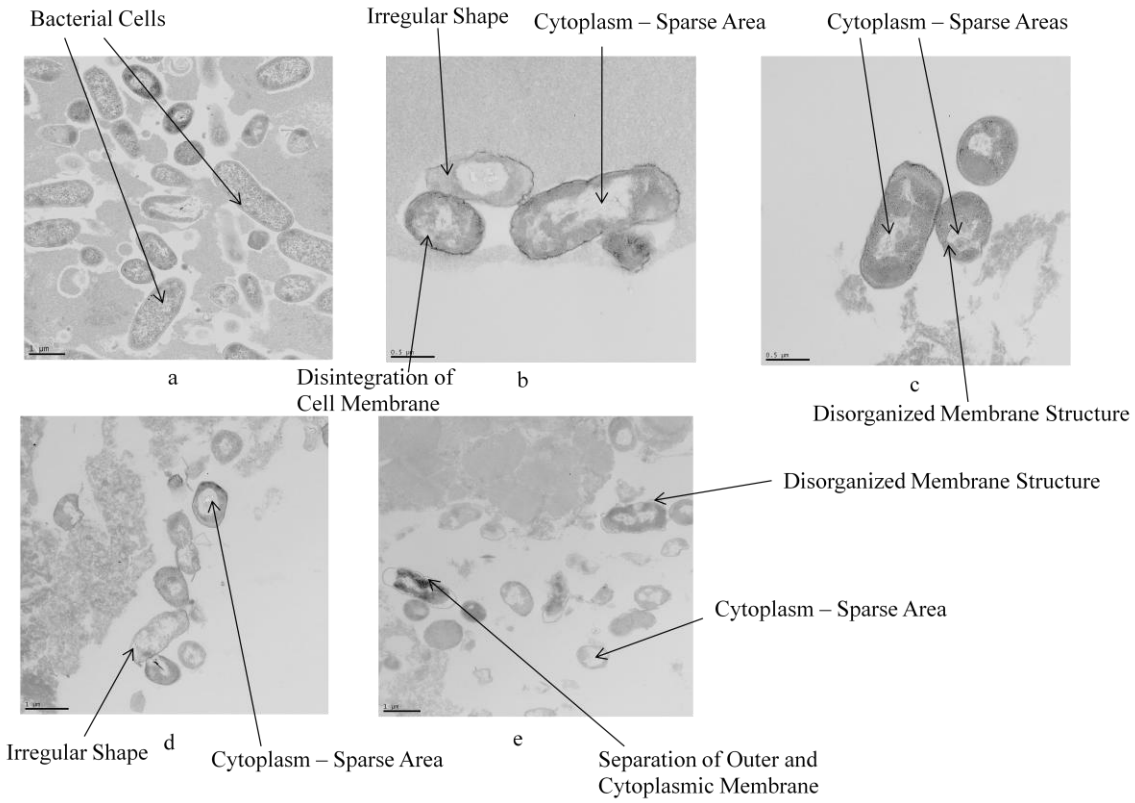


Figure 10: Transmission Electron Micrograph of Ground and Formed Beef Jerky Strip Inoculated with *E. coli* O157:H7, (**Treatments: a - Control**, **b - TM**, **c - MM**, **d - AATM**, **e - TWTM**) [Day 28]

In the case of *S.* Typhimurium, the control cells display uniform shapes with smooth membrane structure on day 1 (**Figure 11a**), even at the end of day 28, the control cells retained their uniform shapes with smooth continuous membranes and an evenly distributed cytoplasm within the intact cell membranes (**Figure 12a**). The cells treated with TM exhibit cytoplasm-sparse areas within few cells on day 1 (**Figure 11b**) and at the end of day 28, the cells clearly depict irregular shapes with some cytoplasm-sparse areas within the cell. Membrane structure is disorganized with a partial disintegration of cell membrane (**Figure 12b**). The cells treated with MM display uniform shapes with

smooth membrane structure, but with cytoplasm-sparse areas in most of the cells on day 1 (**Figure 11c**) and at the end of day 28, the cells clearly exhibit irregular shapes with some cytoplasm-sparse areas within the cell. Membrane structure is disorganized with a partial disintegration of cell membrane (**Figure 12c**). The cells treated with AATM depict uniform shapes with smooth membrane structure with cytoplasm-sparse areas in some of the cells on day 1 (**Figure 11d**). By the end of day 28, the cells demonstrate irregular shapes with cytoplasm-sparse areas within the cell. Membrane structure is disorganized with partial separation of outer and cytoplasmic membranes (**Figure 12d**).

The cells treated with TWTM depict uniform shapes with smooth membrane structure with cytoplasm-sparse areas in some of the cells on day 1 (**Figure 11e**), however, by the end of day 28, the cells exhibit irregular shapes with cytoplasm-sparse areas within the cell. Membrane structure is disorganized with partial separation of outer and cytoplasmic membranes (**Figure 12e**).

