THE IMPACT OF BACTERIAL SPOILAGE AND FOODBORNE PATHOGENS ON BEEF INDUSTRY AND APPLICATION OF ANTIMICROBIAL INTERVENTIONS

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Enas Abdal Hadi Khadem

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

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SUPERVISORY COMMITTEE:

Dr. Birgit Prüβ
Chair

Dr. Neil Dyer

Dr. Teresa Bergholz

Approved:

3/29/2019
Date

John McEvoy
Department Chair

ABSTRACT

The beef industry continues to face concerns regarding the hygiene and the safety of its products. A wide range of microorganisms from various sources can grow on meat surfaces that are rich in fluid and nutrients. This paper was conducted to better understand the common spoilage microflora and the most threatening foodborne pathogens (*E. coli* O157:H7 and *Salmonella spp.*) in ground beef and the role of the virulence factors that allow pathogens to persist in the host. In addition to the above, this paper addresses the effects of using antimicrobial interventions on the ground beef products. Despite using innovative antimicrobial interventions to eliminate or reduce spoilage bacteria and common foodborne pathogens, there is still a need for new antimicrobial technologies to control the industry's sanitary hurdles and to understand their affects on meat quality and sensory characteristics.

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LITERATURE REVIEW

Meat is considered a rich nutrient matrix for microbial growth and one of the most common sources of protein for humans worldwide (Heinz and Hautzinger, 2007). According to the National Health and Nutrition Examination Surveys, from 2003-2004, the average estimated consumption of meat was 128g/day per person in the United States. Despite the decrease in meat consumption in the last few years, people in the United States still consume red meat at a higher rate than any other country (Carrie et al., 2011).

Many companies have found that an increasingly high number of meat products become spoiled due to different microorganisms. In addition to the food sector concern caused by meat spoilage, the potential economic losses due to wasted products are enormous. About 3.5 billion kg of poultry and meat products are lost at retailers and food services (Kantor et al, 1997). Furthermore, approximately 7.6 billion pounds of meat and poultry were wasted due to microbial spoilage in 1995 (Kantor et al., 1997).

From farm to table, the animal body passes several steps before arriving at a grocery store, including the loading of animals, transportation, slaughtering, processing, and production (Cerveny, Meyer, and Hall, 2009). Poor handling or misuse of technique in any of these operations is one of many causes that lead to wasted meat products, a decrease in its quality, and the spoilage of meat (Dave and Ghaly, 2011).

The storage temperature of ground beef plays an important role in the growth of spoilage bacteria. Though the USDA meat regulations section was recommend the storage of perishable food such as meat should be 4.4ºC (40º F or below) to maintain quality and to prevent meat

spoilage (USDA, 2016a). Gill et al. (2002) showed that temperatures higher than 10ºC are common and not unusual during transportation, plant storage, and consumer handling.

This abusive temperature will impact the quality of the meat and reduce the shelf life of the product. Increasing the shelf life of meat products has been challenging in the last few years. Researchers have investigated the effect of different interventions to maintain the product's shelf life and their effect on the characteristics of ground beef. Martin et al (2013) found that storage length and storage temperature affected the stability and the shelf life of ground beef. As a result, efforts need to significantly improve in the meat industry during slaughtering, processing, production and testing practices to reduce the growth of microorganisms in slaughterhouses. Furthermore, new preservative methods or techniques are required to prevent meat spoilage.

Meat Spoilage

Meat spoilage due to microbes occurs due to the microbial activity that produces a change in meat color, flavor, or appearance (Cerveny, Meyer, and Hall, 2009). The changes in the food product during spoilage occur due to the growth of spoilage microorganisms and the activity of endogenous enzymes. Food spoilage is characterized by off odor and off flavors, slime production, changes in the food texture, discoloration, and gas production (Ferrocino et al., 2013). The degree of spoilage in the meat depends on intrinsic and extrinsic factors which include the presence of microorganisms; the pH of the meat's surface; meat constituents; storage and temperature (Lambert et al., 1991).

Meat primarily consists of fat, protein, minerals, carbohydrates, and water. Any pre-slaughter stress will reduce the glycogen content of animal muscles, which then has an effect on the pH level depending on the production level of lactic acid (Rahman and Perera, 1999).

Temperature is considered one of the extrinsic factors that has an impact on the growth of spoilage bacteria. Reducing the storage temperature results in limited bacterial growth and extension of the products' shelf life (Marshal and Ba'al, 2001; Nychas et al., 2005). Microbial spoilage is caused by a variety of microflora (bacteria, yeasts, and molds) that are found in the intestinal tract and the skin of the animal (Jay, Loessner, and Golden, 2005).

Microorganisms that are responsible for spoilage can colonize the meat surface through various stages of meat production. The presence of these microorganisms depends on distinctive features including pH, oxygen availability, storage temperature, and the presence and competition of other bacteria (Ellis and Goodacre, 2001).

One of the primary microbial sources of meat and meat product contamination are the animal hide and the content of the digestive tract during the skinning, slaughtering, and processing stages. The majority of the microorganisms that are frequently found on fresh meat are *Acinetobacter, Alteromonas, Aeromonas, Pseudomonas, Brochothrix, Flavobacterium, Psychrobacter, Enterobacter, Moraxella, Microbacterium, Staphylococcus,* and lactic acid bacteria (Gill, 1986; Lambert et al., 1991; Kamenik, 2013). *Pseudomonads, Enterobacteriaceae,* lactic acid bacteria (LAB), and *Brochothrix thermosphacta* are among several Gram-negative and positive bacteria that contribute to the meat spoilage (Casaburi et. al.,2015). Among these bacteria *Pseudomonas spp*. are the most dominant bacteria which have the ability to grow in vacuum packed meat stored at 5º C (Garcia-Lopex et. al., 1998).

When spoilage microflora reaches at a high level (10^7 Colony Forming Unit (CFU)/cm^2) or more, these microbes will cause several biochemical alterations to the perishable food. Off-odor, unacceptable appearance and slime formation are mainly caused by high microbial growth (Huis in't Veld, 1996; Russell et al, 1995). However, there are some beneficial properties that

have been recognized for microflora such as the prevention of colonization by pathogenic bacteria (Berry and Well, 2010).

Glucose, lactic acid, nitrogen compounds, and free amino acids are some nutrients that meat provides for spoilage bacteria to grow. The concentration of these compounds is playing an important role in the rate of spoilage (Skandamis and Nychas, 2002). In spite of the presence of many microorganisms on fresh meat, different factors are responsible for the growth of spoilage bacteria such as temperature, and the time of the storage and packaging (Ercolini et al, 2006).

Pseudomonas spp. play an important role in the spoilage of fresh meat and are considered the most predominant microflora in the meat stored aerobically at refrigeration temperature. *Pseudomonas spp*. are Gram-negative microorganisms that have the ability to grow faster than any other microorganisms on contaminated meat at a temperature between 2°C and 15°C (Gill and Newton, 1977). *Pseudomonas* produce, like many spoilage bacteria, proteolytic enzymes that hydrolyze proteins in meat. These enzymes will cause harm putrefaction of the meat due to metabolizing of amino acids to produce putrescine and cadaverine which are responsible for a very foul smelling. Furthermore, *Pseudomonas* produce a lipolytic enzymes which responsible for the rancidity in spoiled meat (Nychas, Drosinos, & Board, 1998). Meat spoilage due to *Pseudomonas* occurs when the bacterial populations exceed 10^7- 10^8 CFU/g (Gill and Newton, 1977).

Pseudomonas consumes lactate and glucose at a higher rate than LAB especially in stored beef, whether it's packaged or unpackaged (Tsigarida and Nychas, 2001). *Pseudomonas* agar base is used with added glycerol and *Pseudomonas* C-F-C supplement in isolating this bacteria from food. *Pseudomonas* C-F-C supplement contains cetrimide, fucidine, and

cephalosporine to differentiate *Pseudomonas spp*. from other spoilage bacteria that may be found in meat (Goto and Enomoto, 1970; Lowbury, Collins, 1955).

Brochothrix thermosphacta is another spoilage bacterium that is isolated from slaughterhouses and is dominant in meat stored aerobically (Labadie, 1999). It is responsible for the off-odor in spoiled meat. *B. thermosphacta* is a Gram-positive, rod-shaped, and facultatively anaerobic, nonmotile, and non-spore forming bacterium. *B. thermosphacta* has been first known as *Microbacterium thermosphactum* (McLean & Sulzbacher, 1953). Its importance in the food industry came from its ability to grow at low temperatures (4°C) in vacuum-packed meat. Furthermore, *B. thermosphacta* can utilize glucose and glutamate and produce undesirable volatile substances during food spoilage processes, such as acetoine and acetic acid during aerobic metabolism; that are responsible for the off-odor and discoloration in the meat (Stanley, Shaw, and Egan,1981; Pin, DeFernando, and Ordonez, 2002). Streptomycin-thallous acetate-actidione has been developed as a selective media because of the characteristic resistance of *B. thermosphacta* to streptomycin and thallous acetate (Gardner, 1966).

LAB are another bacterial group that grows in vacuum packed meat stored at chilled temperatures (Borch et. al., 1996). It is responsible for the slimy appearance, off- odor, milky exudate, and sour taste of the meat (Samelis et al. 1998). LAB are Gram-positive coccobacilli that grow anaerobically. Currently, there are four genera including *Streptococcus, Leuconostoc, Pediococcus*, and *Lactobacillus* (Hugas, 1998).

LAB are divided into two groups depending on the end products from glucose fermentation. Homofermentative are include *Pediococcus, Streptococcus,* and *Lactococcus.* This group has the ability to convert all glucose to lactic acid. *Leuconostoc, Cornobacterium* and some *Lactobcilli* are considered to be heterofermentative are convert glucose to carbon dioxide,

ethanol, and lactic acid. These end products produce turbidity, free juice in meat package, slim formation, gas production, and discoloration (Jay et al., 2005).

All Purpose Tween agar is considered as nonselective media, but it has been used for cultivating LAB that require high thiamine medium from food products (Harrigan, 1998).

Enterobacteriaceae are among the frequent bacteria that cause spoilage in meat (Leori et al., 2001). Danity, Edwards, and Hibbard (1985) mentioned that all species under *Enterobacteriaceae* ferment glucose as the source of carbon and produce volatile acids and alcohol that are responsible for spoilage. Mossel et al.(1985) developed Violet Red Bile Glucose Agar (VRBG) which contains lactose for cultivation and identification of *Enterobacteriaceae* from food that is contaminated with this bacteria.

Ultimately, the presence of the spoilage bacteria can affect the sensory properties of the meat such as odor, color, and taste. Therefore implementing good hygiene strategies in the slaughterhouses together with antimicrobial interventions during handling, slaughtering, processing, and transportation will reduce the growth of spoilage microflora to acceptable levels without affecting the quality of the ground beef.

Foodborne Pathogens

Foodborne illnesses are defined as any illness caused due to consumption of food contaminated with bacteria, virus, parasite, toxins, and chemicals (CDC, 2018). According to CDC (2018), 48 million people get sick annually due to contaminated food, 128,000 are hospitalized, and 3,000 die. There are 31 foodborne pathogens in the United States annually caused 9.4 million foodborne illness, 55,961 hospitalization, and 1,351 death (Scallan et al., 2011). Most illnesses caused by Norovirus, in the first place, followed by *Salmonella*. Some pathogens cause illness that lead to hospitalization such as *E. coli* O157:H7 (CDC, 2018).

Ground beef and its products are common source of *E. coli* O157:H7 and *Salmonella spp.* (Smith et al., 2005). In addition to the danger caused by the foodborne pathogens on public health, the economic costs impact on the meat industry is enormous. Recall plus treatment due to food contaminated with *Salmonella* and *E. coli* O157:H7 are cost $3.6 billion and $ 271million respectively (USDA-ERS, 2014).

Escherichia coli O157:H7

History and Characteristics

Escherichia coli was first identified by Theodor Escherich in 1885. It is a Gram-negative, rod-shaped, and a facultative anaerobic bacterium (Escherich, 1885). It can be found harmlessly in the gastrointestinal tract of most warm-blooded animals. *E. coli* serotypes are differentiated by somatic antigen (O) and flagella (H) (Kaper, Nataro, and Mobley, 2004). Among *E. coli* strains, some produce Shiga toxin and are known as Shiga toxin-producing *E. coli* (STEC). These are the largest threat to public health (Zhao et al., 2001).

E. coli O157:H7 and non O157 STEC strains (O26, O45, O103, O111, O121, and O145) are part of the *Enterobacteriaceae* family which include other pathogens such as *Salmonella, Shigella, Vibrio,* and *Haemophilus* (CDC, 2014a). Approximately, 112,752 illnesses have been reported due to food contamination with non O157 in the US (Scallan et al., 2011).

E. coli O157:H7 first became known during an outbreak in Michigan and Oregon in 1982 as a human pathogen which caused bloody diarrhea (Wells et. al., 1983). *E. coli* O157:H7 infections did not get reported in any State until 1987 when Washington became the first state to authorize its reporting to public health authorities (Ostroff et. al., 1989). In 1993, the Jack in the Box outbreak occurred and was linked to undercooked beef patties contaminated with *E. coli* O157:H7. More than 700 cases were hospitalized, and 4 children died during this outbreak which

led to dramatic changes in the food and meat industry (Davis et al., 1994). In 1994 and in response to this outbreak, The Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA) identified *E. coli* O157:H7 as adulterant in raw ground beef and beef products and began to test samples of raw meat products for *E. coli* O157:H7 in federal and retail stores (Code For Federal Regulation, 1996). In 2012, six non O157 Shiga toxin producing *E. coli* were added to the list of the adulterants in beef (Wheeler et al., 2014). The FSIS implemented the "Zero Tolerance Rule" to eliminate any possible contamination of beef carcasses with milk, ingesta, or fecal materials. Moreover, the FSIS identified that implementing Hazard Analysis and Critical Control Points (HACCP) regulatory plan has a benefit in reducing and control foodborne pathogens in meat and poultry products (USDA-FSIS, 2014; Wheeler et al.,2014).

Outbreaks and Recalls

E. coli O157:H7 is a global problem in both developed and developing countries. The Jack in the Box outbreak brought the first attention toward this important pathogen. A massive outbreak of *E. coli* O157:H7 was reported in Japan in 1996 in an elementary school due to the consumption of contaminated white radish sprouts. More than 6000 students were hospitalized due to developing diarrhea or bloody diarrhea, resulting in 11 deaths (Michino et al., 1999; Watanabe et al., 1999).

Since that time, *E. coli* O157:H7 was linked to many outbreaks and is considered a global public health concern to this day. An estimated 73,000 infections, 2,200 hospitalizations, and 61 deaths per year reported by the Centers for Disease Control and Prevention are caused by *E. coli* O157:H7 in the United States (CDC, 2016a). A 2003 study on the prevalence of *E. coli* O157:H7 in livestock was conducted in 29 counties and 3 large States agricultural fairs in the United

States. It was found that *E. coli* O157: H7 could be isolated from 13.8% of beef cattle, 4.1% of dairy cattle, 1.2% of pigs, 4.4% of sheep, and 1.8% of goats (Keen et.al., 2006). The most common reservoir of *E. coli* O157:H7 is cattle as the ground beef is the most frequently identified vehicle of transmission to humans. Furthermore, STEC does not make the animals that carry it ill (Doyle and Schoeni, 1987).