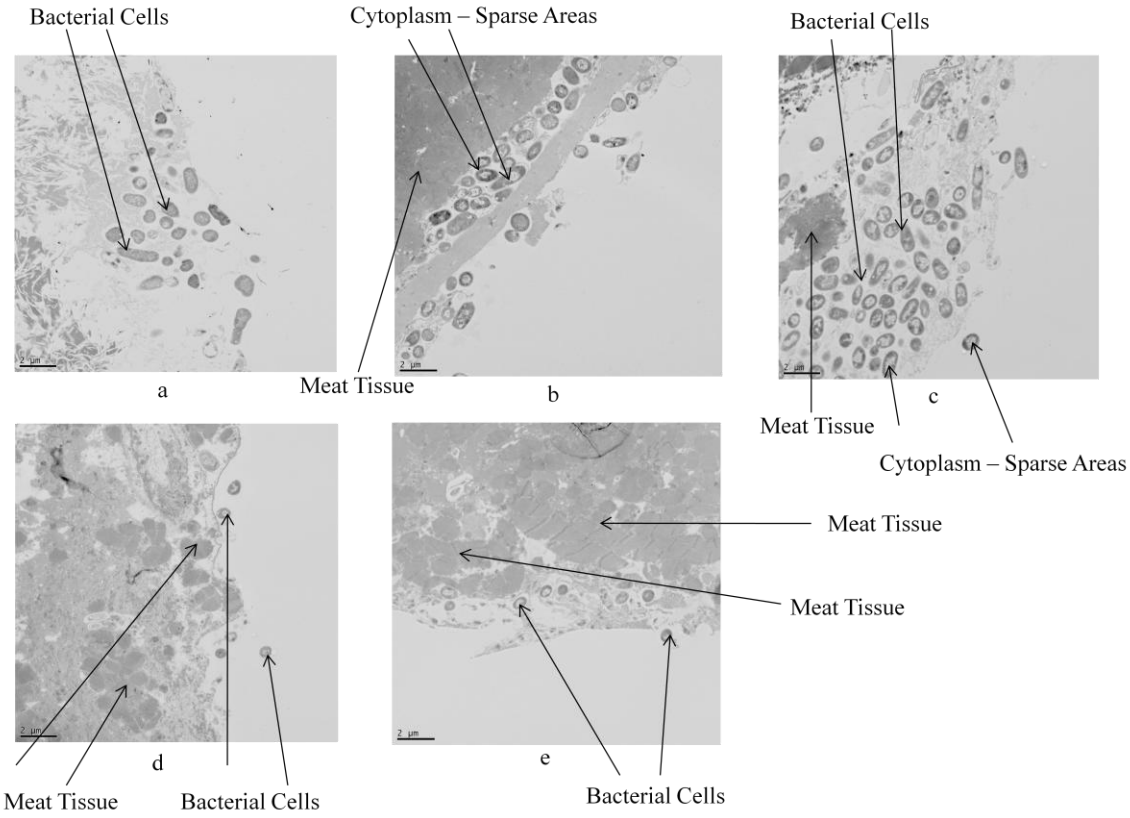


Figure 11: Transmission Electron Micrograph of Ground and Formed Beef Jerky Strip Inoculated with *S.* Typhimurium, (**Treatments: a - Control**, **b - TM**, **c - MM**, **d - AATM**, **e - TWTM**) [Day 1]

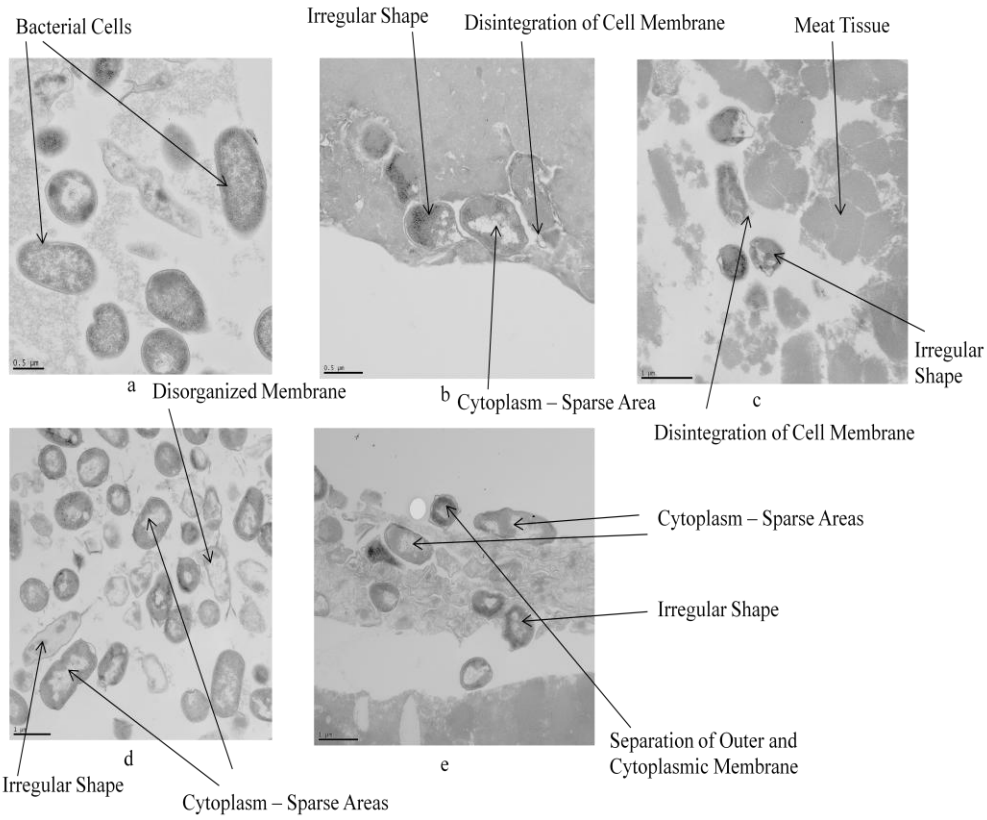


Figure 12: Transmission Electron Micrograph of Ground and Formed Beef Jerky Strip Inoculated with *S.* Typhimurium, (**Treatments: a - Control**, **b - TM**, **c - MM**, **d - AATM**, **e - TWTM**) [Day 28]

In the case of *S. aureus*, the control cells exhibit uniform shapes with smooth membrane structure on day 1 (**Figure 13a**), even by the end of day 28, the control cells retained their uniform shapes with smooth continuous membranes and an evenly distributed cytoplasm within the intact cell membranes (**Figure 14a**). The cells treated with TM exhibit uniform shapes, smooth continuous membrane structure with cross wall formation indicative of cell division (**Figure 13b**), even by the end of day 28, the cells display cell division. However, the cytoplasm is inhomogeneous along with cytoplasmic membrane disruptions (**Figure 14b**). The cells treated with MM exhibit uniform shapes

with smooth continuous membrane structure, but cytoplasm-sparse areas in some cells on day 1 (**Figure 13c**), even by the end of day 28, the cells depict uniform shapes with smooth continuous membrane structure, but inhomogeneous cytoplasm (**Figure 14c**). The cells treated with AATM depict uniform shapes with smooth continuous membrane structure on day 1 (**Figure 13d**), however, by the end of day 28, the cytoplasm is inhomogeneous along with cytoplasmic membrane disruptions (**Figure 14d**).

The cells treated with TWTM display uniform shapes with smooth continuous membrane structure on day 1 (**Figure 13e**), even by the end of day 28, the cells depict uniform shapes with smooth continuous membrane structure, but inhomogeneous cytoplasm (**Figure 14e**).