According to a study published in 2005, 183 outbreaks between 1982 and 2002 were due to foodborne illness transmitted via ground beef. Beef, raw and undercooked has the highest rate of hospitalization (Rangel et. al., 2005).

What makes *E. coli* O157:H7 remarkably dangerous is the low infectious dose, and the relative high resistance to the acidity of the stomach. *E. coli* O157:H7 in ground beef that is only slightly undercooked can result in infection (Kassenborg et. al., 2004). Contaminated food and water are among many sources of the entrance of *E. coli* O157 to the human body besides direct contact between persons and through contact with animals or their environment (Rangel et al., 2005). A recall is an immediate action taken by the retail or the company if they find *E. coli* O157:H7 in a beef product (CDC, 2013). In 2014, a multistate outbreak with *E. coli* O157:H7 led to recall of approximately 1.8 million pounds of ground beef products that might be contaminated. In this outbreak 12 cases were reported with infection in 4 different States (CDC, 2014a). Among the recent outbreaks in 2015, two separate multistate outbreaks of *E. coli* O26 (STEC O26) occurred. 55 cases were reported in the initial outbreak; 21 ill people among them were hospitalized. The other outbreak with a different strain of *E. coli* O26 caused five cases and one individual to be hospitalized. The investigation revealed that these two outbreaks belonged to Chipotle Mexican grill (CDC, 2016a). In December 2016, beef, veal, and bison products were recalled from five States due to contamination with *E. coli* O157:H7. 11 cases were reported and

seven ill people were hospitalized (CDC, 2016b) which indicates that *E. coli* O157:H7 still poses a burden to the public health.

Symptoms

E. coli O157:H7 infection can be asymptomatic or produce symptoms like watery diarrhea, bloody diarrhea, Hemolytic Uremic Syndrome (HUS), thrombotic thrombocytopenia purpura, and death (Besser et al., 1999). After three to four days of exposure with *E. coli* O157:H7, patients begin to develop watery diarrhea. 25-75% of patients recover with no problems, however, if the disease progresses, bloody diarrhea begins on day two or three (Besser et. al., 1999).

HUS is a condition resulting from the abnormal premature destruction of red blood cells, usually induced by Shiga toxins released from the bacteria. Damaged red blood cells can clog the filtering system in the kidneys ultimately resulting in life-threatening kidney failure. HUS is characterized by acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia. HUS is the main cause of acute renal failure in children and may occur with or without diarrhea (Banatvala et. al., 2001). Children and the elderly are more susceptible to developing severe clinical symptoms like HUS, which is the most common characteristic of *E. coli* O157:H7 (Heiman et al., 2015). The higher susceptibility among children and the elderly may be a consequence of an immature immune system of children and a degrading immune system among the elderly.

A study done by Wong et al. (2000) explained the action of antibiotic administration as a risk factor for the development of HUS in children infected with *E. coli* O157:H7. They confirmed that administering a sulfa-containing antibiotic to children increased the risk to develop HUS. However, antibiotic treatment is used to limit the duration of symptoms after

infection and preventing secondary infection (Wong et al., 2000). Antibiotics encourage replication and expression of *stx* genes that are responsible for lysis of the *E. coli* O157:H7, cell envelope, and the release of Shiga toxin into the gastrointestinal tract (Paton and Paton, 1998).

Prevention

In order to keep the zero-tolerance rule, many control measures have been investigated to reduce *E. coli* O157:H7 without negatively impacting the sensory characteristic of beef products. Pohlman et al. (2002) identified that using cetylpyridinium chloride and trisodium phosphate effectively reduced *E. coli* growth in ground beef without changing its color or quality. Beneficial bacteria have caused reduction of some harmful bacteria, the goal of this treatment is to provide a competitive environment between beneficial microbes and pathogenic bacteria. *Lactobacillus spp*. were identified as a direct-fed microbial and reduced the colonization and carriage of *E. coli* O157:H7 and *Salmonella* in cattle (Callaway et. al., 2009). Another study regarding the effect of using high levels of background flora to inhibit *E. coli* O157:H7 under aerobic and anaerobic conditions revealed that the presence of a large number of background bacteria in ground beef stored at 12º C inhibited the growth of *E. coli* O157:H7 (Vold et. al., 2000). A study using organohalamine derivatives which are widely used as a disinfectant with hot water reduced population of *E. coli* O157 and *Salmonella*. Although this spray wash showed an effect on the beef carcasses by reducing the pathogens, one of the disadvantages is the high cost of maintaining the high water temperature (Kalchayanand et. al., 2009).

There is a list of substances that are approved by USDA and considered as GRAS (generally recognized as safe); this includes acetic, citric, and lactic acids, an aqueous solution of peroxyoctanic acids and many more. These antimicrobials are mostly used before harvest, during harvest or during ground beef processing (USDA-FSIS, 2017). Commercial phages have been

recently used as control measures to reduce contamination of slaughterhouses with *E. coli* O157:H7 (Sillankorva, Oliveira, & Azeredo,2012). Since the CDC (2015) reported that *E. coli* O157:H7 is a dangerous foodborne pathogen transmitted through beef, the presence of this pathogen in cattle will continue to be a threat to public health. The effort to produce a free *E. coli* ground beef will continue by using a combination of interventions and implement proper strategies.

E. coli O157: H7 in Cattle

Cattle are the natural reservoir of *E. coli* O157:H7, therefore outbreaks occurred due to consumption of bovine-derived products contaminated with the bacteria. Consuming meat, milk, and dairy product (Armstrong et al., 1996), direct contact with cattle or infected people (Rowe et al, 1993; Rangel et al., 2005) water or unpasteurized apple drinks and vegetable (Cody et al. 1999, Hilborn et al., 1999; Olsen et al., 2000). Some cattle shed the pathogen in their feces more than others and are called super shedders. The super shedders are responsible for more than 95% of human *E. coli* O157:H7 cases (Omisakin et al., 2003, Chase-Topping et al., 2007). Shedding viable *E. coli* O157:H7 and contaminated grass consumed by other cattle transmit the asymptomatic infection to more cattle and subsequently increase the risk for human infection. Because of *E. coli* O157:H7 has been a major concern for decades, understanding the survival and colonization of this pathogen in cattle can be an aid to limit the shedding and limit sources of beef contamination eventual human infection in addition to the importance to develop new strategies for prevention and control. Therefore, it is important to know the factors that *E. coli* O157:H7 use to colonize and survive inside the host.

a. Surviving the Acidic Barrier of the Cattle Stomach:

In general, all the *E. coli* has the ability to breach the acidic barrier of the stomach by using the acid resistance systems. Some pathogenic *E. coli* such as *E. coli* O157:H7 may be more resistant than other foodborne pathogens to the acidic environment in the rumen (Tilden et al., 1996).

So far, *E. coli* O157:H7 has three AR systems: AR 1 system (glucose-repressed), AR 2 system (glutamate-dependent), and AR3 system (arginine-dependent) (Lin et al., 1995; Lin et al., 1996; Hersh et al., 1996) (Fig 1).