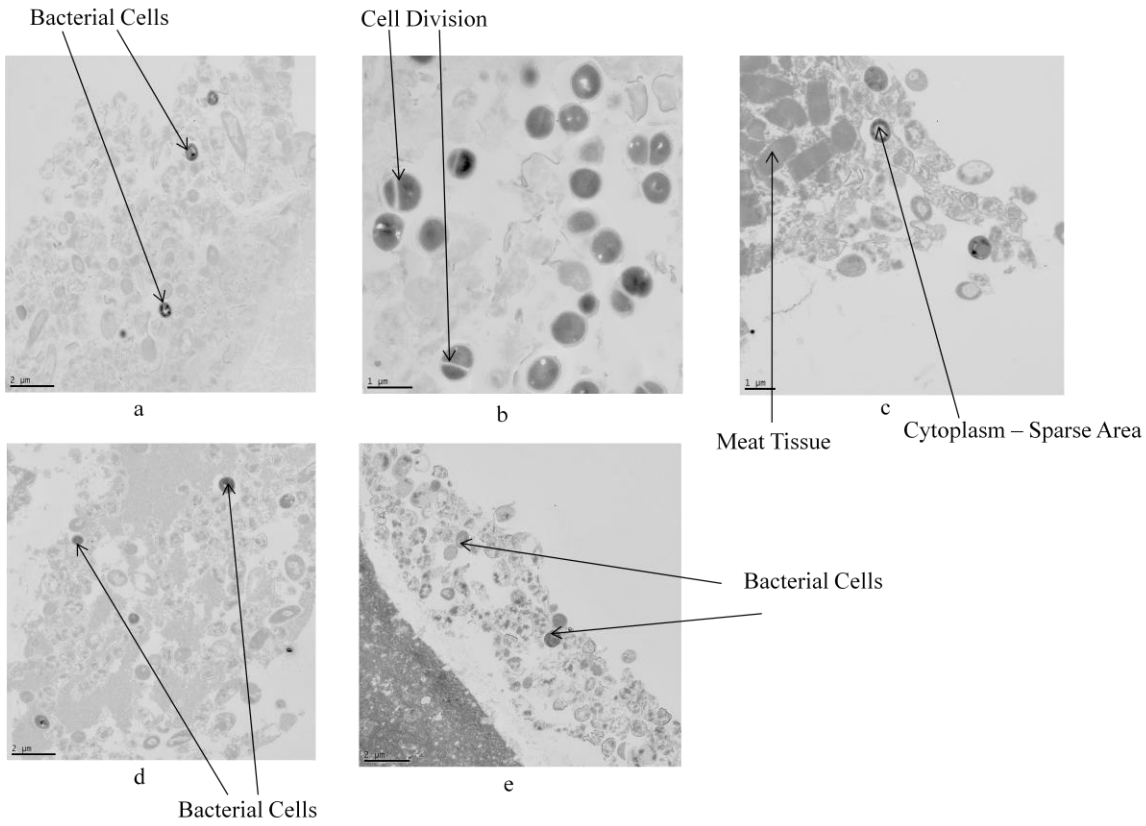


Figure 13: Transmission Electron Micrograph of Ground and Formed Beef Jerky Strip Inoculated with *S. aureus*, (**Treatments: a - Control**, **b - TM**, **c - MM**, **d - AATM**, **e - TWTM**) [Day 1]

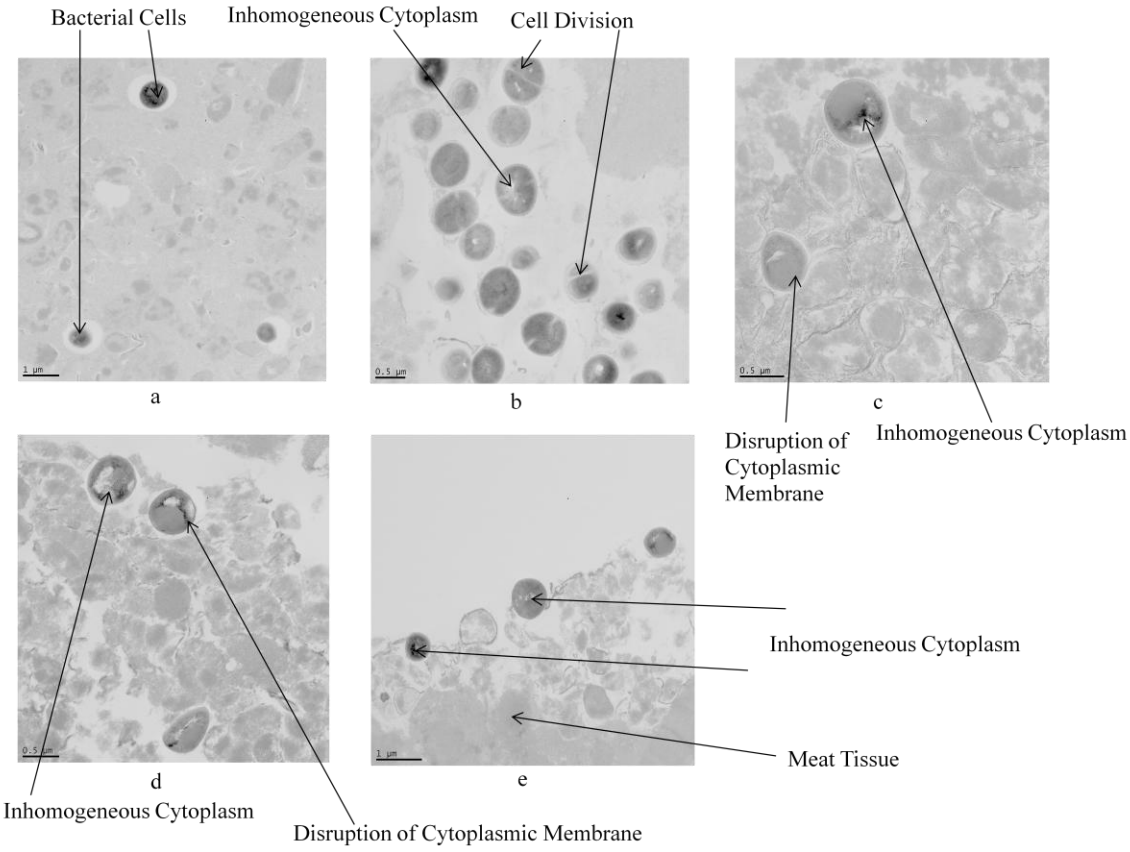


Figure 14: Transmission Electron Micrograph of Ground and Formed Beef Jerky Strip Inoculated with *S. aureus*, (**Treatments: a - Control**, **b - TM**, **c - MM**, **d - AATM**, **e - TWTM**) [Day 28]

In the case of *L. monocytogenes*, control cells exhibit uniform shapes with smooth membrane structure on day 1 (**Figure 15a**), even by the end of day 28, the control cells retained their uniform shapes with smooth continuous membranes and an evenly distributed cytoplasm within the intact cell membranes (**Figure 16a**). The cells treated with TM exhibit cytoplasm-sparse areas within few cells on day 1 (**Figure 15b**). By the end of day 28, the cells clearly display irregular shapes with some cytoplasm-sparse areas within the cell. Membrane structure is disorganized with a partial disintegration of cell membrane (**Figure 16b**). The cells treated with MM display uniform shapes with smooth

membrane structure, but with cytoplasm-sparse areas in some of the cells on day 1 (**Figure 15c**). By the end of day 28, the cells clearly exhibit irregular shapes with some cytoplasm-sparse areas within the cell. Membrane structure is disorganized with a partial separation of outer and cytoplasmic membranes (**Figure 16c**).

The cells treated with AATM depict uniform shapes with smooth membrane structure with cytoplasm-sparse areas in some of the cells on day 1 (**Figure 15d**). At the end of day 28, the cells demonstrate irregular shapes with cytoplasm-sparse areas within the cell. Membrane structure is disorganized with a partial disintegration of cell membrane (**Figure 16d**). The cells treated with TWTM display uniform shapes with smooth membrane structure with cytoplasm-sparse areas in some of the cells on day 1 (**Figure 15e**). By the end of day 28, the cells exhibit irregular shapes with cytoplasm-sparse areas within the cell. Membrane structure is disorganized with partial separation of outer and cytoplasmic membranes (**Figure 16e**).

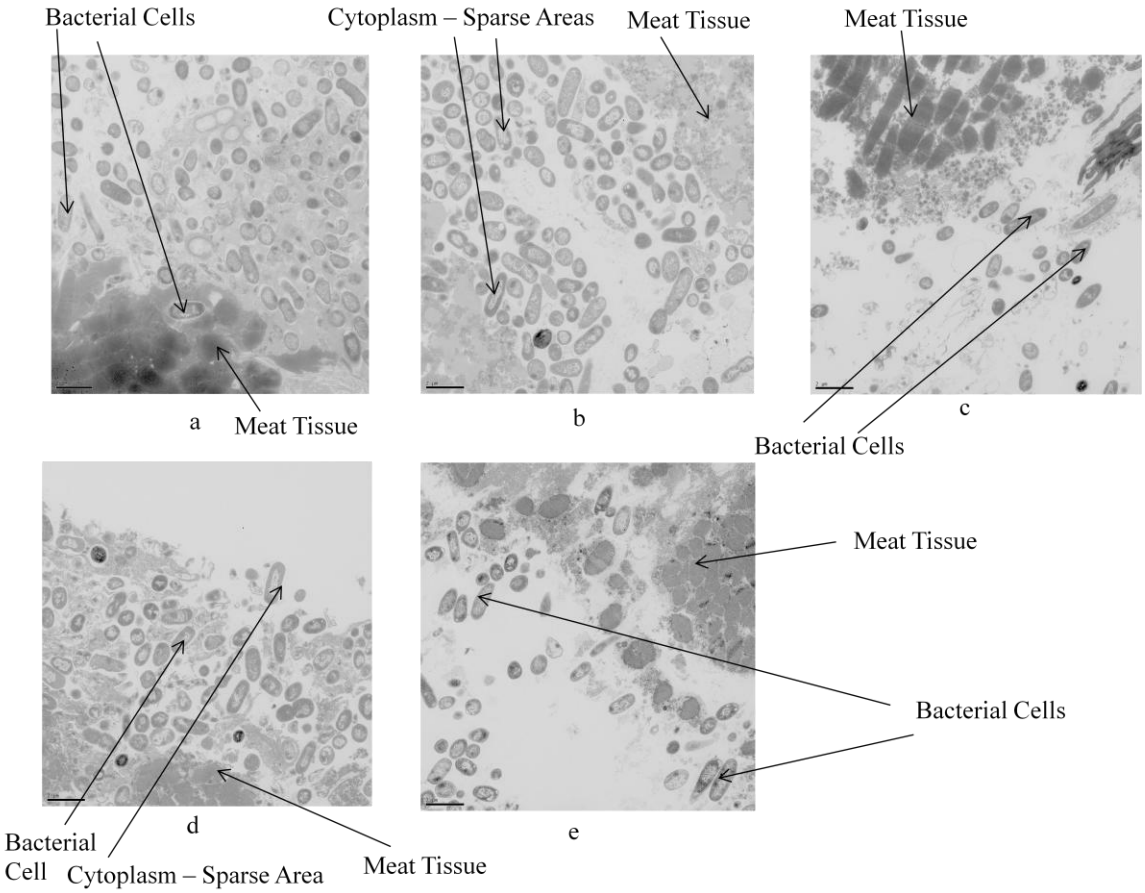


Figure 15: Transmission Electron Micrograph of Ground and Formed Beef Jerky Strip Inoculated with *L. monocytogenes*, (**Treatments: a - Control**, **b - TM**, **c - MM**, **d - AATM**, **e - TWTM**) [Day 1]

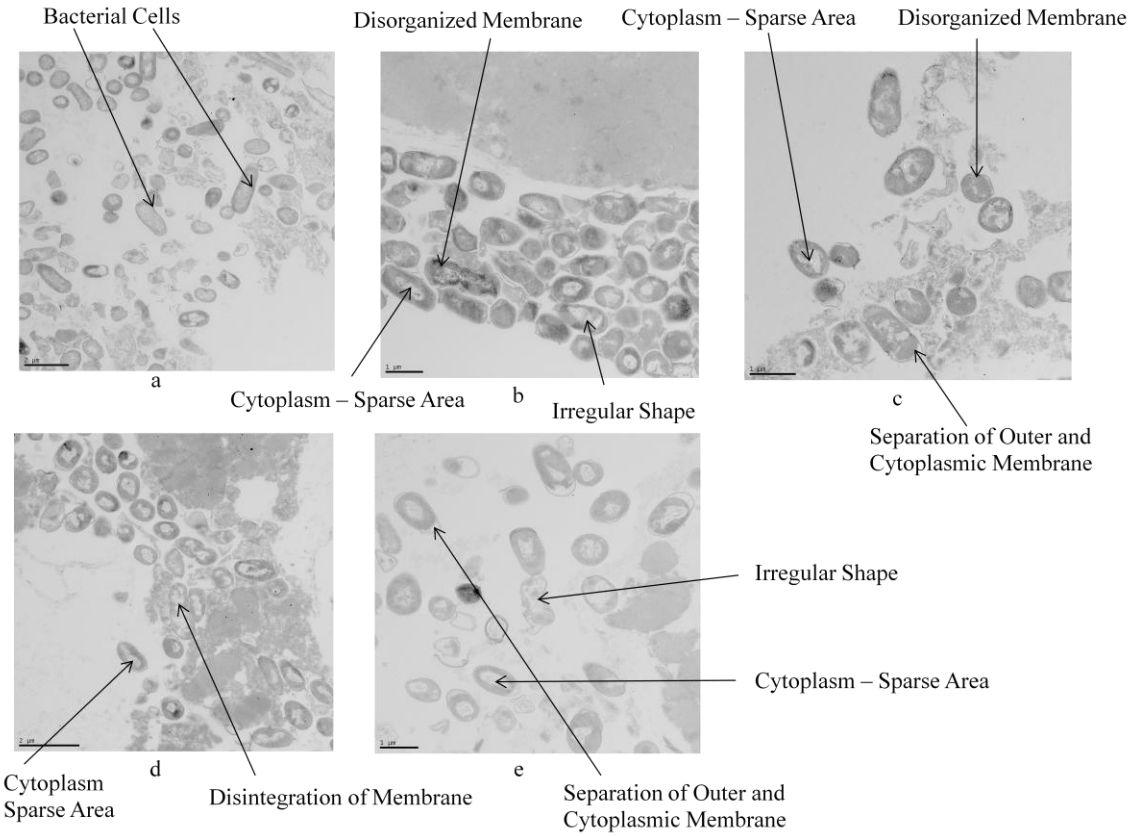


Figure 16: Transmission Electron Micrograph of Ground and Formed Beef Jerky Strip Inoculated with *L. monocytogenes*, (**Treatments: a - Control**, **b - TM**, **c - MM**, **d - AATM**, **e - TWTM**) [Day 28]