AR 1 system is necessary for *E. coli* O157:H7 acid resistance in the stomach and in acidic foods like apple cider (Price et al., 2004). AR 1 system is activated in the stationary phase in LB broth and repressed by adding glucose, hence the name glucose-repressed. Two regulators activate the system: cAMP receptor protein (CRP) and the stress response alternative sigma factor RpoS (Castanie-Conrnet et al., 1999). The *rpoS* mutant showed an inability to resist the acid and colonize GI tract of cattle (Price et al., 2000).

The glutamate and arginine-dependent systems, AR 2 and AR3, have a similar mode of action. The glutamate decarboxylase and arginine decarboxylase convert glutamate or arginine to Ɣ-amino butyric acid (GABA) or agmatine, respectively displacing the α-carboxyl group of the amino acids with a proton from the environment into the cell (Castanie-Cornet et al., 1999). The protons reduced the internal pH, *E. coli* O157:H7 will pump out protons out of the cell and increase the internal pH of the cytoplasm, thus maintaining pH homeostasis.

b. Regulation of Attaching and Effecting Lesions:

The formation of attaching and effecting lesions (A/E lesions) in the recto-anal region (RAJ) in cattle is a crucial step for *E. coli* O157:H7 to persist in the animal. Furthermore,

repressing this mechanism while the bacteria is in the hostile environment of the rumen is very important for the *E. coli* O157:H7 survival.

The *E. coli* O157:H7 chromosomal pathogenicity island contains the locus for enterocyte effacement (LEE). The LEE consist of about 41 genes required for the formation of A/E lesions. These genes encode for type 3 secretion system (T3SS), regulatory proteins, and other effector proteins (Elliott et al., 1998) (Fig 2).

The first step in the development of A/E lesions is injecting the translocated intimin receptor (Tir) protein into the epithelial cell using T3SS. Second, Tir is inserted as a hairpin-like structure in the host cytoplasmic membrane with the central part of the Tir interacting with the LEE encoded surface protein intimin to form a strong attachment of *E. coli* O157:H7 to the target cell (Kenny et al., 1997; Deibel et al., 1998). Third, another effector protein called EspFu is also injected into the host cell and works with Tir protein. These proteins recruit certain host proteins causing actin polymerization. Subsequently, actin will accumulate beneath the *E. coli* O157:H7 attachment and lead to the development of the pedestal-like structure characterizing A/E lesions (Campellone et al., 2004; Weiss et al., 2009).

E. coli O157:H7 contains the transcriptional regulator SdiA (Kanamaru et al., 2000; Hughes et al., 2010) and does not have the ability to synthesize Acyl homoserine lactones (AHLs). However, the SdiA in *E. coli* O157:H7 can sense AHLs produced by other bacteria in the rumen of cattle (Hughes et al., 2010). After sensing AHLs, the SdiA activates the *gad* genes (the acid fitness genes) (Kanamaru et al., 2000; Hughes et al., 2010) which regulates the acid resistant systems (AR).

Activation of SdiA when the bacteria passes through the rumen will downregulate the genes required for A/E lesions formation. Thus, no bacterial attachment on the rumen mucosa

will happen. The attachment of the bacteria to the RAJ lead to the destruction of microvilli and formation of pedestal-like structure cupping the bacteria due to the accumulation of polymerized actin beneath the site of attachment (Nataro and Kaper, 1998).

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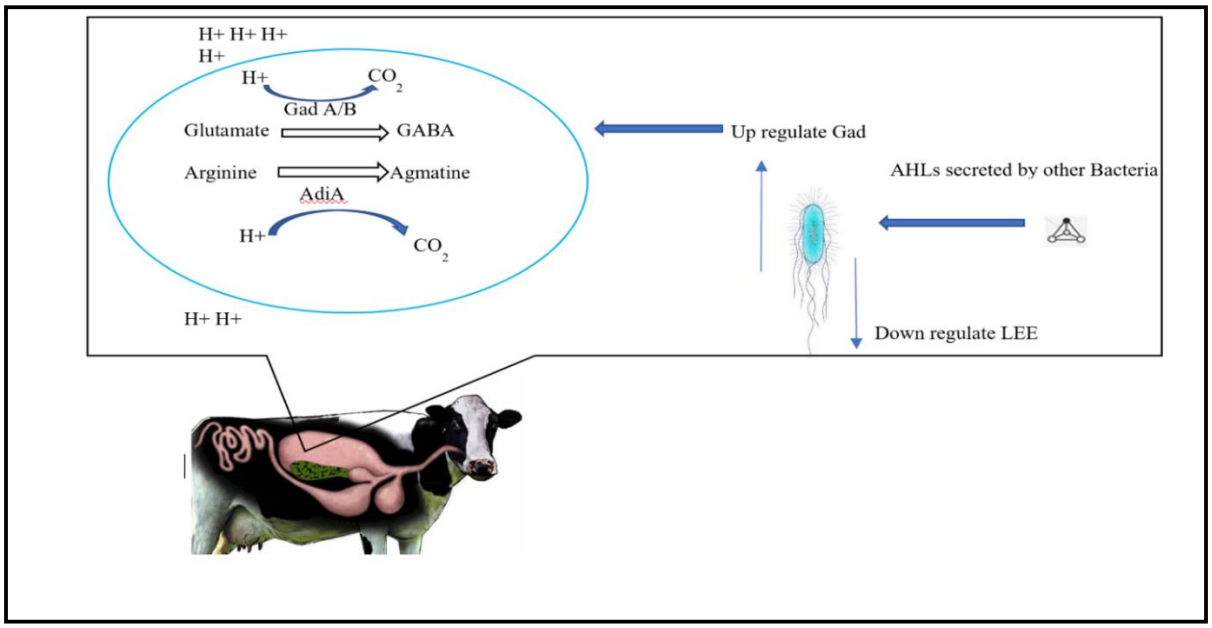

Figure 1. Acid resistant system 2 and 3 used by *E. coli* .