CHAPTER 5

DISCUSSION

5.1 pH and Water Activity

Both pH and water activity (a_w) values were low for all the beef jerky samples, whose treatment included acetic acid as its ingredient in comparison to the control for all four bacterial strains used. The overall pH values of jerky strips subjected to the MM (pH 4.45 to 4.69), AATM (pH 4.80 to 5.21), and TWTM (pH 4.95 to 5.32) treatments were lower than those for strips subjected to the control (pH 5.21 to 5.51) and TM (pH 5.33 to 5.78) treatments.

These low pH values of the treatments having acetic acid as an ingredient resulted in the destruction of microbial cells as manifested by the low microbial count observed in the microbiological evaluation of jerky samples treated with MM, ATM and TWTM in comparison to the control samples.

The damage inflicted by this low pH can be apparently seen in the images captured by scanning electron microscopy (SEM) and transmission electron microscopy (TEM)., showing the physical changes caused by the treatments TM, MM, AATM and TWTM in the microstructure of bacterial cells with comparison to the control jerky samples.

The overall water activity values of jerky strips subjected to the MM (0.533 to 0.696), AATM (0.636 to 0.817), and TWTM (0.773 to 0.865) treatments were lower than those for strips subjected to the control (0.891 to 0.923) and TM (0.806 to 0.883) treatments. The a_w values decreased over the period of 28 days of storage because of the

effect of the ingredients of marinade treatments i.e. soy sauce, Worcestershire sauce, vinegar, salt, acetic acid, sodium lactate and Tween 20.

The previous USDA standards required products labeled as jerky to have a moisture : protein Ratio (MPR) of 0.75:1 or lower. In the past meeting this labeling standard was regarded as also ensuring that pathogens wouldn't grow on the product, i.e. the product was shelf-stable. However, more recent guidance from USDA says that shelf stability of jerky products should be evaluated in terms of a_w, not MPR (FSIS 2008).

Lonnecker and others (2005) conducted a research to determine characteristics and production methods of jerky in small and very small plants. Their data provides sufficient correlation to proceed with lethality studies employing a_w of 0.74, NaCl 6.85% and pH 5.85, with additional study around upper and lower limits of each parameter in combination to determine any synergistic affect between parameters.

A water activity critical limit for stabilization of 0.85 or lower should control growth of all bacterial pathogens of concern. Water activity of beef jerky must be no higher than 0.88 if product is to be stored in oxygen-free packaging or no higher than 0.85 if product is to be stored under air (FSIS 2008). The a_w values obtained by our research were close to this limit as all four treatments i.e. TM, MM, AATM and TWTM were able to result in water activity values in the above mentioned range.

Hence, the marinade treatments used in the preparation of jerky strips played the main role in reductions (by way of injury or death) in populations of bacteria inoculated after drying, by causing the reduction in water activity and low pH.

5.2 Microbiological Evaluation

The effect of four different marinade treatments used i.e. TM, MM, AATM and TWTM varied greatly with respect to four bacterial strains used. AATM was the most effective treatment against *E. coli* O157:H7, TWTM against *S. aureus*, MM against *L. monocytogenes* and TM against *S.* Typhimurium. Even though TM did not contain acetic acid, tween 20 or sodium lactate as one of its ingredient like the other three treatments, it was still the most effective treatment against *S.* Typhimurium and against other bacterial strains also it was no that ineffective which is not surprising because two of the main ingredients of TM i.e. soy sauce and Worcestershire sauce have a pH of 4.83 and 3.50 respectively. In addition it also had salt at a concentration of approximately 5.8%, which is good enough to cause damage in microbial cells as shown by the previous research (Hajmeer and others 2005).

In general, the decline in the numbers of all four bacterial populations was faster in beef jerky strips subjected to the MM, AATM, and TWTM treatments than in the strips subjected to control and TM treatments, indicating potential antimicrobial activity arising from the properties of these treatments or the ingredients involved. The strong antimicrobial effects were probably the result of additional hurdles, such as a lower pH. The low-pH environment may have led to a reduced osmolyte accumulation in the bacterial cytoplasm, since the bacteria were under osmotic stress (Ogahara and others 1995). In addition, the disruption of pH homeostasis because of MM (Jordan and others 1999; Casadei and others 2001) or weaker attachment of bacteria to the meat surface because of presence of a surfactant (Tween 20) in the strips (TWTM) (Calicioglu and

others 2002) may have resulted in an increase in the sensitivity of bacteria to hurdles such as low pH.

All three treatments that included acetic acid as their ingredient i.e. MM, AATM, and TWTM caused overall faster declines in bacterial counts than the C and TM treatments did, however, the effectiveness levels of the former three treatments differed in all four bacterial populations.

The inhibition of microorganisms in food products by employing a combination of intrinsic and extrinsic factors (hurdles) such as temperature, pH, a_w (water activity), oxidation-reduction potential, or preservatives is known as the hurdle effect (Leistner 2000). The principle of this concept is that complex interactions between these hurdles or antimicrobial barriers have symbiotic effect, which is detrimental on microbial survival in foods. Jordan and others (1999) reported that the combinations of lactate (50 mmol), ethanol (5%), and high acidity (pH 3.0 by HCl) were successful in reducing the viability of exponential-phase, stationary-phase, and acid-habituated cells (grown to midexponential phase at pH 5.8) of *E. coli* O157:H7 significantly ($P < 0.05$) in TSB. However, the stationary-phase cells and habituated cells were more resistant to these combinations compared to the exponential-phase cells or nonhabituated cells. Their results further pointed out that such combinations of lactate, ethanol, and high acidity killed the cells by disrupting pH homeostasis and leading to changes in gene expression and enzyme activity.

Therefore, this hypothesis was modified and embraced as a predrying treatment (MM) in the present study by utilizing commercially available Soy sauce containing

approximately 5% ethanol and adding 2% of a 60% sodium lactate preparation and acetic acid to TM. The volume of marinade solution was also increased by using double the amount of Soy sauce and Worcestershire sauce to deliver a more highly concentrated marinade solution than TM.