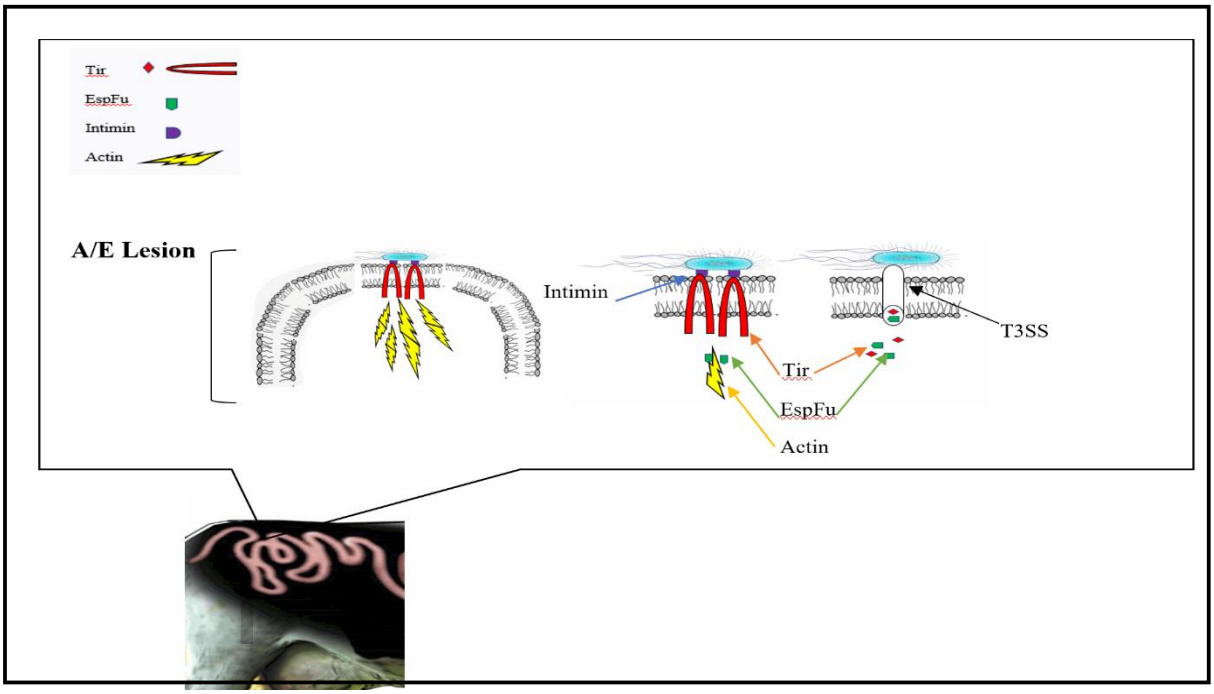

Figure 2. Formation of attaching and effacing lesion (A/E) in the rectal anal junction (RAJ). (1) *E. coli* O157:H7 uses T3SS to inject effector proteins to the host cytoplasm. (2) Tir binds to intimin works with EspFu to recruit host proteins (actin polymerization) (3) actin will accumulate beneath *E. coli* O157:H7 attachment and lead to the development of the pedestal like structure characterizing the attaching and effacing lesion.

Shiga Toxin

E. coli O157:H7 produces Shiga toxin that is responsible for the food poisoning and related symptoms in addition to the life-threatening complication. Shiga toxin is structurally and antigenically similar to the toxin produced by *Shigella dysenteria* type 1(O'Brien and LaVeck, 1983).

Shiga toxin is composed of two subunits: A and B. The B subunit binds to globotriaosylceramide-3 (Gb3), which has an important role in the pathophysiology of the *E. coli* O157:H7 (Lindgwood et al,1987). When *E. coli* O157:H7 produce Shiga toxin in the large intestine, the toxin will bind to the endothelium and subsequently expressing Gb3, permit the

absorption of the toxin in the blood stream and spreading the toxin to the other organs (Sandvig, 2001)

The tissue and cell types expressing Gb3 are different among different host (Pruimboom-Brees et al., 2001). In humans, dissemination of the toxin in the renal glomerular endothelium will express high levels of Gb3, the kidney suffers from acute renal failure, thrombocytopenia, and hemolytic anemia (HUS) due to the production of the Shiga toxin (Karmali et al., 1985). On the other hand, cattle lack the vascular expression of Gb3. Nevertheless, Gb3 is expressed in the kidney and the brain of cattle and the toxin cannot reach to these sites since the inability to bind to the blood vessels in the GI (Pruimboom-Brees et al., 2000). Ultimately, cattle are more tolerant to the *E. coli* O157:H7 and act as an important reservoir for the pathogen.

Salmonella spp.

History and Characteristics

In 1880, Ebreth had isolated typhoid bacillus from spleen and mesenteric lymph node of a patient who died due to typhoid fever (Eberth, 1880). In 1886, Salmon and Smith identified *Salmonella* as a pathogen during their investigation for the cause of swine fever (hog cholera) and named as *S. choleraesuis* (Salmon and Smith, 1886).

Salmonella spp. are classified in the *Enterobacteriaceae*, they are Gram-negative, non-spore forming, and facultative anaerobes (Wray and Davies 2000). More than 2,000 *Salmonella* serotypes have been identified. *Salmonella* is divided into two species *S. enterica*, that poses a serious risk for public health, and *S. bongori* (Brenner et al., 2000). S. *enterica* has 6 subspecies that are differentiated depending on their flagellar, carbohydrate, and lipopolysaccharide (LPS): *S. enterica, salamae, arizonae, diarizoniae, houtenae,* and *indica* (Fierer & Guiney, 2001). *Salmonella* serovars Dublin, Typhimurium, and Choleraesuis cause disease in humans and

animals while serovars Typhi, Paratyphi, and Sendi are common in humans only cause enteric fever (Fierer and Guiney, 2001). *S.* Typhimurium, Enteritidis, Newport, and Javiana are considered the most common serotypes that are related to foodborne illnesses (CDC, 2013). *Salmonella* is estimated to cause more than one million illnesses annually, with 19,000 hospitalizations and 380 deaths each year in the US (CDC, 2017). *Salmonella spp*. are similar to *E. coli* in transmission and cause similar symptoms. Non-typhoidal *Salmonella* including all *Salmonella* serotypes except Typhi and Paratyphi A, Paratyphi B, and Paratyphi C. *S.* Typhi and *S.* Paratyphi are the main cause of typhoid fever. People with typhoid fever have a high fever (39 - 40°C), stomach pain, headache, loss of appetite, a rash of flat, and rose-colored spots (CDC, 2016).

Outbreaks and Recalls

The majority of *Salmonella* infections are attributed to the consumption of animal-derived food such as eggs, chicken, pork, beef, and turkey as well as fresh fruits and vegetables (Brunette, 2017). Between 1998-2008, 1,1491 outbreaks were reported to the Foodborne Diseases Outbreaks Surveillance System of the CDC due to contamination of *Salmonella*. 595 outbreaks were linked to animal-derived food contaminated with different serotypes of *Salmonella* (Jackson et al., 2013). In 2012, 423 among 579 outbreaks bacteria were the most cause of the outbreaks. *Salmonella* was in the second place after Norovirus which cause 106 outbreaks (CDC, 2014b). In July 2012, Cargill Meat Solutions recalled 29,339 pounds of fresh ground beef products due to contamination with *S. enteritidis*. A total of 46 persons were infected in nine States and12 people were hospitalized (CDC, 2012). Another multistate outbreak was reported in 2013 due to the contamination of ground beef products with *S.* Typhimurium with a total of 22 persons infected and seven people hospitalized. Approximately 500 pounds of

ground beef was recalled from Gab Halal Foods in Michigan state in 2012 (USDA-FSIS, 2013a). From March 2013 to July 2014, a large outbreak with 634 cases included seven outbreak strains of *S.* Heidelberg which were identified in 29 States and Puerto Rico. About 38% of all sick individuals were hospitalized. After an investigation, the source of this outbreak was determined to be due to the consumption of contaminated chicken from Foster Farms (CDC, 2014b).

Symptoms

The infectious dose of *Salmonella* in the human host is over 100,000 bacterial cells (Harrison et al., 2004). People who are infected with *Salmonella* develop diarrhea, fever and abdominal cramps between 12-72 hours after infection and the illness typically lasts four to seven days (CDC, 2016). Once the pathogen enters the body, symptoms like gastroenteritis, acute diarrhea, abdominal pain, fever and sometimes vomiting are developed. Bacteremia develops in 5% of ill people. Salmonellosis may cause dehydration as a complication due to a long-term of diarrhea and vomiting which causes an imbalance of electrolytes in the human body. In rare cases, *Salmonella* can cause reactive arthritis, a pain in people joints. Reactive arthritis can last in a month or develop chronic arthritis (CDC, 2016). Complications like dehydration and death due to invasive infection may vary depending on age, strain, and immune system (Brunette, 2017).

Prevention

The presence of *Salmonella* in the lymph nodes of cattle make the contamination more difficult to contain during meat processing. Furthermore, most of the lymph nodes are buried in thick layers of fat and muscles that make using any antimicrobial interventions difficult to perform during meat processing. Samuel et al. (1980) reported that 54% of the mesenteric lymph

nodes were positive for *Salmonella* in Australia. A similar study identified the presence of *Salmonella* in subiliac lymph nodes in feedlot cattle prepared for slaughter (Gragg et. al., 2013).