The effectiveness of an acetic acid dip as a predrying treatment can be explained by the results of a recent report by Shadbolt and others (2001) which demonstrated that an initial low pH shock (in TSB, pH 3.5 with HCl, for 24 hrs) followed by an exposure to low a_w (0.90) was significantly more effective in reducing numbers of bacteria compared with an initial exposure to low a_w followed by exposure to low pH. These authors hypothesized that initial acid stress may lead to large energy depletion of the cells and subsequently may sensitize cells to other environmental stresses such as low a_w or heat.

On the other hand, an initial exposure to a low a_w environment may cause cells to synthesize osmoprotective compounds, so that they remain in a state of low metabolic activity with a lower expenditure of energy, which may lead to an increased resistance to secondary stresses such as acidity.

Therefore, in the present study, bacterial cells were exposed to acetic acid (shock) first (AATM) and then to reduction of water activity (drying), imitating the same study in our project. It is known that bacterial cells attached to the surface of meat become more resistant to stress factors such as heat than non-attached cells as demonstrated by Humphrey and others (1997).

Another study showed that prespraying beef carcasses inoculated with high levels of *E. coli* O157:H7 with 5% Tween 20 followed by spraying with 2% lactic acid resulted in

significantly higher reduction of the pathogen compared to spraying with lactic acid alone or water (Calicioglu and others 2002). The speculation is that Tween 20 may loosen bacterial cells or prevent their cellular attachment on the meat surface by way of its surfactant and hydrophobic effects, thus making the cells more vulnerable to the effect of subsequent acid exposure.

This suggested carcass decontamination method was implemented as a predrying treatment in the present study (TWTM) prior to acetic acid dip and marinating with a TM recipe. Tween 20 is permitted to be used in food as an adjuvant (Code of Federal Regulations 2001). The use of all three treatments used in this project i.e. MM, AATM, and TWTM imposed an osmotic stress on all the four bacterial populations, and it is well documented that the osmotic stress of low a_w environments causes bacterial cells to undergo a series of metabolic changes.

A well-documented response of bacteria under hyperosmotic conditions is to synthesize a de novo protein (Shadbolt and others 1999) or accumulate various compounds in their cytoplasm to increase intracellular osmolarity against extracellular hyperosmolarity. Such compounds determined in *E. coli* include potassium ions, proline, glycine betaine, trehalose, glutamate and taurine (Csonka and Hanson 1991; Mclaggan and Epstein 1991; Ogahara and others 1995).

However, the level of accumulation and types of these compounds are dependent on external pH. Ogahara and others (1995) reported that *E. coli* accumulated higher levels of glutamate and potassium ions in a high pH medium (e.g., 8.0) than in a lower pH medium (e.g., 6.7), indicating that lower pH rendered bacteria more vulnerable to the adverse

effects of osmotic stress. Shadbolt and others (2001) reported that an initial acid stress may lead to a large energy depletion of bacterial cells and subsequently may sensitize them to other environmental stresses such as low a_w or heat. From a more practical point of view, it is well documented that survival of *E. coli* O157:H7 in acidic foods such as fermented sausage (pH 4.8) dramatically decreases during storage at an ambient temperature (25°C) compared to lower temperatures (4°C) (Clavero and Beuchat 1996; Ryu and others 1999; Calicioglu and others 2001).

In an another study, Mendum and Smith (2002), reported that glycine betaine uptake by *L. monocytogenes* was dependent on storage temperature. More specifically, osmoresistance or glycine betaine uptake of *L. monocytogenes* was high at temperatures between 5 and 7°C and maximum at 12°C, while it decreased significantly as the temperature increased to 25°C and no uptake occurred at 30°C.

These studies may suggest that an effective use of osmotic stress resistance mechanisms by bacteria are controlled by environmental conditions such as temperature and pH. In light of these findings, it can be proposed that the conditions inflicted by all three marinade treatments i.e. MM, AATM, and TWTM in jerky strips (a_w 0.533 - 0.865, pH 4.45 - 5.32, storage at 25°C) reduced the use of osmoprotective mechanisms by all four bacterial populations, leading to a faster decline in their numbers during storage.

5.3 Scanning Electron Microscopy (SEM)

Scanning electron microscopy was performed to confirm the results of microbiological evaluation by visual observation of pathogens on the surface of beef

jerky strips. In general, the cells from all four bacterial populations treated with the marinade treatments appeared wrinkled and unlike the control, some fibrils connecting the cells to each other or attaching to the membrane filter began to appear. These fibrils were believed to be from cytoplasmic leakage. It is also possible that exoploysaccharide material on the outer membrane of the cells were untangled and released due to marinade treatments (Slavik and others 1994).

These changes in the microstructure of bacterial cells were caused by a combination of the individual effect of each ingredient of the different marinade treatments used. Ingredients such as vinegar (pH 2.41), Worcestershire sauce (pH 3.50), soy sauce (pH 4.83) and salt. Not a lot of study has been done using SEM and TEM to study the damage caused by these ingredients on the bacterial cells by visually observing the morphological changes in bacterial cells. The closest match to our study was research done by (Slavik and others 1994) and (Hajmeer and others 2005). Both these groups used salt at different concentrations as their treatments and then employed SEM and TEM to study the morphological changes in different bacterial cells.

The effect of high salt concentration on bacterial cells is that it exerts hyperosmotic shock on bacterial cell causing an instantaneous efflux of water from the bacterial cells, accompanied by a concomitant decrease in cytoplasmic volume, which causes considerable shrinkage of the bacterial cells resulting in the death of cells (Csonka 1989). This process is known as plasmolysis and can be detected as an increase in the turbidity of the bacterial cultures upon introduction of the cells into media of increased osmolarity (Csonka 1989).

5.4 Transmission Electron Microscopy (TEM)

A typical method to assess the impact of antimicrobial treatments on bacterial populations is using selection of microbiological plating media and techniques for detection, isolation, identification and enumeration of the bacterial population to ensure that correct counts of the microorganisms is reported (Hartman 1979; Ray and Adams 1984; Kang and Fung 1999).

Although enumeration of bacterial populations before and after treatment (e.g. different marinades) provides information on the treatment effect on viability, limited information is available on the type or extent of morphological or physical damage that occur to the bacterial cells. Visual information is extremely useful in providing an insight on the microstructure of the cell. It can also assist in characterizing the type and magnitude of changes that occur to cell composition in response to the treatment, and it enhances our understanding of how and why a given treatment is effective/non-effective against a particular microorganism.