According to the CDC (2017), antibiotics are not required for the treatment of Salmonellosis. Intravenous fluid and fluid rehydration are the best way to treat diarrhea. People with severe diarrhea, high fever, or bacteremia or people at severe risk such as infants, elderly or immunocompromised patients must be treated with antibiotics.

Like many foodborne pathogens, reduced food contamination and consumption besides additional education efforts are significant in reducing Salmonellosis. Harris et al. (2006) found that using antimicrobial interventions, acidified sodium chlorite, acetic and lactic acids were promising in reducing foodborne pathogens in beef trim prior to grinding.

Another study suggested using plant extracts against some foodborne pathogens including *Salmonella* Typhimurium. This study found that herb extracts provide minimal protection (by 1 $\log_{10}$ reduction) on intact beef lean stored under refrigerated or vacuum-packaged conditions (Cutter 1999).

Molecular Aspects of Virulence

Salmonella enterica Typhimurium infection in human and neonatal calf models lead to enterocolitis, which is characterized by an increase in vascular permeability, neutrophils influx, mucosal edema, and necrosis of ileal mucosa (Zhang et al., 2003). Subsequently, leakage of extravascular fluids and neutrophils transmigration into the intestinal lumen occurs. Since *Salmonella* infection in mice is dramatically different from a human infection in intestinal pathology, ligated ileal loops from bovine have been used to study *Salmonella enterica* Typhimurium pathogenesis (Zhang et al., 2003). In vivo studies in neonatal calves and human volunteers using oral infection route showed that patients and animals developed necrotizing

enterocolitis and fibrino-purulent exudate with large neutrophils infiltrations. (Blaser and Newman,1982; Tsolis et al., 1999).

Salmonella has many virulence factors required for the invasion of intestinal cells, such as T3SS-1, effector proteins (SopB, SopE2, SipA, SopD, SopA, AvrA, SptP, SlrP), translocase (SipB, SipC, and SipD), membrane ruffling (SipA, SopB, SopE2, SptP), and cytoskeletal change effector proteins (SipA, SopD, SopA) (Fig 3).

Genes encoding for T3SS-1 are located on *Salmonella* Pathogenicity Islands (SPI-1) (Schmidt and Hensel, 2004). The SPI-1 to SPI- 5 gene clusters are found in the genome of entire *Salmonella* genus. On the other hand, certain SPIs are present in certain serotypes like SPI-7, which is present in *Salmonella enterica* serovar Typhi (Schmidt and Hensel, 2004; Hensel, 2004). T3SS-1 is a needle like structure which facilitates the penetration of the host membrane and translocates bacterial proteins into the host cell (Jones et al, 1998; Jung et al., 1995).

Secreted effector proteins are transported from *Salmonella* to the host cells by T3SS-1. These effectors will stimulate the host cell to take up the bacteria (Ibarra and Steele-Mortimer, 2009). Translocase includes: SipB, SipC, and SipD. These proteins are first transported to the host cell and form a translocation complex in the eukaryotic membrane called translocase SipBCD. The SPI-1 has the genes that are responsible for the encoding of SipBCD (Kaniga, Trollinger, and Galán. 1995; Kaniga et al., 1995). The function of the translocase is in the delivery of other effector proteins into the host cell cytoplasm (Hardt and Galán, 1997; Collazo and Galán1997; Wood et al,1996). Genetic studies have shown that mutation in *SipB* strongly reduces the pathogenicity of the bacteria in neonatal calves (Zhang et al., 2002) (Fig 4).

In order to invade the host cell, *Salmonella* induces actin-rich membrane ruffles which is a cytoplasmic projection from the host cell. It eventually surrounds the bacteria and forms membrane-bound vacuoles, hence bacterial internalization (Finely and Falkow, 1997).

Next, *Salmonella* induces cytoskeletal changes in the host cell. These processes are mediated by the effector proteins SipA, SipC, SopE2, and SptP which is translocated by T3SS-1(Zhou and Galán, 2001). Genes which encode SipA and SptP are located on SPI-1 (Kaniga, Trollinger, and Galán. 1995; Kaniga et al., 1995), while the remaining effector proteins are encoded by genes located outside SPI-1 (Hardt, Urlaub, and Galán,1998; Miao et al., 1999; Galyov et al., 1997). *Salmonella* proliferates inside the host cells in *Salmonella* containing vacuoles (SCV) away from the host immune system (Salcedo et al.,2001).

Salmonella infection in human and neonatal calve models are characterized by enterocolitis which feature acute inflammatory response which lead to increases in vascular permeability leading to edema and necrosis of upper most ileal mucosa (Day, Mandal, and Morson, 1978; McGovern, and Slavutin. 1979; Tsolis et al., 1999).

Necrosis and injury to the intestines occur due to the influx of polymorphonuclear neutrophilic leukocytes (PMNs). Necrosis may result from the formation of pseudo membranes in the ileum and colon (Tsolis et al., 1999). *Salmonella* invades enterocyte of absorptive villi and epithelial cells after forming TTSS-1. First, the bacteria encounter the host cell, then translocate the effector proteins using TTSS-1, which leads to the ruffling of the brush border of the enterocytes. Eventually, bacterial internalization occurs and the infected M cells will carry the bacteria to Peyer's patch (Salcedo et al., 2001). Bacterial infection will stimulate a massive influx of PMNs through the follicle associated epithelium in the lumen leading to necrosis and fluid accumulation (Santos et al., 2002). Neutrophils transmigration into the intestinal lumen is

accompanied with hemorrhage followed by detachment of epithelial cells leading to increased intestinal permeability and protein loss.

The mechanism by which TTSS-1 effector proteins induce the inflammation response and subsequent damage is by directly stimulating proinflammatory signals in the host cells. Previous studies showed that the effector proteins SipA, SopB, SopD, and SopE2 trigger chemokines released from the macrophages and epithelial cells of the host (Criss et al., 2001; Lee et al., 2000) (Santos and Bäumler, 2004; Santos et al., 2002; Zhang et al., 2002).

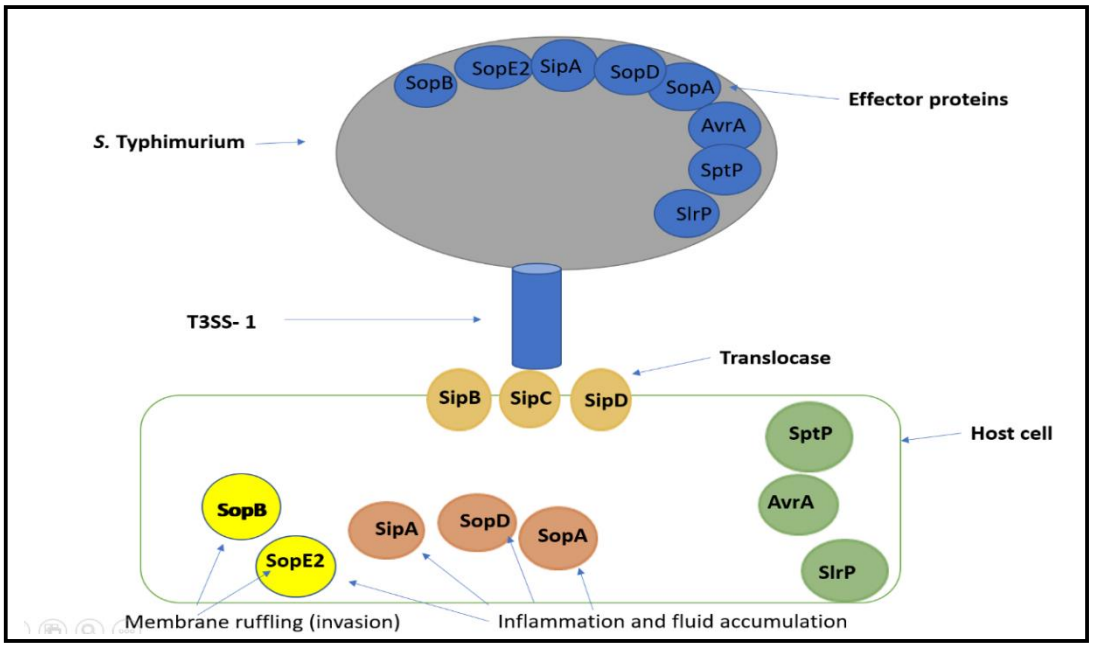

Figure 3. T3SS-1 forms a needle like crossing the membrane of the *Salmonella* to transport effector proteins into the target cell by translocation complex (SipB, SipC, and SipD).