Additionally, visual information can also impact the decision regarding the selection of microbiological plating media for bacterial enumeration studies and cell recovery. It is well known that bacteria grow and increase in number (exponential phase) in an environment with sufficient nutrients until the environment becomes limiting or inhibitory to microbial growth leading to cell fatigue, exhaustion, damage or lysis and increased death rate (McMeekin and others 1993). In this study all the bacterial populations retained their structural integrity at day 1 indicating that the growth environment was still suitable for microbial proliferation. However, as the storage time of

cells was extended to 28 days, an environmental stress was exerted onto the bacterial populations probably due to accumulation of waste metabolites and toxins resulting in morphological damage and plausible death of cells. Hence, it is vital to consider the effect of storage time on bacterial cells, because the antimicrobial effectiveness of a given decontamination method may be overestimated as both time and stress play a role relating to the impact of the treatment on the microbial cell.

All three bacterial populations including *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* were less tolerant to marinade treatments as compared to *S. aureus*, which agrees with the literature (Stein 2000). The osmotolerance of *S. aureus* to different marinade treatments is portrayed in its ability to maintain its outer membrane structural integrity. *S. aureus* can cope with osmotic environments by accumulating osmoprotectants such as proline and glycine betaine in the cells under osmotic stress (Neidhardt and others 1990).

Several compounds have been found to accumulate in *S. aureus* cells such as L-proline, proline betaine, choline, taurine and especially glycine betaine enhancing staphylococcal growth under osmotic stress (Jablonski and Bohach 1999). Townsend and Wilkinson (1992) reported that *S. aureus* cells had a 21-fold increase in proline in less than 3 minutes after exposure to high salt concentration.

Distinct separation of the cytoplasmic cellular material from the cell membrane of damaged cells was observed. Irregularities in cell morphology such as elongation of cells and partial disintegration of cell membrane and loss of cell integrity due to marinade exposure were also observed. Nevertheless, some cells were not as severely damaged as

the others, they just tended to elongate or filament rather than totally collapse and disintegrate. The control cells displayed an evenly distributed cytoplasm within the intact cell membranes. In contrast, the cytoplasm was irregularly scattered within the treated cells, showing an uneven distribution and condensation. Cytoplasm within the cells became thin or condensed, perhaps due to increased membrane porosity and the effect of free radicals (Brandt and others 1962; Mrigadat and others 1980). The inhomogeneous appearance of the cytoplasm may also be the result of segregation of the internal organelles (Kim and others 2007).

The death of *E. coli* O157:H7 and *S.* Typhimurium was more rapid, whereas the death of *L. monocytogenes* and *S. aureus* was much slower. Gram-negative bacteria may be more susceptible to low pH because of their very thin peptidoglycan layer, which is only about 2 to 3 nm thick (Murray and others 1965). In contrast, the peptidoglycan layer of gram-positive bacteria is about 30 nm thick (Shockman and Barrett 1983). This peptidoglycan layer stabilizes the cytoplasmic membrane of intact bacterial cells against turgor pressure exerted by the cytoplasm (Csonka 1989). Therefore, the very thin peptidoglycan layer of gram-negative bacteria may be less capable of preventing the cytoplasmic membrane from bursting, once it is weakened by low pH. In contrast, the presence of a thick, rigid peptidoglycan layer in gram-positive bacteria may prevent a weakened cytoplasmic membrane from expanding and bursting.

CHAPTER 6

CONCLUSIONS

6.1 Summary and Conclusions

Every marinade used in this study was effective in reducing pathogen survival on inoculated beef jerky when compared to the control, which was evident by low microbial count in treated jerky samples compared to control. However, the effect of four different marinade treatments used i.e. TM, MM, AATM and TWTM varied greatly with respect to four bacterial strains used. AATM was the most effective treatment against *E. coli* O157:H7, TWTM against *S. aureus*, MM against *L. monocytogenes* and TM against *S.* Typhimurium.

Scanning electron microscopy was performed to confirm the results of microbiological evaluation by visual observation of pathogens on the surface of beef jerky strips. In general, the cells from all four bacterial populations treated with the marinade treatments appeared wrinkled and unlike the control, some fibrils connecting the cells to each other or attaching to the membrane filter began to appear. These fibrils were believed to be from cytoplasmic leakage. It is also possible that exopolysaccharide material on the outer membrane of the cells were untangled and released due to marinade treatments (Slavik and others 1994).

The response of *E. coli* O157:H7, *S.* Typhimurium, *S. aureus* and *L. monocytogenes* to marinade-induced stress as observed from TEM micrographs was in agreement with the results of microbiological evaluation, and enhanced the understanding of behavior of bacterial cells under marinade-stress conditions and assisted in analyzing the data

obtained via quantitative techniques. Therefore, TEM observations can be powerful tools for researchers to better understand the impact of a stressor such as marinade treatments on bacterial cells and eventual performance or adaptability.

In order to reduce the survival of pathogens that are introduced to the surface of beef jerky post-processing, a combination of marinade reformulation, effective thermal processing, and avoidance of cross - contamination is considered ideal for ensuring the safety of beef jerky for consumers.

6.2 Significance

Traditional meat items like jerky are not well-studied because they have enjoyed such a long history of success without causing foodborne illness. Since that paradigm has changed, there is a need to identify new treatments and practices that can restore scientific reasoning to the preservation of beef rather than the quaint art that was formerly practiced. The main significance was to provide jerky processors with necessary facts so that they may achieve compliance with recent regulatory guidelines and requirements to verify safety of commercial processes is possible.

There are over 120 small to medium-sized meat processing companies in Missouri and they represent a key audience for the information that was determined by this project. Since, these small meat processors are unable to implement challenge studies with pathogenic microorganisms in a commercial environment, this project provides them a unique opportunity to verify the effectiveness of practical processing modifications and ingredients. The model implemented in this project can provide alternative processes to

commercial jerky manufacturers, and thus, impact future methods for production of safe jerky products.