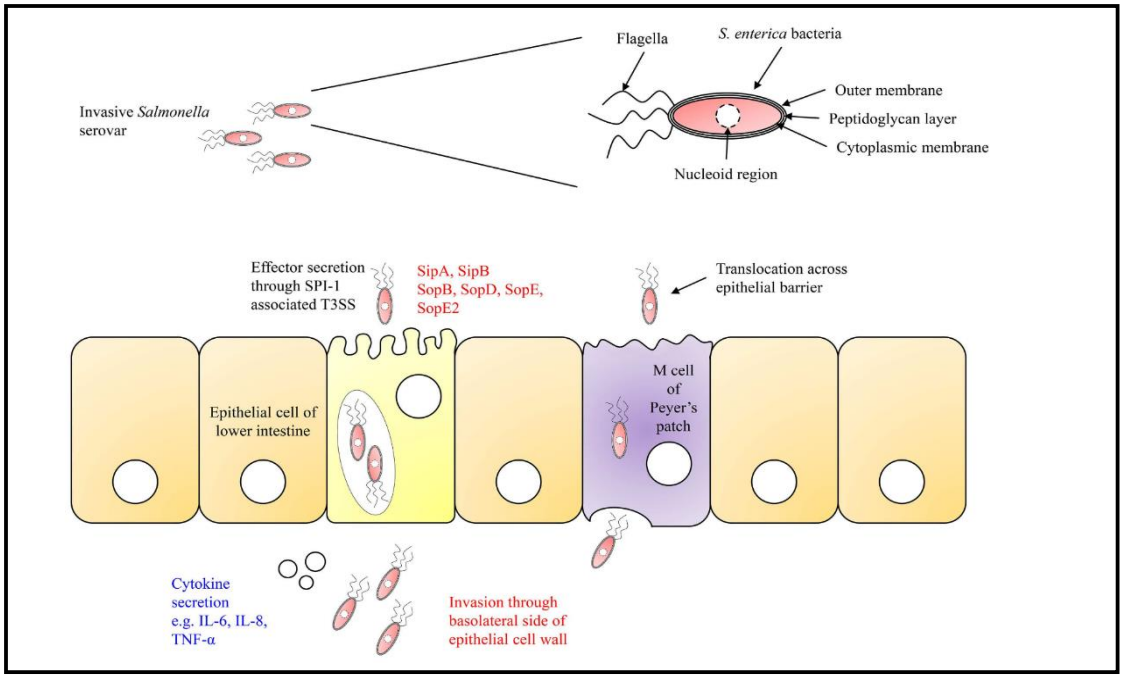

Figure 4. T3SS and effectors proteins associated with the invasion of *Salmonella spp*. to the epithelial cells of the intestine (Reprint with permission of Hurley et al., 2014).

Chemokines released during *Salmonella* infection in bovine ligated ileal loops include: interleukin 8 (IL-8), granulocyte chemotactic protein 2 (GCP-2), and growth-related genes α and Ɣ (GRO- α, GRO- Ɣ) which cause acute infiltration of neutrophils (Santos et al., 2002). The bacterial infection to the host cells induces a Ca^{2+} response (Pace and Galán, 1994; Gewirtz et al.,2000) which is responsible for the activation of many signaling pathways inside the cells (Yoshihara and Montana, 2004; Van Haasteren et al., 2000). An increase in Ca^{2+} during *Salmonella* infection will lead to activation of chemokine gene expression (Gewirtz et al., 2000).

Antimicrobial Interventions

The main source of contamination is the animal itself during the slaughtering, hiding, and handling of the carcass. However, over the past years, the meat industry has made a significant improvement in food safety when food companies developed different strategies to reduce or

eliminate microbial contamination on meat surfaces. Thus, treating the carcass before the chilling step will reduce the contamination of ground beef before reaching the consumer. Food safety interventions include physical, chemical, and biological (Brashears and Chaves, 2017). One main consideration for using these interventions is to extend the shelf life of the perishable food; which has become more important in recent years due to the globalization of the food industry (Ouattara et. al., 1997a). Even with these interventions to decrease the microbial load in fresh meat, the risk of microbial contamination is still in place (Dave and Ghaly, 2011).

The USDA listed a thorough list of interventions that are used to decrease contamination by microorganisms in the meat industry (USDA-FSIS, 2017a). Among these interventions, this paper will focus on the organic acids (acetic, citric, and lactic) as examples of chemical interventions that are widely used in slaughterhouses. The basic principle on the mode of action of organic acids is the undissociated forms (non-ionized) which target the microorganism via penetration of the bacterial cell wall and disrupt the metabolic functions of a certain type of bacteria (Lues and Theron, 2012). Furthermore, the acid will lower the internal pH leading to impair or stop the bacterial growth (Cetin-Karaca, 2011).

Ouattara et. al. (1997b) examined the inhibitory effect of organic acids in most bacteria that caused spoilage. Another study examined the effect of acetic, lactic, propionic, and formic acid at (1, 1.5, and 2%) concentrations as a spray wash on the meat surface in reducing the growth of *E. coli* O157 and *Staphylococcus.* Their findings showed a reduction in the growth of both bacteria after using these acids. 1.25 and 1.35 log reduction of *E. coli* O157 after using acetic and lactic acids respectively (Raftali et al., 2009).

Combining multiple mitigation techniques to control or even minimize pathogens in food products is termed as “the hurdle techniques” (Morgan et al., 2018). Since the beef industry has

many steps that allowing the pathogens to contaminate the meat such as hiding, skinning, processing and washing that does not provide any kill step for pathogens. Adding antimicrobial interventions, heating, irradiation, and postharvest interventions are a helpful approachs to eliminate pathogens in the meat but any increase in the dose of any of these technologies may affect the sensory characteristic of beef and beef products (Sohaib et al., 2016). Thus, applying hurdle technonlgy by using a combination of additives or preservatives can be surround this problem. The hurdle method can be applied at any processing steps i.e postharvest, preharvest, and post processing (Sohaib et al., 2016). Zhou et al. (2010) were applying an example of hurdle method by using the active packaging combining with heating or irradiation to the packaged products to improve the physical antimicrobials in eliminating pathogens. however, using the hurdle methods must have no effect on the stability, shelf life, sensory characteristic, and nutrient properties of the beef and its products (Sohaib et al., 2016).