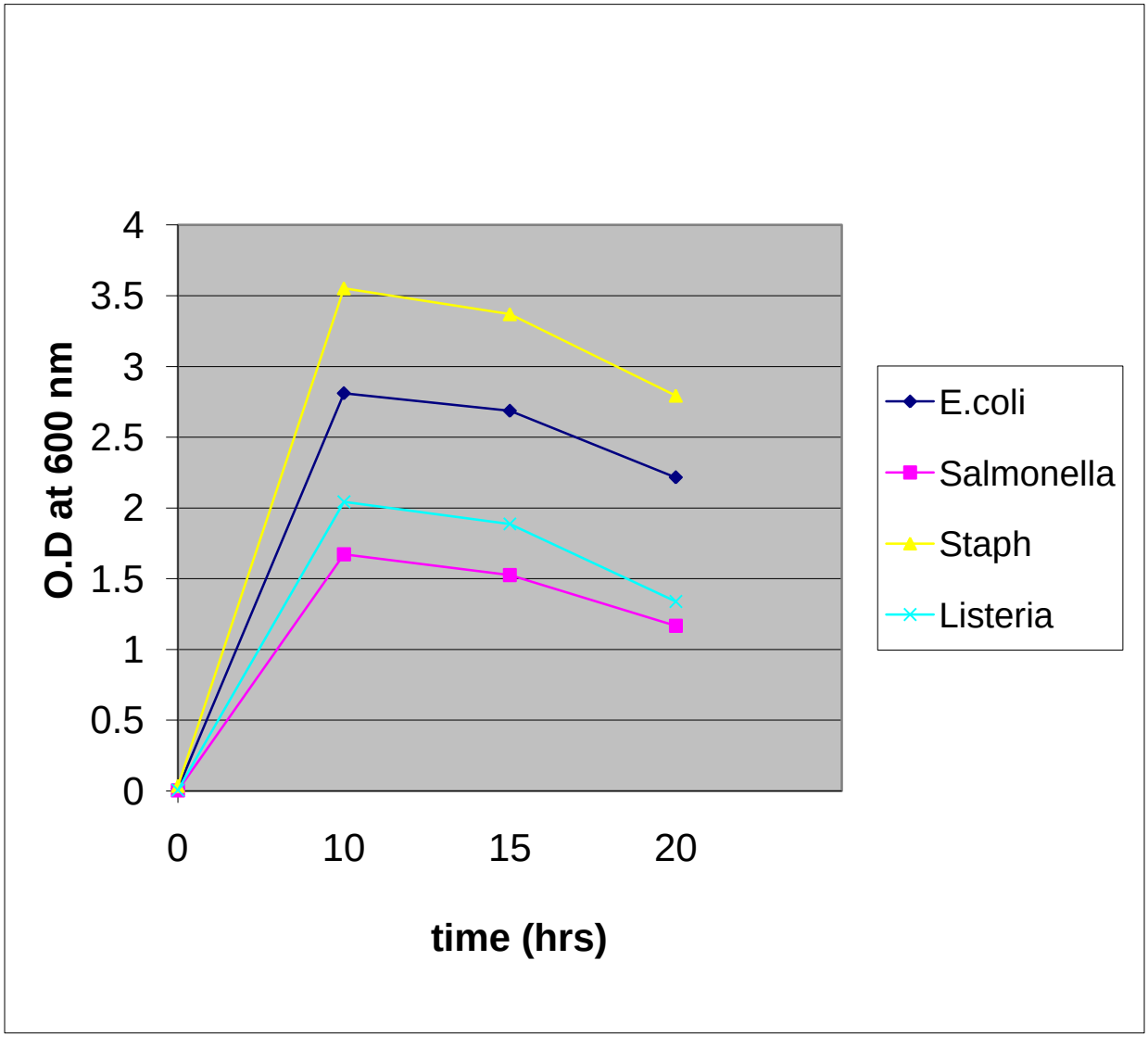
6.3 Future Recommendations

The future work on this project involves conducting a sensory study to ascertain the acceptability of palatability of marinade-treated jerky, as the ultimate aim of any processed meat product is to sell it to the consumers and the initial response in our lab indicated that the taste of jerky was acceptable, however, a detailed sensory study would be really helpful in establishing the acceptability of its flavor.

Since this study concentrated on the examination of microstructural changes of bacterial cells using electron microscopy, future experiments involving microbial physiology are needed to explain the underlying bacterial destruction mechanisms of marinade treatments for future application of marinade treatments in the meat industry.

APPENDIX

APPENDIX 1: Determination of Optical Density (O.D.) of *E. coli* O157:H7, *S.* Typhimurium, *S. aureus* and *L. monocytogenes* at Various Time Points



APPENDIX 2: Analysis of Variance (ANOVA) TABLES

ANOVA Table for pH (*E. coli* O157:H7)

Effect	Numerator DF	Denominator DF	F Value	Pr > F
trt	4	8	4910.68	< 0.0001
day	4	40	70.31	< 0.0001
trt*day	16	40	40.99	< 0.0001

ANOVA Table for pH (*S.* Typhimurium)

Effect	Numerator DF	Denominator DF	F Value	Pr > F
trt	4	8	6470.92	< 0.0001
day	4	40	111.06	< 0.0001
trt*day	16	40	71.00	< 0.0001

ANOVA Table for pH (*S. aureus*)

Effect	Numerator DF	Denominator DF	F Value	Pr > F
trt	4	8	4811.47	< 0.0001
day	4	40	148.04	< 0.0001
trt*day	16	40	59.25	< 0.0001

ANOVA Table for pH (*L. monocytogenes*)

Effect	Numerator DF	Denominator DF	F Value	Pr > F
trt	4	8	11475.3	< 0.0001
day	4	40	124.80	< 0.0001
trt*day	16	40	50.34	< 0.0001

ANOVA Table for water activity (*E. coli* O157:H7)

Effect	Numerator DF	Denominator DF	F Value	Pr > F
trt	4	8	15168.2	< 0.0001
day	4	40	403.29	< 0.0001
trt*day	16	40	238.08	< 0.0001

ANOVA Table for water activity (*S.* Typhimurium)

Effect	Numerator DF	Denominator DF	F Value	Pr > F
trt	4	8	7662.64	< 0.0001
day	4	40	60.56	< 0.0001
trt*day	16	40	52.20	< 0.0001

ANOVA Table for water activity (*S. aureus*)

Effect	Numerator DF	Denominator DF	F Value	Pr > F
trt	4	8	16582.6	< 0.0001
day	4	40	2125.99	< 0.0001
trt*day	16	40	433.22	< 0.0001

ANOVA Table for water activity (*L. monocytogenes*)

Effect	Numerator DF	Denominator DF	F Value	Pr > F
trt	4	8	11191.8	< 0.0001
day	4	40	382.74	< 0.0001
trt*day	16	40	97.11	< 0.0001

ANOVA Table for microbial count (*E. coli* O157:H7)

Effect	Numerator DF	Denominator DF	F Value	Pr > F
trt	4	50	34.94	< 0.0001
day	4	50	184.72	< 0.0001
trt*day	16	50	4.52	< 0.0001

ANOVA Table for microbial count (*S*. Typhimurium)

Effect	Numerator DF	Denominator DF	F Value	Pr > F
trt	4	50	6.16	0.0004
day	4	50	298.68	< 0.0001
trt*day	16	50	0.70	0.7851

ANOVA Table for microbial count (*S. aureus*)

Effect	Numerator DF	Denominator DF	F Value	Pr > F
trt	4	50	20.68	< 0.0001
day	4	50	156.02	< 0.0001
trt*day	16	50	1.89	0.0443

ANOVA Table for microbial count (*L*. *monocytogenes*)

Effect	Numerator DF	Denominator DF	F Value	Pr > F
trt	4	50	5.17	0.0015
day	4	50	217.03	< 0.0001
trt*day	16	50	0.53	0.9164

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