Lactic Acid

Lactic acid is one of the organic acids that is most commonly used in the meat industry because of its low cost and high effectiveness. It is generally recognized as safe (GRAS) and approved by the FDA (Wheeler et. al., 2014). An early study found that applying water wash and lactic acid (500 ml of 4% L-lactic acid, 55°C) on hot carcass before chill reduced *E. coli* O157:H7 and *S. typhimurium* contamination by 5 logs. Additional reduction in *E. coli* was noticed at the post chill acid treatment of 2 to 2.4 logs (Castillo et al., 2001). A study by Alakomi et al. (2000) showed the effect of lactic acid to cause sublethal injury to *E. coli* when they suggested that such injury occurred due to the disruption of the LPS layer of *E. coli*. They investigated the effect of lactic acid on the membrane permeability of *E. coli* O157:H7, *Pseudomonas aeruginosa* and *S. enterica* serovar Typhimurium by using a fluorescent- probe

uptake assay. Their results showed that lactic acid damaged the permeability of the outer membrane more than any other substances used (EDTA and HCL). Similarly, beef trim at 4°C treated with 5% lactic acid reduced the population of *E. coli* O157:H7 by 1.3 log CFU/cm^2. Beside the pathogenic bacteria, lactic acid has the ability to inhibit the growth of food spoilage bacteria (Dolores, 1993). Lactic acid penetrates the cytoplasmic membrane of gram-negative bacteria, decreases the intracellular pH and disrupts the transmembrane proton force (Ray and Sandine, 1992). External agents that release components from the outer membrane or intercalate in the membrane can damage the solidity of the outer membrane and then shed the permeability barrier function (Vaara, 1992). While lactic acid showed a significant effect in reducing pathogenic and spoilage bacteria, its impact on the quality of the meat is noticeable. Changing in the red color of meat to light or pink color due to using lactic acid was proven in a study which showed the impact of lactic acid on reducing *E. coli,* but at the same time, it reduced the quality of ground beef and its redness (Stivarius et. al., 2002b).

Acetic Acid

Acetic acid is another organic acid that is widely used in antibacterial treatment in the beef industry (Wheeler, 2014). Acetic acid is GRAS and mostly used as a spray on the carcass and a variety of meats and trimmings (USDA-FSIS, 2013b). Acetic acid at a 3% concentration showed an effect in reducing *Enterobacteriaceae* by 1.5 logs when used to sanitize the beef surface at 70°C (Anderson, Marshall, and Dickson, 1991). A combination of lactic acid and/or acetic acid showed an inhibitory effect on *Listeria monocytogenes, S. typhimurium,* and *E. coli* O157:H7 in low acid foods (Cagri, Ustunol, and Ryser, 2001). The mechanism approach of acetic acid is similar to lactic acid and other organic acids. It enters the microbial cells and decreases the pH of the cytoplasm rapidly which can lead to an imbalance of energy.

Furthermore, the organic acid application can lead to an accumulation of free acid anions that lead to the killing of these microbes by a high level (Gonzalez-Fandos and Herrera, 2014). Several studies were conducted to verify the effect of organic acids on pathogenic bacteria in the meat industry. Zhou et al. (2007) identified the antimicrobial effect of acetic acid (0.10%) in combination with thyme (100 mg/ml) and carvacrol (100 µl/ L) to inhibit the growth of *S. typhimurium* in poultry meat without affecting flavor. They have also reported that microorganism resistance to these acids decreased at a moderately low temperature (10°C). Another study reported that using different concentrations of lactic acid and acetic acid spray on surfaces of lean beef inoculated with *E. coli* O157:H7 (3.5 logs CFU/ml) reduced the growth by 1.3 logs at 2% lactic acid (Laury et al., 2009).

Chlorine

Chemicals such as chlorine have been widely used in the beef industry due to its lower cost and easy application. Multiple studies have identified the benefits of using chlorine against Gram-positive and negative bacteria when implementing multiple hurdles methods (Sohaib et al., 2016). Stivarius et al. (2002a) investigated the effect of chlorine dioxide on ground beef inoculated with *E. coli* and *S. Typhimurium.* They used 200 ppm of chlorine dioxide to reduce several bacterial Species. However, treatment with chlorine caused ground beef to become lighter in color. Another study showed the effect of different concentrations of chlorine on the reduction of *E. coli* O157:H7 and *Salmonella* (Carlson et al., 2008). This study revealed that potassium cyanate (2.4%), sodium sulfide (6.2%) and sodium hydroxide washing with 0.02% of chlorinated water reduced *E. coli* O157 inoculated in beef hide (5.4, 4.7, and 5.2 logs CFU/ cm^2) by 5.1, 4.8, and 5 logs CFU/cm^2 respectively.

Chlorine is GRAS and is mostly used in potable water for carcass decontamination. It has the ability to decrease the uptake of nutrients and the oxygen of bacterial cells along with the oxidation of sulfhydryl enzymes (Yang et al., 2009). Despite its beneficial effects, there are some limitations in using chlorine in slaughterhouses. Chlorine has to be used after dehiding of the animal because of its ability to be neutralized by organic materials involve with hides (Sohaib et al., 2016).

N-halamine is another chemical compound which was used as a part of antimicrobial packaging to prevent the spoilage bacteria and to extend the shelf life. N-halamine is a nitrogen compound that has the ability to reduce microbial growth by 1 log CFU/g and to extend the shelf life of refrigerated raw beef (Ren et. al., 2018).

Previous work has been done in our lab and determined that β-phenylethylamine (PEA) acted as a biofilm inhibitor for *E. coli* O157:H7 grown in a liquid beef broth and incubated at 10º C (Lynnes, et. al., 2014). PEA is found naturally in cheese and chocolate at levels of 100 mg/kg (Halasz et. al., 1994). During food fermentation, PEA forms as a result of the activity of tyrosine decarboxylase by the bacteria (Millichap and Yee, 2003). PEA has a positive impact on human health and an inhibitory effect on microorganisms. Due to the increase in food consumption in meat in particular and the demand for safer meat with a longer shelf life, the need for new, safe, antibacterial additives is obvious. Using safe and effective antimicrobial interventions will reduce the risk of spoilage due to natural microflora, which found normally on the meat surfaces, and other foodborne pathogens.

CONCLUSION

The rich nutrient composition and the high moisture content of the meat allow the growth and survival of a wide variety of microorganisms. Even with the availability of developed technology and programmed protocols to reduce the incidence of microbes in the meat industry, still, it is a big concern for meat producers, retailers, and consumers. Eliminating or minimizing the growth of common foodborne pathogen as well as microorganisms that cause meat spoilage is a major goal of the meat industry. In addition to the danger caused by the foodborne pathogens on public health, the economic costs impact on the meat industry is enormous. Recall plus treatment due to food contaminated with *Salmonella* and *E. coli* O157:H7 are cost $3.6 billion and $ 271million respectively (USDA-ERS, 2014). Slaughtering, fecal material, carcass to carcass contact, animal hiding, processing, packaging, and transportation are some of the potential sources of many microorganism's contaminations.

Our understanding of the factors crucial for survival, colonization, and the virulence determinants of *E. coli* O157:H7 and *Salmonella spp*. and other common foodborne pathogens will assist in the development of new technologies to prevent bacterial contamination into the food and eventual human infection. Knowing the mechanism of bacterial enterance and invasion will aid in implementing a good decontamination technology to prevent preharvest contamination with the most common foodborne pathogens.

It should be kept in mind that there is no single technology that can enhance the safety of meat without adversely affecting the quality characteristics of the meat and meat-related products. Therefore, new studies combining the use of decontamination interventions along with implementing good hygiene technologies, and proper meat handling are necessary for reducing

the risk of contamination in the general public. Further research is needed by using natural substances that already GRAS and approved by FDA to minimize meat contamination.

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